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Development of the Brain in the Oegopsid Squid, *Todarodes pacificus*: An Atlas from Hatchling to Juvenile

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ABSTRACT—Post-hatching development of the brain in the oegopsid squid, *Todarodes pacificus* was described using conventional histological and Cajal's silver impregnation methods. The oegopsid squids spend a specific paralarval period before attaining adult-like juveniles. In the just-hatched paralarvae, the brain lobes (lower and intermediate motor centers) are differentiating only in the ventral part of the brain (subesophageal mass, SBM), and development of the dorsal part of the brain (supraesophageal mass, SPM) shows a heterochronic delay. In the SPM, an arched bundle of axonal tracts (transverse arch, TA) crosses the region over the oral ingrowth. In the early paralarval period, the basal lobes and precommissural lobe (higher motor centers) begin to develop along the TA. A little later, a pair of longitudinal axonal tracts (supraesophageal ladder, SPRL) elongates anteriorly from the TA, and accessory lobes (centers for memory and learning) and superior buccal lobes begin to differentiate along the SPRL. In the mid paralarval period, the lobes of the olfactory center and the peduncle lobe develop well in each optic tract region. In the late paralarvae, all brain lobes become identifiable and the brain shows substantially the same organization as that in the adults. The dorsal-most region of the SPM largely increases in volume with striking growth of the accessory lobes. The SBM elongates in anterior and posterior directions and the rostral end (anterior SBM) separates from the middle SBM. The optic lobes become very large with neuropils arranged in layers. In the juveniles, the neuropils increase in relative volume to the perikaryal layers, and neuronal somata enlarge markedly in some lobes. The retarded development of higher motor centers during paralarval development suggests that the early paralarvae of *T. pacificus* are not active predators but suspension feeders.

Key words: cephalopods, central nervous system, paralarva, neuropil, hetero chrony

INTRODUCTION

The cephalopod nervous system represents a peak of evolution in invertebrates. In the comprehensive anatomical studies of the brain, Young (1974, 1976, 1977b, 1979) and Messenger (1979) have defined about 30 brain lobes in *Loligo* and presumed the function of each lobe. Development of lobes has been described in the embryonic brain in some cephalopod species: *Octopus vulgaris* (Marquis, 1989), *Loligo vulgaris* (Meister, 1972), *Todarodes pacificus* (Shigeno *et al*., 2001b), and *Sepioteuthis lessoniana* (Shigeno *et al*., 2001c), but development of the cephalopod brain during post-hatching period has fragmentarily been examined. Nixon and Mangold (1996) have summarized post-embryonic development of the brain in *Octopus vulgaris* by reviewing scattering data from hatchlings (Frösch, 1971; Marquis, 1989), planktonic larvae (Packerd and Albergoni, 1970; Giuditta *et al*., 1971; Giuditta and Prozzo, 1974), and adults (Wirz, 1959; Young, 1971) together with their own findings in settled juveniles. In *Sepia officinalis* post-embryonic maturation of the accessory lobes (vertical lobe system) has been studied (Messenger, 1973, 1979; Dickel *et al*., 1997). In loliginid species, juvenile brains have incidentally been dealt with in neuroanatomical studies of the adult nervous system (Young, 1974, 1976,

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1977b, 1979; Messenger, 1979; Dubas *et al.*, 1986a, b; Novicki, *et al.*, 1990; Budelmann and Young, 1987, 1993).

Differences in the brain structure among cephalopods reflect not only phylogenetic relationships but also variety in life styles (Young, 1977a, 1988; Maddock and Young, 1987). Though cephalopods do not have true larvae, the post-hatching individuals show various modes of growth. The hatchlings of the typical sepioids and some octopods, e.g. *Octopus briareus*, begin benthic or nekto-benthic life as miniatures of the adults (Boletzky 1977). However, the post-embryonic individuals in the teuthoid squids and some other octopods including *O. vulgaris* are called paralarvae, which are different in shape from the adults and spend planktonic life for a while (Boletzky, 1977; Young and Harman, 1988). The paralarvae in loliginid squids are active predators, but those in ommastrephid squids (rynchoteuthion paralarvae) are very premature with the arm crown, buccal mass, and digestive organs undeveloped (Naef, 1928; Hamabe, 1962; O'Dor *et al.*, 1982, Watanabe *et al.*, 1996; Shigeno *et al.*, 2001a), and are estimated to be suspension feeders (O'Dor *et al.*, 1985, Vidal and Haimovici, 1988). Development of behaviors and changes in life styles during growth must be related to post-embryonic development of brain lobes. The nekto-benthic hatchlings of *Sepia officinalis* have more developed brain than the planktonic hatchlings of *Loligo vulgaris*, particularly in the vertical lobe system concerning the tactile and visual memory (Frösch, 1971). Comparative studies of brain development among cephalopods will bring us an insight into function of brain lobes as well as evolution of the cephalopod brains.

In the preceding paper (Shigeno *et al.*, 2001b), we have established an atlas of the developing brain up to hatching stage in *T. pacificus*. We have described the process of brain formation from a circumesophageal cluster of ganglia that arise separately through ingression of neuroblasts (cf. Fig. 10). We have revealed that two longitudinal columns of neuropil bridged with some commissural tracts make a ladder-like framework for construction of the ventral part of the brain (subesophageal mass). The brain is much premature at the time of hatching in *T. pacificus* than in other cephalopod species such as *O. vulgaris* (Marquis, 1989) and *Sepioteuthis lessoniana* (Shigeno *et al.*, 2001c). Though most brain nerves have already radiated from the brain at the time of hatching, brain lobes are differentiating only in the subesophageal mass, and the dorsal part of the brain (supraesophageal mass) is hardly developed. In the present paper, we describe the post-embryonic development of the brain from the paralarvae to the juveniles in *T. pacificus*.

MATERIALS AND METHODS

Specimens

Paralarvae 1, 2, and 3 (see below), and juveniles up to 45 mm in mantle length (ML) of the Japanese common squid, *Todarodes pacificus* (Teuthoidea, Oegopsida, Ommastrephidae) were used. The paralarvae were collected during a cruise of the Choukai-Maru of Yamagata Prefectural Kamo Fisheries Senior High School and the Mizunagi of Kyoto Prefectural Marine Senior High School around the Sea of Japan. The paralarvae 1 were for the most part raised from the embryos obtained by artificial insemination on board (Ikeda *et al.*, 1993; Shigeno *et al.*, 2001a). Some of the paralarvae 1, and all of the paralarvae 2 and 3 were collected by oblique hauls of a cylinder-core ring net or a long NORPAC net from a depth of ca. 75 m. The paralarvae of *T. pacificus* (rynchoteuthion paralarvae) were easily distinguishable from other concomitant cephalopod paralarvae by a short proboscis and the characteristic umbrella-shaped mantle (Okutani, 1965; Sweeney *et al.*, 1992). Juveniles of 38 and 45 mm in ML were caught by set-nets in Kyoto and Shimane Prefecture, respectively. The post-embryonic stages of *T. pacificus* were identified on the basis of Watanabe *et al.* (1996) and Shigeno *et al.* (2001a).

Histology

The paralarvae 1 raised from fertilized eggs were fixed in Bouin's solution dissolved in seawater for 12 hr and stored in 70% ethanol at 4°C. The samples obtained in the field were fixed in 5–10% formalin/sea water and preserved in 5% formalin/phosphate buffer (pH 7.4) at room temperature. Specimens were embedded in Paraplast (Oxford). Sections were stained with Mayer's hematoxylin and eosin or Masson's trichrom. Some juvenile samples were stained by Cajal's silver impregnation technique modified for cephalopod nervous systems (Stephens, 1971). Three dimensional maps of embryonic brains were reconstructed from serial sections of 3–5 μm in thickness. In order to confirm structural details, some specimens were embedded in Spurr's resin, and semithin sections (ca. 1 μm thick) were stained with 1% toluidine blue.

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. We have introduced some new terms to the nervous system in *T. pacificus* embryos (Shigeno *et al.*, 2001b).

RESULTS

Post-embryonic development of *T. pacificus*

We briefly summarize the post-embryonic development of *T. pacificus* described by Shigeno *et al.* (2001a). The hatchlings of *T. pacificus* spend a planktonic period as paralarvae before they become juveniles. The paralarva has an umbrella-like mantle (Fig. 1) and a large funnel, floating without showing active swimming behaviors. Shigeno *et al.* (2001a) have divided the paralarval period into 3 stages, paralarva 1, 2, and 3. The paralarva 1, measuring about 1 mm in mantle length (ML), has a pair of small buds of arm 4 at the ventral-most portion in the arm crown, and a proboscis that is formed by fusion of the paired anlagen of tentacles soon after hatching. Since the paralarva 1 retains inner yolk, we can keep it without feeding in the laboratory. Watanabe *et al.* (1996) defined 9 stages (stages 26–34) in the paralarvae 1 maintained in the laboratory aquarium for 7 days after hatching at 20°C. Disappearance of the inner yolk is the criterion of the end of the period of paralarva 1. The paralarva 2 measures ca. 2 mm in ML. Cilia around the mouth (lip cilia) and a specific head withdrawal behavior characterize the paralarva 2, and disappearance of these characters is the criterion of the end of the period of paralarva 2. In the paralarva 3, a pair of arms 3 elongates and the proboscis begins to separate again from the base toward the tip. The paralarva 3 grows up

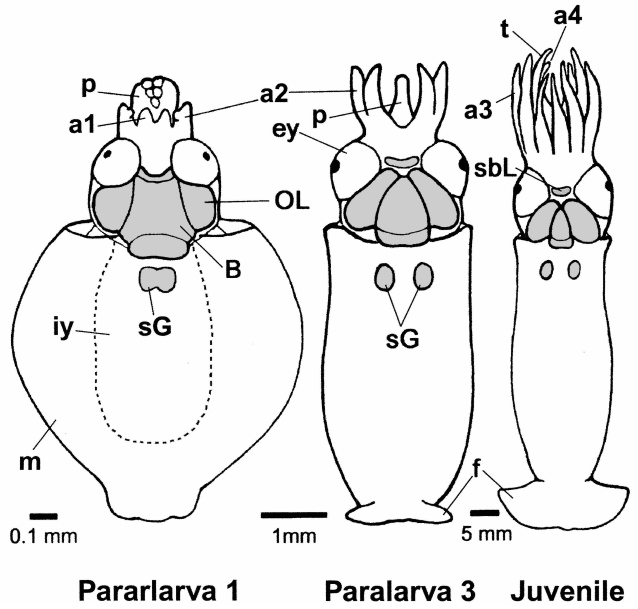

Fig. 1. Dorsal view of paralarva 1, paralarva 3, and juvenile. Top, anterior. The proboscis (p) in the paralarvae has separated into a pair of the tentacles (t) in the juvenile. The funnel located in the ventral side is not shown in this figure. a1–a4, arm 1- arm 4; B, brain; ey, eye; f, fin; iy, inner yolk; m, mantle; OL optic lobe; sbL, superior buccal lobe; sG, stellate ganglion.

to 30 mm in ML. The complete separation of the proboscis is the criterion of beginning of the juvenile period. In the juvenile, the mantle transforms to a slender corn with a pair of triangular fins near the tapering posterior end. The juvenile is considered to be an active predator, growing rapidly and migrating in the coastal zone. The individual becomes an adult (ca. 200 mm in ML) with sexual maturation.

Outline of the adult central nervous system

To facilitate understanding of descriptions on developing brains, we provide a brief sketch of the adult brain. Fig. 2 shows the lateral view of the brain and associated structures in the head region of *T. pacificus* (Sasaki, 1925). The cephalopod brain is penetrated by the esophagus, consisting of the ventrally located subesophageal mass (SBM) and the dorsally located supraesophageal mass (SPM). The esophagus ends anteriorly in the buccal mass, on which the superior (sbL) and the inferior buccal lobes (ibL) are present. The subesophageal mass is subdivided into the anterior (ASM), middle (MSM), and posterior (PSM) subesophageal masses. All the brain nerves radiate from the SBM; anteriorly each brachial nerve (bN) connects the ASM with the intrabrachial ganglion (ibG) in each arm and tentacle, and posteriorly the pallial nerves (pN) elongate to the stellate ganglia and the visceral nerves (vsN) innervate visceral organs. The large oval-shaped optic lobes (OL) are situated on the lateral sides of the brain (Fig. 1). The optic tract regions (OTR) of the SPM laterally connect the brain with the OL. Each region of the brain is subdivided into many brain lobes, which consist of the neuropils including axons and glial fibers, and the perikaryal layers containing

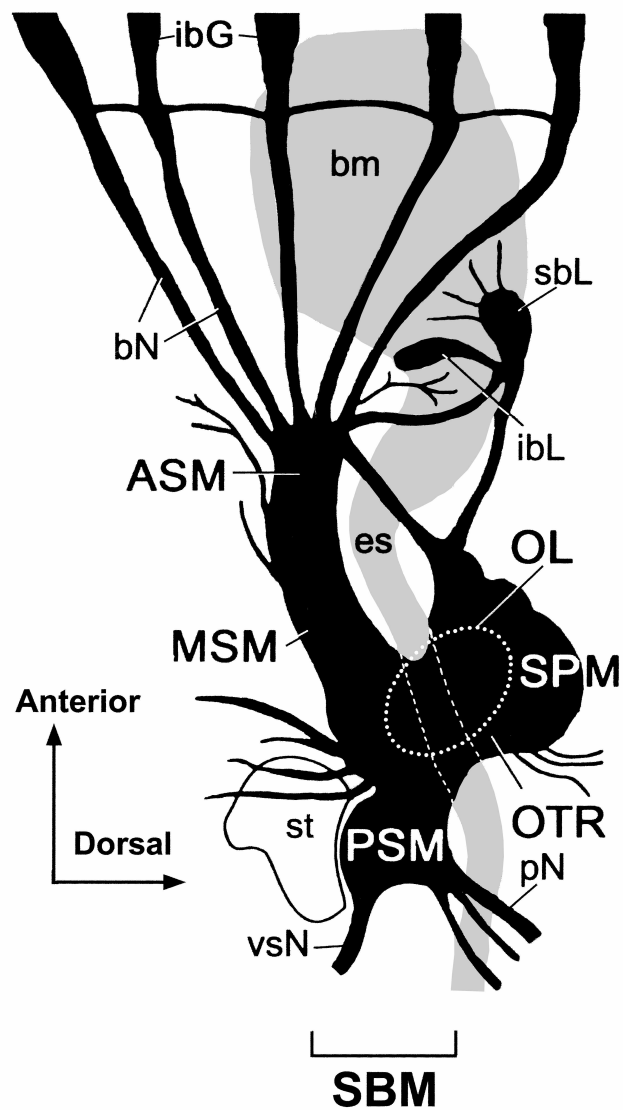

Fig. 2. Lateral view of the adult brain and associated structures in *T. pacificus* (modified from Sasaki, 1925). The brain pierced by the esophagus (es) is divided into the subesophageal mass (SBM) and the supraesophageal mass (SPM). The SBM is subdivided into the anterior (ASM), middle (MSM), and posterior (PSM) subesophageal masses. The optic lobe (OL) is situated on the lateral side of the optic tract region (OTR) in the SPM. bm, buccal mass; bN, brachial nerve; ibG, intrabrachial ganglion; ibL, inferior buccal lobe; pN, pallial nerve; sbL, superior buccal lobe; st, statocyst; vsN, visceral nerve.

the somata of neurons and glial cells. In *Loligo*, Young (1974, 1976, 1977b, 1979) and Messenger (1979) have morphologically defined about 30 brain lobes and presumed their function. The lobes in the SBM (e.g. pedal lobes, palliovisceral lobes, and magnocellular lobes) mainly control muscles in each body part. The lobes concerning olfaction are present in the OTR. The basal lobes (anterior, median, and lateral basal lobes, and interbasal lobe) in the SPM integrate the lower motor centers in the SBM, and the accessory lobes (vertical lobe, subvertical lobe, superior and inferior frontal lobes) are the centers of tactile and visual memory and learning (for a review, see Budelmann, 1995).

Post-embryonic development of the brain in *Todarodes pacificus*

Early paralarva 1 (stages 26–27) (Fig. 3A): The brain in this period retains almost the same feature as that in the embryos just before hatching (stage 25). Since we have already described the brain structure at the time of hatching in the preceding paper (Shigeno *et al.*, 2001b), we briefly summarize the structure of the brain in the early paralarva 1. For the diagrammatic drawing of the brain in this period, see Fig. 10 in Shigeno *et al.* (2001b).

Brain lobes are differentiating only in the subesophageal mass (SBM), which is a flat plate in shape in this period and not yet discretely subdivided into the ASM, MSM, and PSM. The intrabrachial ganglia are continuous to the anterior end of the SBM. Neuropils are present only in the middle to the posterior region of the SBM. In the middle region of the SBM, the posterior pedal lobe (ppL) is prominent but the anterior pedal lobe is not yet fully developed. The neuropil of the pallioviscerial lobe occupies a large area in the posterior region of the SBM. A series of 3 magnocellular lobes [the ventral (vmL), dorsal, and posterior magnocellular lobes] encompasses the ventro-lateral margin of the SBM (the periesophageal region). Three main commissures in the SBM [the middle pedal (mpC, Fig. 3A), ventral magnocellular (vmL), and posterior magnocellular commissures] and the brachio-pedal connective are already established. Most brain nerves have been formed at the time of hatching, radiating from the SBM.

The supraesophageal mass (SPM) is an arched belt of neuroblasts covering dorsally the oral ingrowth (buccal mass, bm) (Fig. 3A). In the SPM, a thick commissural bundle runs transversely across the region on the epithelium of the oral ingrowth (Fig. 3A). It contains 3 commissural tracts connecting the left and right optic tract regions (OTR) (optic, olfactory, and peduncle commissures), and the suprapedal commissure (spC) arising from the left and right posterior pedal lobes (Fig. 3A). We will refer to this bundle of commissures crossing the SPR as the transverse arch (TA). In the SPM, no neuropils are visible except for the transverse arch, but axons are diffusely occurring in the lateral region of the SPM (Fig. 3A). This is the first sign of differentiation of the basal lobes.

Diffusely occurring axons are also visible in the OTR. The peduncle lobes and the lobes of the olfactory system (the olfactory and dorso-lateral lobes) differentiate later in this region. A thick olfactory nerve from each olfactory organ has reached the OTR.

In the optic lobe (OL), axons are diffusely scattered (Fig. 3A) except for a circular profile of the neuropil forming the plexiform zone.

Mid paralarva 1 (stages 28–29) (Figs. 3B–F, 4): The SPM is increasing in thickness and brain lobes begin to differentiate in the dorsal and the lateral regions of the SPM. The small neuropils of the posterior basal lobe (pbL) and the precommissural lobe at first appear along the transverse arch (TA) in the SPM at stage 28 (Fig. 3B). At stage 29, the neuropils of the posterior basal lobes and the precommissural lobe (prL) gradually increase in volume in the posterior region of the SPM. The posterior basal lobe gradually differentiates into the dorsal basal (dbL), median basal (mbL), lateral basal (lbL), and interbasal lobes (itL), and these lobes are arranged along the TA in order of the dbL, mbL, lbL, and itL from dorsal to ventral, and the neuropil of the itL is continuous to that of the posterior pedal lobe (ppL) in the SBM (Fig. 4A–C). At this stage, another arch of neuropil crossing the region over the esophagus appears in the anterior position of the neuropil of the precommissural lobe (Figs. 3C, E, F and 4A). This arch of neuropil forms the anterior basal lobe (abL). A pair of longitudinal axonal tracts begins to elongate in an anterior direction from the neuropil of the TA. The neuropil of the precommissural lobe transversely connects the left and right longitudinal axonal tracts. Thus a ladder-like structure of neuropil consisting of two longitudinal axonal tracts bridged by a transverse neuropil is formed in the SPM. We will refer to this structure as the supraesophageal ladder (cf. Fig. 11). A little later than the appearance of the precommissural lobe, and the posterior and anterior basal lobes, the neuropils of the superior frontal lobe (sfL) and inferior frontal lobe (ifL) begin to differentiate along the supraesophageal ladder (Fig. 3E, F). The anterior end of the SPM protrudes anteriorly, covering the dorsal surface of the buccal mass (bm) (Fig. 3E, F). The supraesophageal ladder elongates into this region, and a pair of the neuropils of the superior buccal lobes (sbL) occurs at the anterior end of the ladder. A pair of the inferior buccal lobes (ibL) originating from isolated ganglionic bodies is located between the posterior surface of the buccal mass and the esophagus as isolated ganglionic bodies. Many axons are entering from the supraesophageal ladder and the transverse arch into the dorsal-most region of the SPM (Fig. 4A–C). This is the first sign of differentiation of the vertical (vtL) and the subvertical (svL) lobes, and small neuropils of these lobes become recognizable in this region at the end of this period (Fig. 3E, F).

In each OTR a slender neuropil of the peduncle lobe (pdL) becomes identifiable, but the olfactory and dorso-lateral lobes are not distinguishable from each other (Fig. 3D).

The SBM is also increasing in thickness, becoming oval in shape. The neuropil in the SBM extends anteriorly and subdivision of the SBM into the ASM, MSM, and PSM becomes evident (Fig. 3F). In the MSM, the middle pedal commissure (mpC) makes a distinct boundary between the anterior pedal lobe (apL) and the posterior pedal lobe (ppL). The posterior magnocellular commissure (pmC), ventral magnocellular commissure (vmC), and the brachio-pedal connectives (bpC) are also evident in the SBM (for the position of the commissures and the connectives, see Fig. 7).

In the OL, irregular profiles of neuropils appear in the zone (tangential zone, tz) proximal to a clear layer of neuropil of the plexiform zone (pz) (Fig. 3D). The OL sends out many thin axonal tracts (the optic-peduncle tracts) to the neuropil of the peduncle lobe.

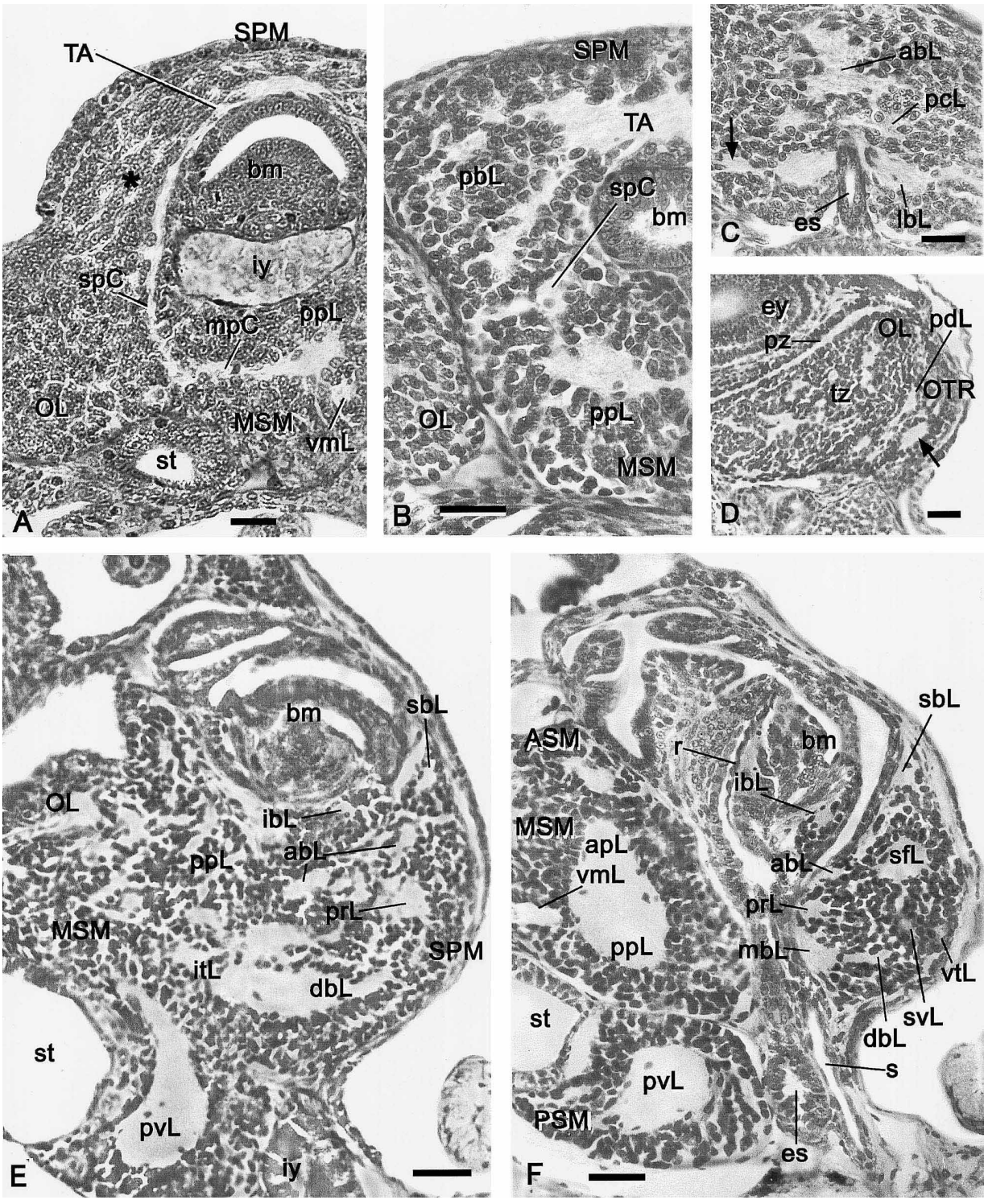

Fig. 3. Light micrographs of sections of the developing brain in the early to mid paralarva 1 at (**A**) stage 27, (**B**) stage 28, and (**C–F**) stage 29. (**A, B**) Transverse section. Top, dorsal. Axons are diffusely present in the region (*) where the posterior basal lobe (pbL) begins to differentiate. (**C**) Horizontal section. Top, anterior. (**D**) Para-sagittal section through the optic lobe (OL) and the optic tract region (OTR). Top, anterior; right dorsal. The arrows in (**C**) and (**D**) indicate the anlage of the olfactory and dorso-lateral lobes before separation. (**E**) Para-sagittal section and (**F**) mid-sagittal section of the same brain. Top, anterior; right, dorsal. Staining: (**A**, **D–F**) hematoxylin and eosin; (**B**, **C**) Masson's trichrom. abL, anterior basal lobe; apL, anterior pedal lobe; ASM, anterior subesophageal mass; bm, buccal mass; dbL, dorsal basal lobe; es, esophagus; ey, eye; ibL, inferior buccal lobe; itL, interbasal lobe; iy, inner yolk; lbL, lateral basal lobe; mbL, median basal lobe; mpC, middle pedal commissure; MSM, middle subesophageal mass; pbL, posterior basal lobe; pcL precommissural lobe; pdL, peduncle lobe; ppL, posterior pedal lobe; pvL palliovisceral lobe; pz, plexiform zone; sbL, superior buccal lobe; SPM, supraesophageal mass; PSM, posterior subesophageal mass; r, radula; a, salivary duct; sfL, superior frontal lobe; spC, suprapedal commissure; st, statocyst; svL, subvertical lobe; TA, transverse arch; tz, tangential zone; vmL, ventral magnocellular lobe; vtL, vertical lobe. Bar, 50 μm.

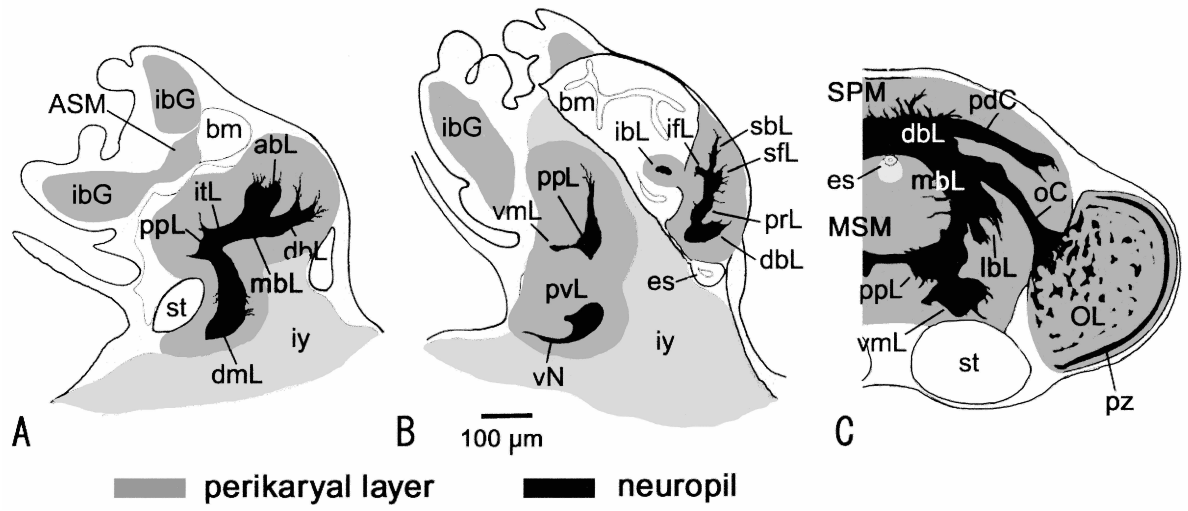

Fig. 4. Tracing of histological sections of the brain in the mid paralarva 1 at stage 29. (**A**) Para-sagittal and (**B**) mid-sagittal section of the same brain. (**C**) Transverse section. ASM, anterior subesophageal mass; bm, buccal mass; dbL, dorsal basal lobe; dmL, dorsal magnocellular lobe; es, esophagus; ibG, intrabrachial ganglion; ibL, inferior buccal lobe; ifL, inferior frontal lobe; itL, interbasal lobe; iy, inner yolk; lbL, lateral basal lobe; mbL, median basal lobe; MSM, middle subesophageal mass; oC, optic commissure; OL, optic lobe; pdC, peduncle commissures; ppL posterior pedal lobe; prL, precommissural lobe; pvL, palliovisceral lobe; pz, plexiform zone; sbL superior buccal lobe; sfL, superior frontal lobe; SPM, supraesophageal mass; st, statocyst; vmL, ventral magnocellular lobe; vN, visceral nerve.

Late paralarva 1 (stages 30–34) (Figs. 5, 6A, B): Most brain lobes except for some minor ones become identifiable in the brain in this period. In the lateral to the dorsal region of the SPM, the neuropils of the precommissural lobe (prC), anterior basal lobe (abL), and posterior basal lobes [dorsal basal (dbL), median basal (mbL), lateral basal (lbL), and interbasal (itL) lobes] show clear outlines, occupying a large volume (Figs. 5 and 6A). In the dorsal region of the SPM, the superior frontal lobe (sfL) also increases in volume. It continues to the precommissural lobe through a pair of broad channels of neuropil (Fig. 6B). In the dorsal region over the superior frontal lobe and the precommissural lobe, differentiation of the vertical (vtL) and subvertical (svL) lobes is evident, though they are very small in size in this period (Figs. 5A, B, D and 6A). The neuropil of the vertical lobe is a thin transverse plate in the dorsal-most region of the SPM (Fig. 5A, B). In the anterior end of the SPM, the paired anlagen of the superior buccal lobe (sbL) fuse with each other into a single mass including a continuous neuropil. A pair of axonal tracts originating from the longitudinal columns of the supraesophageal ladder runs from the precommissural lobe to the superior buccal lobe via the inferior frontal lobes (ifL) (Fig. 5A).

In the OTR, the slender neuropil of the peduncle lobe (pdL) is distinct (Fig. 6B), and differentiation of the olfactory (ofL) and the dorso-lateral (dlL) lobes becomes evident (Fig. 5A, E).

In the PSM, the large palliovisceral lobe (pvL) is subdivided into 3 lobules (central, latero-ventral, and posterior palliovisceral regions). Around the palliovisceral lobe, the neuropils of a pair of the fin lobes (fL), a pair of the posterior chromatophore lobes (pcL), and a single visceral lobe (vsL) begin to differentiate, though they are only faintly visible in this period (for the position of these lobes, see Fig. 7).

In the OL, neuropils of the tangential zone are still irregular and unorganized.

*Paralarva 2 (Fig. 6C)***:** The brain keeps almost the same feature as seen in the late paralarva 1, except that neuropils increase in volume to some degree. In the SPM, the neuropil of the precommissural lobe (prL) is the largest, and the neuropils of the vertical (vtL) and subvertical (svL) lobes are still small in volume. The vertical lobe somewhat increases in thickness, showing an oval profile in the mid-sagittal section.

The OTR increases in size. Since the OL remains small in size in the paralarva 2, the relative volume of the OTR to the OL is the largest in this period. In each OTR, the neuropil of the peduncle lobe (pdL) shows a characteristic crescent-shaped profile, and those of the olfactory (ofL) and the dorso-lateral lobes (dlL) also show distinct outlines (Fig. 6C).

In the PSM, the visceral lobe (vsL), fin lobes (fL), and posterior chromatophore lobes (pcL) become gradually distinct.

In each OL the semicircular profile of the plexiform zone (pz) is very prominent. The inner plexiform zone (ipz) becomes discernible in the layer proximal to the plexiform zone (Fig. 6C). Neuropils in the tangential zone (tz) show clear and smooth outlines, but columnar arrangement of neuropils is not yet seen in the OL.

Paralarva 3 (Fig. 6D, E, 7): The brain shows marked changes in the outer shape (Figs. 6D and 7), and the basic arrangement of the brain lobes completes internally. The dorsal region of the SPM rises into a large dome-shaped mass. The SBM elongates both in anterior and posterior directions (Fig. 6D), becoming flat in shape. The ASM separates from

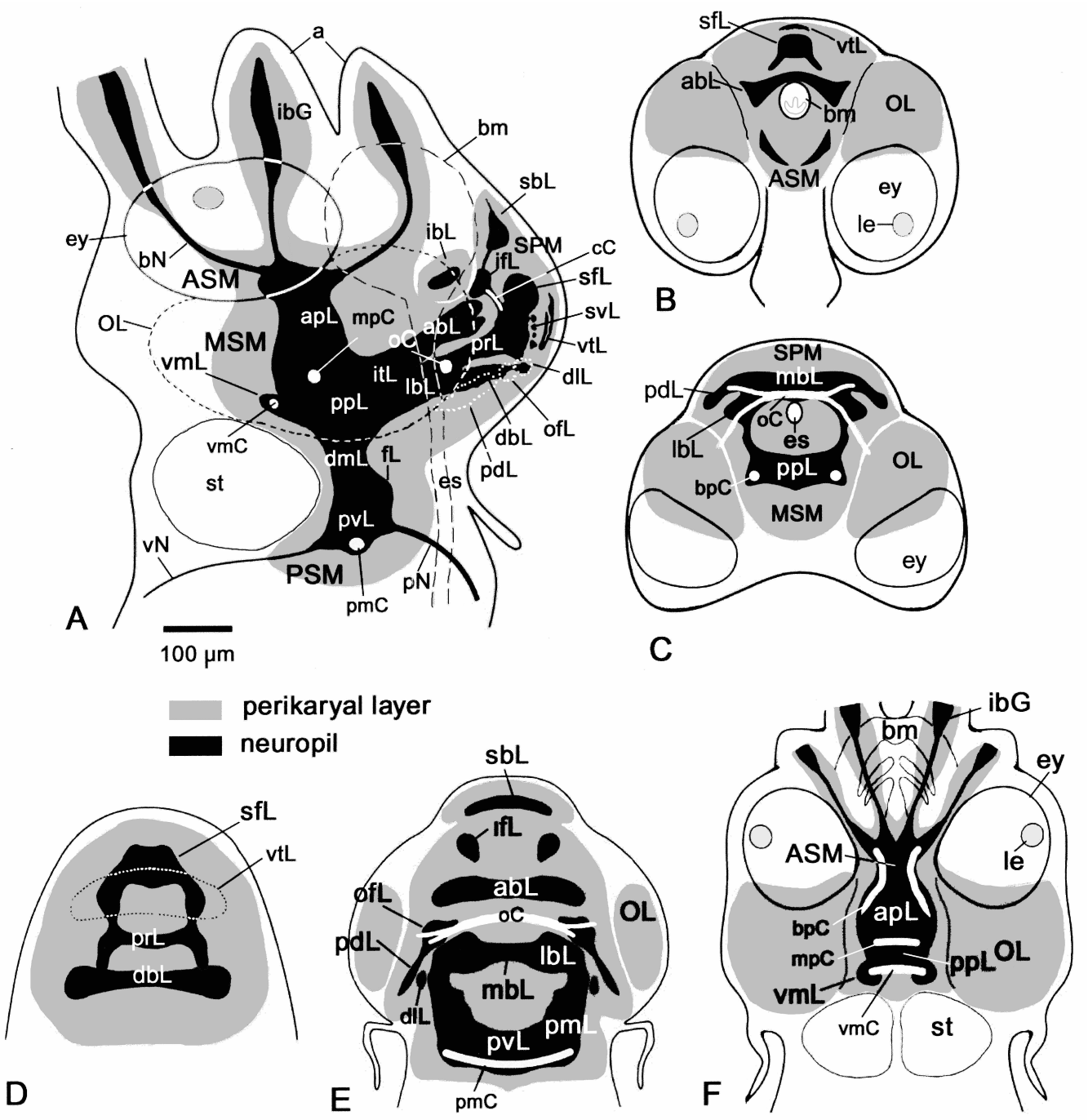

Fig. 5. Histological reconstruction of the brain in the late paralarva 1 at stage 30. (**A**) Lateral view. Top, anterior; right, dorsal. (**B, C**) Frontal views through an anterior (**B**) and a middle (**C**) part of the brain. (**D–F**) Horizontal views through a dorsal (**D**), middle (**E**), and (**F**) ventral level of the brain. Dotted lines show the structures out of the sectional plane. The neuropils in the optic lobe (OL) are not shown, a, arm; abL, anterior basal lobe; apL, anterior pedal lobe; ASM, anterior subesophageal mass; bm, buccal mass; bN, brachial nerve; bpC, brachio-pedal connective; cC, cerebral connective; dbL, dorsal basal lobe; dlL, dorso-lateral lobe; dmL, dorsal magnocellular lobe; es, esophagus; ey, eye; fL, fin lobe; ibG, intrabrachial ganglion; ibL, inferior buccal lobe; ifL, inferior frontal lobe; itL, interbasal lobe; lbL lateral basal lobe; le, lens; mbL, median basal lobe; mpC, middle pedal commissure; MSM, middle subesophageal mass; oC, optic commissure; olL, olfactory lobe; OL, optic lobe; pdL, peduncle lobe; brachial lobe; pmC, posterior magnocellular commissure; pmL posterior magnocellular lobe; pN, pallial nerve; ppL, posterior pedal lobe; prL, precommissural lobe; PSM, posterior subesophageal mass; pvL, pailliovisceral lobe; sbL, superior buccal lobe; sfL, superior frontal lobe; SPM, supraesophageal mass; st, statocyst; svL, subvertical lobe; vmC, ventral magnocellular commissure; vmL, ventral magnocellular lobe; vtL, vertical lobe; vN, visceral nerve.

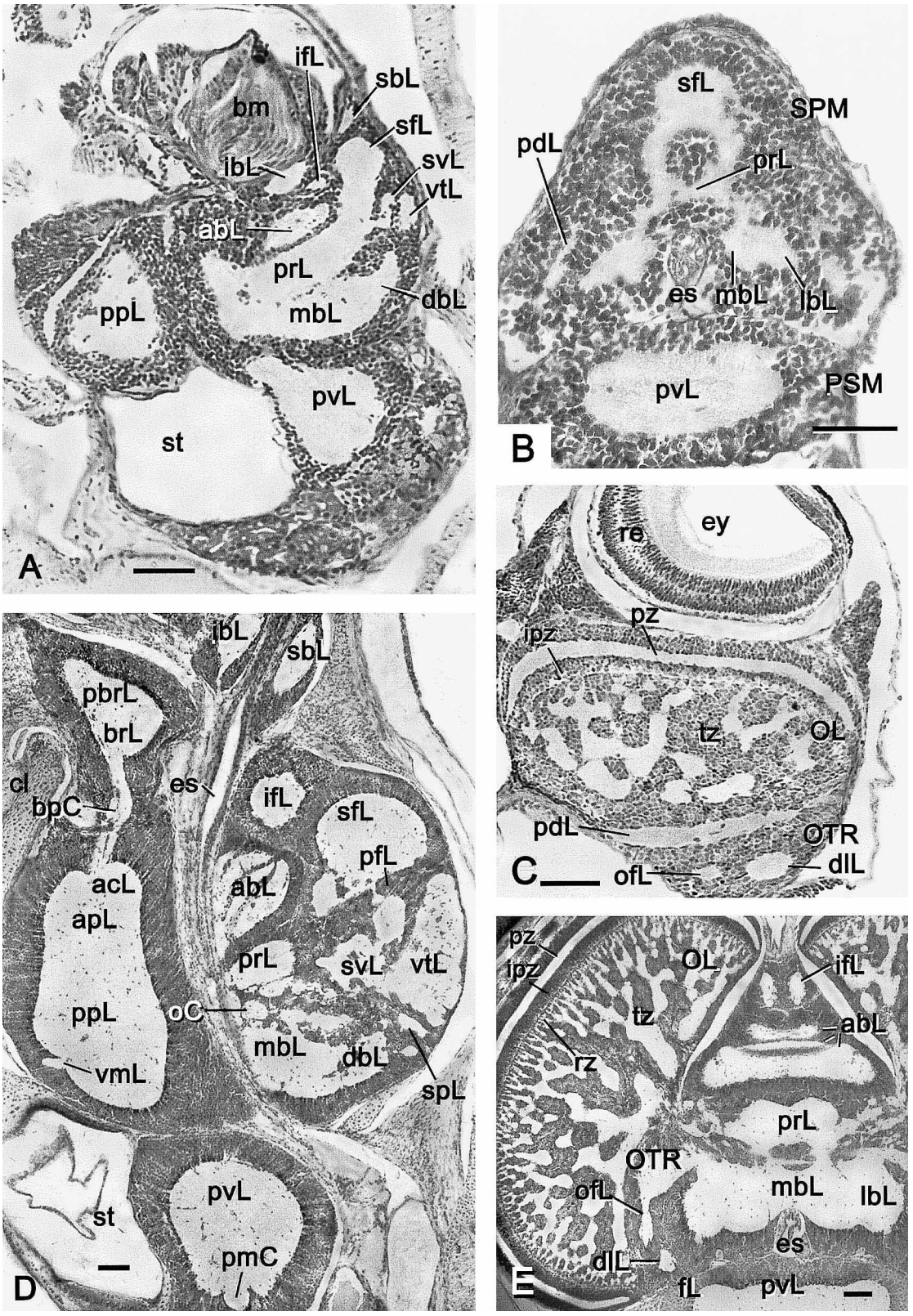

Fig. 6. Light micrographs of the developing brain in the mid to late paralarva 1. (**A, B**) Late paralarva 1 at stage 31. (**C**) Paralarva 2. (**D, E**) Paralarva 3 (3.9 mm in mantle length). (**A**, **C**) Para-sagittal section. Top, anterior; right, dorsal. (**B**, **E**) Horizontal section. Top, anterior. (**D**) Mid-sagittal section. Top, anterior; right, dorsal. Staining (**A**, **C–E**) hematoxylin and eosin; (**B**) Masson's trichrom. acL, anterior chromatophore lobe; abL, anterior basal lobe; apL, anterior pedal lobe; bm, buccal mass; bpC, brachio-pedal connective; brL, brachial lobe; cl, cephalic cartilage; dbL, dorsal basal lobe; dlL, dorso-lateral lobe; es, esophagus; ey, eye; fL, fin lobe; ibL, inferior buccal lobe; ifL, inferior frontal lobe; ipz, inner plexiform zone; lbL, lateral basal lobe; mbL, median basal lobe; oC, optic commissure; ofL, olfactory lobe; OL, optic lobe; OTR, optic tract region; pbrL, pre-

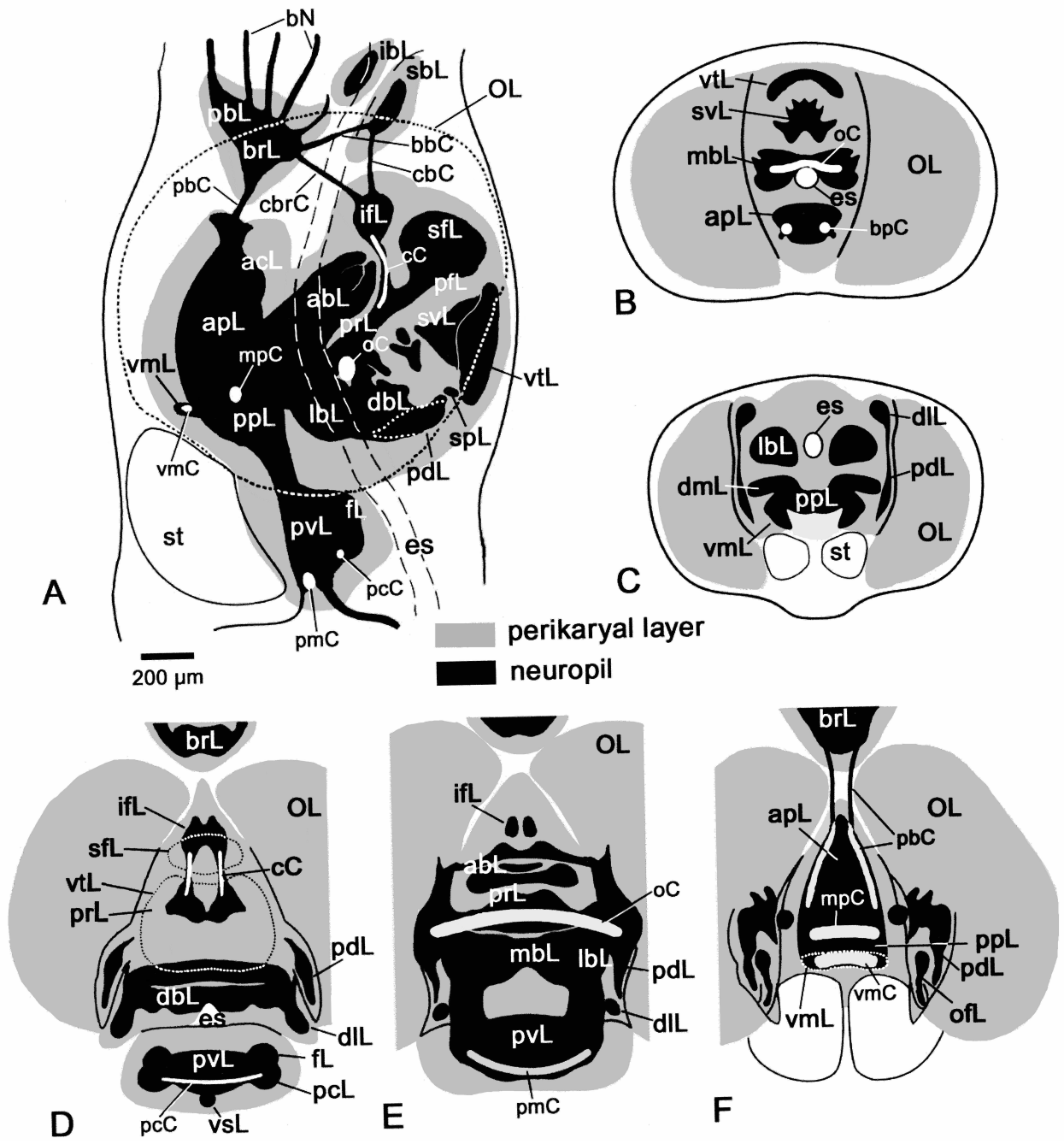

Fig. 7. Histological reconstruction of the brain in paralarva 3. (**A**) Lateral view. Top, anterior; right, dorsal. (**B, C**) Frontal views through a middle (**B**) and posterior (**C**) part of the brain. Top, dorsal. (**D–F**) Horizontal views through a dorsal (**D**), middle (**E**), and ventral level (**F**) of the brain. Top, anterior. Dotted lines show the structures out of the plane. The neuropils in the optic lobe (OL) are not shown. abL, anterior basal lobe; acL, anterior chromatophore lobe; apL, anterior pedal lobe; bbC, buccal-brachial connective; bN, brachial nerve; bpC, brachio-pedal connective; brL, brachial lobe; cbC, cerebro-buccal connective; cbrC, cerebro-brachial connective; cC, cerebral connective; dbL, dorsal basal lobe; dlL, dorsolateral lobe; dmL, dorsal magnocellular lobe; es, esophagus; fL, fin lobe; ifL, inferior frontal lobe; lbL, lateral basal lobe; mbL, median basal lobe; mpC, middle pedal commissure; oC, optic cominissure; ofL, olfactory lobe; pbL pre-brachial lobe; pcL, posterior chromatophore lobe; pdL, peduncle lobe; brachial lobe; pmC, posterior magnocellular commissure; ppC, posterior chromatophore commissure; ppL, posterior pedal lobe; prL, precommissural lobe; pvL, palliovisceral lobe; sbL, superior buccal lobe; sfL, superior frontal lobe; spL, subpedunculate lobe; st, statocyst; svL, subvertical lobe; vmC, ventral magnocellular commissure; vmL, ventral magnocellular lobe; vtL, vertical lobe; vsL, visceral lobe.

brachial lobe; pdL, peduncle lobe; pfL, post-frontal lobe; pmC, posterior magnocellular commissure; ppL, posterior pedal lobe; prL, precommissural lobe; PSM, posterior subesophageal mass; pvL, palliovisceral lobe; re, retina; pz, plexiform zone; rz, zone of radial columns of medula; sbL, superior buccal lobe; sfL, superior frontal lobe; SPM, supraesophageal mass; spL, subpedunculate lobe; st, statocyst; svL, subvertical lobe; tz, tangential zone; vmL, ventral magnocellular lobe; vtL, vertical lobe. Bar, 100 µm.

the MSM. The cerebro-brachial connectives (cbrC) and the brachio-pedal connectives (bpC) link the ASM with the SPM and the MSM, respectively (Fig. 7A, F). The intrabrachial ganglion in each arm and tentacle leaves from the ASM with brachial nerves running from the ASM to each ganglion (Fig. 7A). The superior buccal lobe (sbL) also becomes an isolated ganglionic body apart from the SPM (Fig, 6D) on the postero-dorsal surface of the buccal mass, and the cerebro-buccal connective (cbC) and the buccal-brachial connective (bbC) link the superior buccal lobe with the SPM and the ASM, respectively (Fig. 7A). The OL markedly increases in volume and becomes a kidney-like shape with the anterior end filling the space enclosed by the ASM, MSM, SPM and the superior buccal lobe (Figs. 6E and 7D–F).

In each brain lobe, the neuropil increases in volume and the perikaryal layer becomes thick with a marked increase in cell number (cf. Fig. 6D with Fig. 6A). Particularly the accessory lobes (vertical lobe, subvertical lobe, and superior and inferior frontal lobes) show prominent growth in the dorsal region of the SPM (Fig. 6D). The round neuropil of the superior frontal lobe (sfL) occupies the greater part of the anterior dorsal region of the SPM. The neuropil of the vertical lobe (vtL) expands in anterior and posterior directions and its lateral margins extend in a ventral direction, eventually forming a large dome under the dorsal surface of the SPM (Fig. 7A, B, D). The neuropil of the subvertical lobe (svL) shows a very complicated profile with many islands and indentations (Fig. 6D). The left and right inferior frontal lobes (ifL) become continuous to each other through a transverse channel of neuropil (Fig. 7D). A pair of axonal tracts (cerebral connectives, cC) links the inferior frontal lobes to the precommissural lobe (prL). Two small brain lobes, the post-frontal lobe (pfL) and subpedunculate lobe (spL) become identifiable at the posterior portion of the superior frontal lobe and at the posteroventral position of the vertical lobe, respectively (Figs. 6D and 7A). In the lateral region of the SPM, the anterior basal lobe (abL) becomes very large and subdivisions (the anterior anterior basal lobe, lateral anterior basal lobe, and posterior anterior basal lobe) become clear in the neuropil (Fig. 6D, E). The precommissural lobe (prL) does not show prominent growth, becoming a relatively small lobe in the SPM.

In the separated ASM, the brachial (brL) and pre-brachial (pbL) lobes are formed (Fig. 6D). The anterior chromatophore lobe (acL) becomes newly identifiable at the anterior end of the MSM (Fig. 6D). In the ventral margin of the MSM, the neuropil of the ventral magnocellular lobe (vmL) markedly

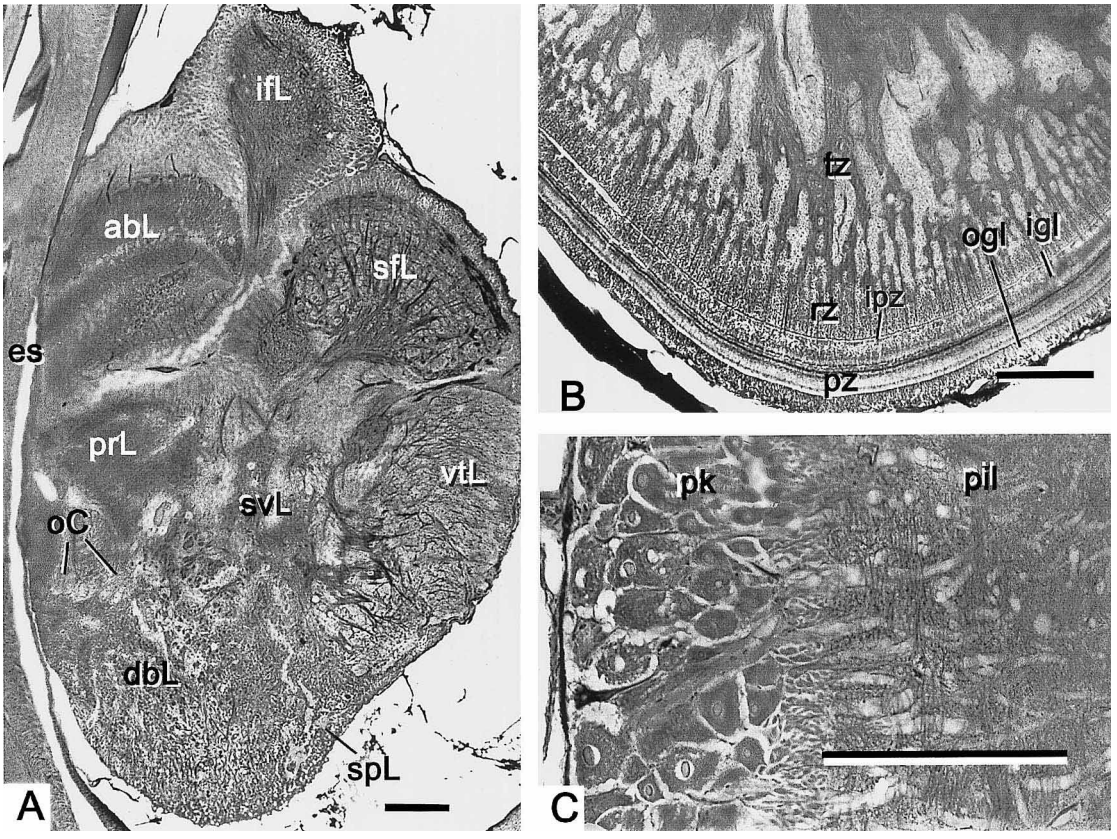

Fig. 8. Light micrographs of the brain in a juvenile (45 mm in mantle length). Cajal's silver staining. (**A**) Mid-sagittal section of the supraesophageal mass. (**B**) Optic lobe. The palisade zone and optic tract zone are not shown. (**C**) Ventral part of the posterior pedal lobe. Note large neuronal cells in the perikaryal layer (pk). abL, anterior basal lobe; dbL, dorsal basal lobe; es, esophagus; ifL, inferior frontal lobe; igl; inner granular layer; ipz, inner plexiform zone; oC, optic commissure; ogl, outer granular layer; pil, neuropil; prL, precommissural lobe; rz, zone of radial columns of medulla; sfL, superior frontal lobe; spL, subpedunculate lobe; svL, subvertical lobe; pz, plexiform zone; tz, tangential zone; vtL, vertical lobe. Bar, 300 μm.

decreases in relative volume.

In the OL, the neuropils increase in volume in the tangential zone (tz), and show columnar profiles arranged in a radial fashion in the zone just inside the frontier zone (zone of radial columns of medulla, rz) (Fig. 6E). In contrast to the marked growth of the OL, the OTR does not grow largely, and the lobes of the olfactory system [olfactory (ofL) and dorsolateral (dlL) lobes] become relatively small in the paralarva 3 (Fig. 6E).

Enlargement of neuronal cells (neuronal gigantism) as usually observed in the cephalopod brains begins to be evident in the paralarva 3. The somata of neurons increase in size in the perikaryal layer in the ASM, and in the fin lobe and posterior chromatophore lobe in the PSM (data shown in the juvenile).

Juvenile (Figs. 8, 9): The brain structure as observed in the adult completes in the early juvenile. Though juveniles grow from 15 mm to 200 mm in mantle length, the brain shows substantially the same feature as that in the paralarva 3 (cf. Fig. 6D with Fig. 8A). The brain itself does not increase in volume in proportion to the growth of the body, but the musculatures and the cephalic cartilage (cl) surrounding the brain are highly reinforced. The main changes observed in the brain of early juveniles are: a marked increase in ratio of the neuropil to the perikaryal layer, a progress of neuronal gigantism, and local modification in some brain lobes.

In the SPM, the accessory lobes [vertical (vtL), inferior (ifL) and superior frontal (sfL), and subvertical (svL) lobes] develop well in particular, occupying the greater part of the dorsal region of the SPM (Figs. 8A and 9A–D). In the dorsalmost region of the SPM, the perikaryal layer forms very thin walls enclosing the neuropils of the vertical (vtL) and superior frontal lobes (sfL) (Fig. 9B–D). In the posterior region of the vertical lobe, many islands of neuropils of the subpedunculate lobe (spL) appear (Fig. 9E). In the OTR, the olfactory lobe subdivides into 3 lobules, the olfactory lobules 1, 2, and 3, each showing a knob-like neuropil (Fig. 9E). In the OL, the layered organization of the neuropil is completed and the 8 layers defined by Young (1974) in *Loligo* are clearly visible (Fig. 8B). The separation of the superior buccal lobe from the SPM, and of the ASM from the MSM becomes more distinct.

Enlargement of the neuronal somata is notable in the perikaryal layers of every lobe in the ASM and MSM (Figs. 8C and 9B, C), the fin lobes and posterior chromatophore lobes in the PSM, and the superior buccal lobe. The enlargement occurs to a lesser extent in the inferior frontal (ifL), anterior basal, and posterior basal lobes in the SPM. The enlarged somata measure maximally about 60 μm in diameter in contrast to usual somata measuring about 10 μm in diameter.

The major commissures [the optic (oC), olfactory, peduncle (pdC), suprapedal (spC), middle pedal (mpC), posterior pedal, ventral magnocellular, and posterior magnocellular commissures] and the major connectives [the cerebro-buc-

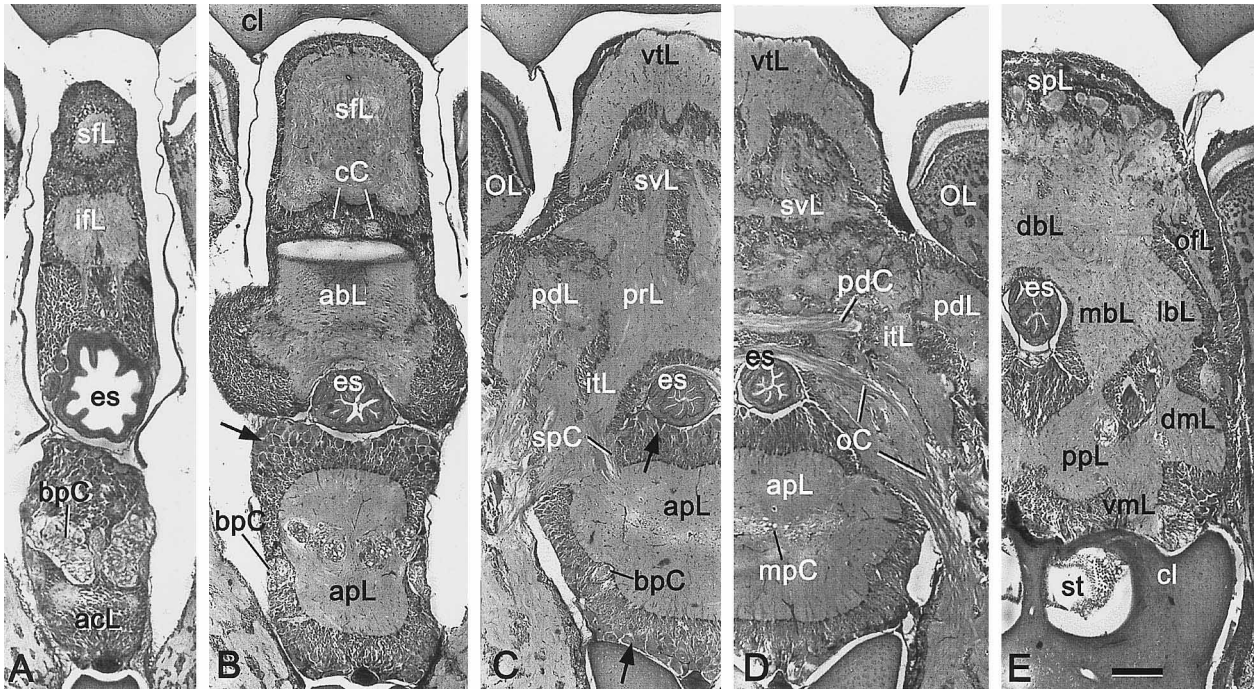

Fig. 9. Light micrographs of cross sections of the brain in a juvenile (38 mm in mantle length). Sections are arranged from rostral (**A**) to caudal (**E**). Masson's trichrom staining. Arrows in (**B**) and (**C**) indicate enlarged neuronal somata in the perikaryal layer. abL, anterior basal lobe; acL, anterior chrornatophore lobe; apL anterior pedal lobe; bpC, brachio-pedal connective; cC, cerebral connective; cl, cephalic cartilage; dbL, dorsal basal lobe; dmL, dorsal magnocellular lobe; es, esophagus; ifL, inferior frontal lobe; itL, interbasal lobe; lbL lateral basal lobe; mbL, median basal lobe; mpC, middle pedal commissure; oC, ventral root of optic commissure; ofL, olfactory lobe; OL, optic lobe; pdC, peduncle comniissure; pdL, peduncle lobe; ppL, posterior pedal lobe; prL, precommissural lobe; sfL, superior frontal lobe; spC, suprapedal commissure; spL, subpedunculate lobe; st, statocyst; svL, subvertical lobe; vmL, ventral magnocellular lobe; vtL, vertical lobe. Bar. 300 μm.

cal, brachio-pedal (bpC), cerebro-brachial, and cerebral (cC) connectives] are intensely fasciculated with various-sized axons (Fig. 9A–D), and some showing very large sectional profiles.

DISCUSSION

In the present and the preceding papers (Shigeno *et al.*, 2001b), we have clarified the morphological process of the brain development from the early embryo to juvenile in *T. pacificus*. Here we summarize it briefly (Fig. 10). The brain originates from placodal thickenings occurring in the ectoderm at the end of epiboly. The neuroblasts ingress from the placodes and assemble into 4 pairs of ganglia under the surface epithelium (Fig. 10A). The ganglia accumulate into a ring encircling the oral ingrowth (Fig. 10B), and gradually form the brain composed of the subesophageal (SBM) and the supraesophageal (SPM) masses. Many brain lobes consist-

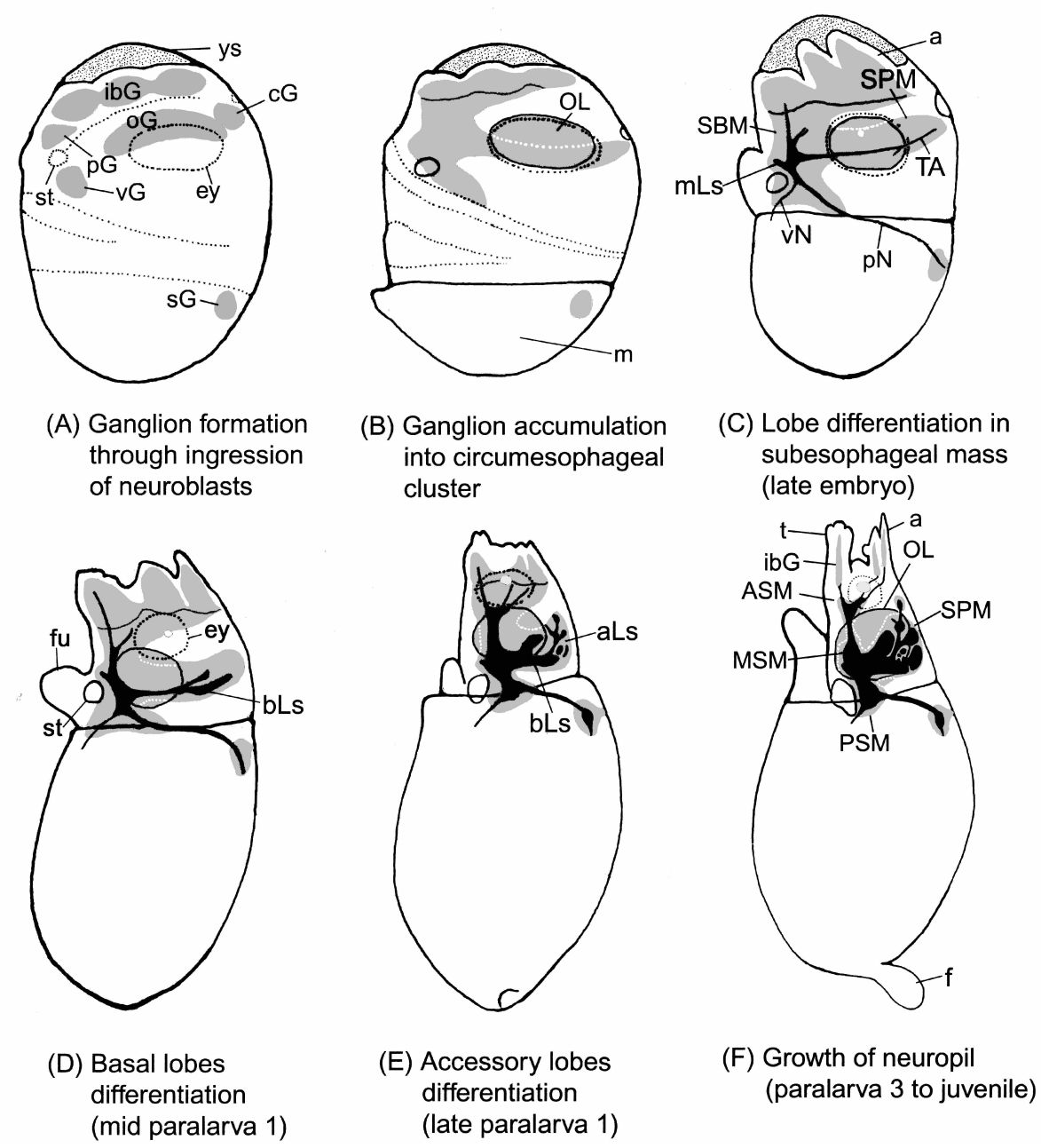

Fig. 10. Summary of the brain development in *Todarodes pacificus*. Left-side view. Top, anterior; right, dorsal. a, arm; aLs, accessory lobes; ASM, anterior subesophageal mass; bLs, basal lobes; cG, cerebral gangion; ey, eye; f, fin; fu, funnel; ibG, intrabrachial ganglion; m, mantle; mLs, magnocellular lobes; MSM, middle subesophageal mass; oG, optic ganglion; OL, optic lobe; pG, pedal ganglion; pN, pallial nerve; PSM, posterior subesophageal mass; SBM, subesophageal mass; sG, stellate ganglion; SPM, supraesophageal mass; st, statocyst; t, tentacle; TA, transverse arch; vG, palliovisceral ganglion; vN, visceral nerve; ys, outer yolk sac.

ing of neuropils and perikaryal layers gradually differentiate in the developing brain. In the embryonic brain before hatching, brain lobes (e.g., pedal lobes and magnocellular lobes) differentiate only in the SBM (Fig. 10C). Differentiation of the lobes in the SPM begins after hatching; basal lobes first differentiate in the early paralarvae (Fig. 10D) and accessory lobes follows (Fig. 10E). The basic arrangements of the brain lobes completes in the late paralarvae. Each lobe grows with an increase in volume of the neuropil from the late paralarvae to the juveniles (Fig. 10F).

It has been suggested in some species, e.g. fruit fry (Therianos *et al.*, 1996; Nassif *et al.*, 1998), zebrafish (Wilson *et al.*, 1990; Chitnis and Kuwada, 1990, Ross *et al.*, 1992) and mouse (Easter *et al.*, 1993), that the complex circuitry of adult brain is conformed to a simple scaffold of axonal tracts in the embryonic brain. We clarified the presence of a simple framework of neuropils before differentiation of brain lobes in the developing brain of *T. pacificus* (Fig. 11). In the early SBM of the embryonic brain, axonal tracts show a simple pattern consisting of bilaterally situated longitudinal columns bridged with some commissural tracts (subesophageal ladder) (Shigeno *et al.*, 2001b). Along the subesophageal ladder, 3 magnocellular lobes, pedal lobes, and palliovisceral lobes gradually differentiate. An arched axonal tract running transversely across the region over the esophagus in the SPM (transverse arch, TA) occurs before hatching (Shigeno *et al.*, 2001b). The neuropils of the posterior basal lobes and the precommissural lobe differentiate after hatching along the TA. The anlage of the anterior basal lobe forms another arch of neuropil anteriorly to the TA. In the SPM after hatching, we found anterior elongation of a ladder-like structure from the TA (supraesophageal ladder). Accessory lobes and the superior buccal lobes differentiate along the supraesophageal ladder. Thus, in the developing brain of *T. pacificus*, the subesophageal and the supraesophageal ladders and two arches across the SPM (Fig. 11) seem to play a role as the scaffold for brain construction. Wildemann *et al.* (1997) have showed in the developing brain of *Drosophila* that circumesophageal arch-like structure at the brain-foregut interface is important in the morphogenesis of embryonic brain. This structure seems to correspond to the TA in *T. pacificus*. The TA itself remains as a thick commissural bundle containing the suprapedal, peduncle, olfactory, and optic commissures in the adult brain. The subesophageal ladder remains as the brachio-palliovisceral connectives and the middle pedal, posterior pedal, and ventral magnocellular commissures in the adult brain. The lateral columns of the supraesophageal ladder seem to become the cerebral tracts and/or the optic to superior frontal tracts in the adult brain.

Heterochronic delay in organogenesis characterizes the development of the ommastrephid squids. Development of the digestive, respiratory, and circulatory organs and two pairs of the arms are markedly retarded in *T. pacificus* as compared with that in the Octopoda, Sepioidea, and Myopsida (Watanabe, 1996). We found that heterochronic retardation also occurred in the differentiation of brain lobes in the SPM

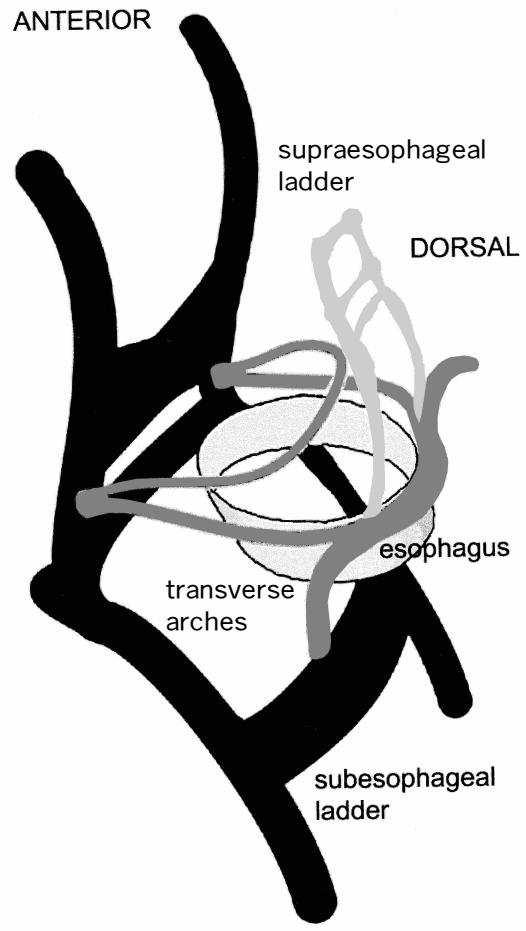

Fig. 11. The presumed scaffold for brain construction in *Todarodes pacificus*. The transverse bridges between the left and right longitudinal columns in the supraesophageal ladder correspond to the neuropils of the superior buccal, inferior frontal, and superior frontal lobes from anterior to posterior, and those bridges in the subesophageal ladder correspond to the neuropils of the posterior pedal, ventral magnocellular and palliovisceral lobes from anterior to posterior. The anterior transverse arch corresponds to the neuropil of the anterior basal lobe, and the posterior one, to the neuropils of the precommissural and the posterior basal lobe.

of *T. pacificus*. In the species examined so far: *Octopus vulgaris* (Frösch, 1971; Marquis, 1989), *Sepia officinalis* (Frösch, 1971), *Loligo vulgaris* (Frösch, 1971), *Sepioteuthis lessoniana* (Shigeno *et al.*, 2001c), and *Idiosepius paradoxus* (Shigeno and Yamamoto, in preparation), the neuropils are formed in almost all brain lobes at the time of hatching. In the hatchlings of *T. pacificus*, neuropils are differentiating in the subesophageal mass (SBM) and the optic tract region (OTR), but are absent in the supraesophageal mass (SPM) except for the axonal bundle forming the transverse arch (TA). In Fig. 12, we compare the timing of neuropil differentiation among *T. pacificus*, *S. lessoniana* (Shigeno *et al.*, 2001c), and *O. vulgaris* (Marquis, 1989) according to the universal embryonic stages determined by Naef (1928) (Naef's stages are usually shown by Roman numerals). In the 3 species, regional differentiation of the brain proceeds in the same sequence:

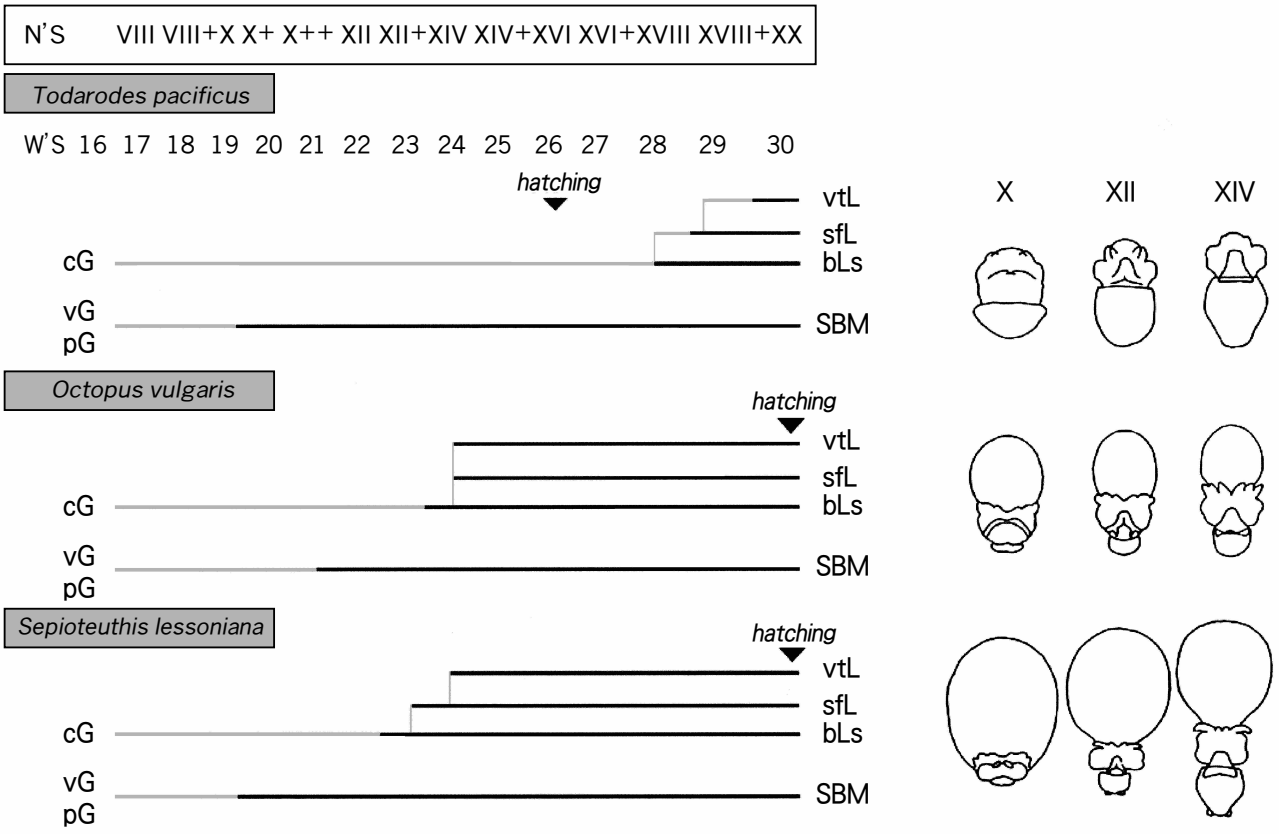

Fig. 12. Comparison of the timing of neuropil differentiation among *Todarodes pacificus*, *Octopus vulgaris* (Marquis, 1989), and *Sepioteuthis lessoniana* (Shigeno *et al.*, 2001b) on the basis of the Naef's universal developmental stages (N's) (Naef, 1928). Thick lines indicate presence of neuropils. WS are developmental stages by Watanabe *et al.* (1996) for *T. pacificus*. Rough drawings of embryos (ventral view) are shown in the right. bLs, basal lobes; cG, cerebral ganglia; pG. pedal ganglia; sfL, superior frontal lobe; SBM, subesophageal mass; vG, palliovisceral ganglia; vtL, vertical lobe.

Neuropils appear in order of the SBM, OTR, and SPM, development advances from posterior to anterior in the SBM, and differentiation of the basal lobes precedes that of the accessory lobes (e.g. superior frontal lobe, vertical lobe) in the SPM. In the 3 species, the first neuropil appears from stage X to X^{++} in the SBM. However, the time when neuropils first appear in the SPM is stage XVIII in *T. pacificus* in contrast to stage XII and XIV in *S. lessoniana* and *O. vulgaris*, respectively.

The post-embryonic development of the brain must be reflected in development of behaviors in the paralarva. Young (1974, 1976, 1977b, 1979) and Messenger (1979) have presumed the function of brain lobes in *Loligo*. The brain lobes in the SBM, which are presumed to be lower and intermedidate motor centers controlling the muscles in each body part (Young, 1976), are differentiating at the time of hatching in *T. pacificus*. The basal lobe group (anterior basal, dorsal basal, median basal, lateral basal, and interbasal lobes), which is presumed to be the higher motor center integrating the brain lobes in the SBM (Young, 1977b), begins to different in the SPM shortly after hatching. The accessory lobe group (vertical, subvertical, superior frontal and inferior frontal lobes), which is presumed to concern the visual and tactile memories and learning (Young, 1979), begins to develop last in the paralarva 1. The early paralarva 1 before formation of the basal lobes (stages 26 and 27) simply repeats vertical leaping using weak jet propulsion (O'Dor, *et al.*, 1986; Bower and Sakurai, 1996). The paralarva with nascent basal lobes (stage 28) begins to show a slight sign of integrated movements. They float in an oblique posture in a constant depth of the water column by regulated movements of the mantle and the funnel. However, they never show such elaborate swimming behaviors as rapid foreword movement, rapid course changes, searching for food, and intimidation to other individuals (Shigeno *et al.*, unpublished data). No data are available on the behavior of the paralarvae after loss of the inner yolk (paralarvae 2 and 3) and juveniles in the ommastrephid squids.

Adult cephalopods are all active predators, but early paralarvae of the ommastrephid squids are estimated to be suspension feeders on the basis of the presence of the cilia around the mouth (lip cilia) and a specific head withdrawal behavior (O'Dor, 1985; Shigeno *et al.*, 2001a). Capturing living prey consisting of a complex of behaviors such as search, pursuit, attack, and seizure (Hanlon and Messenger, 1996) requires highly coordinated movement of the funnel, mantle, arms, and tentacles, and may be possible after completion of the higher centers integrating lower motor centers. In the brain

of the paralarva 2 in *T. pacificus*, the anterior basal lobe remains relatively small and the accessory lobes are poorly developed. This feature suggests that the paralarvae 1 and 2 are suspension feeders without elaborate behaviors of swimming and feeding. The basal lobes and accessory lobes develop markedly in paralarva 3 in *T. pacificus*. The brain lobes of the hatchling in *Loligo* and *Sepia* (Frösch, 1971) have already reached the same degree of development as those of the paralarva 3 in *T. pacificus*. Since hatchlings of *Loligo* and *Sepia* show prey-capturing behaviors, the shift from the suspension feeding to the predatory seems to occur in the paralarva 3 in *T. pacificus*. Kier (1996) has stated that in *Sepioteuthis lessoniana*, start of the prey capturing can be estimated from the ultrastructure of tentacle muscle; the fast-contracting cross-striated muscles differentiate in correlation with commencement of rapid striking movement of the tentacle.

Changes in behaviors can be estimated from changes in relative volume among brain lobes. Nixon and Mangold (1996) have reported in *O. vulgaris* that the neuropils increase in volume in the tactile memory centers but decrease in the swimming centers at the time of settlement of the planktonic paralarvae. In *Sepia officinalis*, changes in relative volume among accessory lobes are related to emergence of predatory pursuits in the nekto-benthic young (Messenger, 1973, 1979; Dickel *et al*., 1997). In *Loligo*, the relative volume increases in the vertical lobe (Young, 1979) but decreases in the olfactory lobe (Messenger; 1979) during the period of the early juvenile. In *T. pacificus*, the lobes in the olfactory system (the olfactory and dorso-lateral lobes in the OTR) develop well in the paralarva 2, but decrease in relative volume in the paralarva 3. In contrast, the optic lobes of *T. pacificus* remain small in the paralarva 2, but they increase strikingly in volume and the layered organization of the neuropils is elaborated in the paralarva 3. This change of dominance between the olfactory and optic lobes also suggests the shift of feeding mode from olfaction-dependent to vision-dependent; the olfactory sense is important in suspension feeding but vision is indispensable for prey capturing.

The present paper with the preceding results (Shigeno *et al*., 2001b) provides morphological bases for future analyses of neural development at the level of axogenesis and gene expression. We found that *T. pacificus* could be a suitable material for the study of neurogenesis. It produces large numbers of small and transparent eggs enclosed by very loose egg jelly, and embryos are readily obtainable by artificial insemination. The histological and cytological observation of neural development is comparatively easy due to a smaller cell number and a smaller quantity of yolk than in the embryos of other model cephalopod species such as *Loligo*, *Sepia*, and *Octopus*. The characteristic delay in the formation of brain lobes to post-hatching stages provides an opportunity to examine relationships between differentiation of a certain lobe and onset of a certain behavior. However, at present no data are available on behaviors of paralarvae 2 and 3, and juveniles of *T. pacificus*. No one has succeeded in rearing the hatchlings obtained by artificial insemination until after disappearance of the inner yolk, or in maintaining field-collected paralarvae in the laboratory aquarium.

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