

Divergence in developmental patterns of larval and adult nervous system in direct-developing holothuria *Eupentacta fraudatrix* suggests their independent evolution

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ABSTRACT: The remarkable distinctions between the larval and adult nervous systems of echinoderms prompt inquiries into their potential developmental continuity and evolutionary origins. To address these questions, it is imperative to discern the conservative aspects of neurogenesis from its adaptive features related to the larval lifestyle. In this study, we explored the influence of direct development on the formation of both larval and adult nervous systems in *Eupentacta fraudatrix*, a species of holothurians with lecithotrophic larvae. Gastrula, vitellaria, and pentactula stages were analyzed via double labeling with antibodies targeting serotonin (5-HT) and acetylated α -tubulin (AcTub). First 5-HT-positive cells of the larval nervous system emerge in the ectoderm of the animal hemisphere during the gastrula stage. By the vitellaria stage, 5-HT-positive cells and fibers become concentrated in a band-like network surrounding the base of the preoral lobe. Notably, this network is exclusively superficial and lacks cell clusters resembling an apical organ. In the late vitellaria stage, AcTub-positive fibers become visible within this network. Upon settlement, 5-HT-positive elements disappear entirely, while some AcTub-positive fibers from the larval network persist in the basiepidermal regions of pentactula. Within the developing adult nervous system, AcTub staining reveals the formation of a circumoral nerve ring and a ventral cord at the vitellaria stage. Subsequently, five radial cords and a caudal nerve ring gradually emerge during settlement, leading to the establishment of a pentaradial nervous system in juveniles. No 5-HT-positive elements were detected among the rudiments of adult nervous system. Despite the coexistence of both larval and adult nervous systems for extended period during the vitellaria stage, no morphological connection between them was observed. Our findings revealed that the structure of the larval nervous system in *E. fraudatrix* exhibits significant differences from planktotrophic larvae of holothurians and shows a closer resemblance to the lecithotrophic larvae of other echinoderms. Meanwhile, the formation of the adult nervous elements in *E. fraudatrix* does not differ from that of holothurians with planktotrophic larvae. Thus, the lecithotrophic lifestyle appears to influence the structure of the larval nervous system, leaving the development of the adult nervous system unaffected. Consequently, these distinct nervous systems demonstrate disparate adaptive plasticity and likely evolved independently.

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KEY WORDS: larval nervous system, vitellaria, pentactula, direct development, nerve cords, holothurians, echinoderms.

Различия в развитии нервной системы личинки и взрослого у голотурии с прямым развитием *Eupentacta fraudatrix* свидетельствуют об их независимой эволюции

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РЕЗЮМЕ: Значительные различия между строением нервных систем личинки и взрослого иглокожего вызывают вопросы об их происхождении и возможной преемственности в процессе развития. Для изучения этих вопросов необходимо отличать консервативные аспекты нейрогенеза от его адаптаций к образу жизни личинки. В нашем исследовании мы оценили влияние прямого развития на нейрогенез иглокожих, исследуя формирование личиночной и взрослой нервной системы у *Eupentacta fraudatrix*, голотурии с лецитотрофной личинкой. Для этого мы провели двойное маркирование личинок антителами против серотонина (5-НТ) и ацетилированного альфа-тубулина (AcTub) на стадии гастролы, вителлярии и пентактулы. Первые 5-НТ-положительные клетки личиночной нервной системы появляются в эктодерме анимального полюса гастролы. К стадии вителлярии 5-НТ-положительные клетки и их отростки концентрируются в нервной сети, опоясывающей основание преоральной лопасти. Данная сеть имеет исключительно рассеянное расположение и не содержит скоплений клеток, напоминающих апикальный орган. На стадии поздней вителлярии в этой сети также появляются волокна, позитивно реагирующие на AcTub. У ювенильных особей 5-НТ-позитивные элементы полностью исчезают, в то время как некоторые AcTub-позитивные волокна из личиночной нервной сети сохраняются в базискожном эпидермальном районе пентактулы. В развивающейся взрослой нервной системе AcTub начинает выявляться в околоторовом нервном кольце и вентральном нервном стволе на стадии вителлярии. В процессе оседания у личинок появляются все пять радиальных стволов и каудальное нервное кольцо, что приводит к формированию пентарадиальной нервной системы у ювенильных особей. Внутри зачатка взрослой особи 5-НТ-положительных структур не обнаружено. Несмотря на то, что обе нервные системы присутствуют в вителлярии одновременно в течение большей части развития, морфологических связей между ними не обнаружено. Наши результаты показали, что личиночная нервная система *E. fraudatrix* сильно отличается от нервной системы планктотрофных личинок голотурий и имеет большое сходство с лецитотрофными личинками других иглокожих. При этом, формирование взрослой нервной системы *E. fraudatrix* остаётся таким же как у голотурий с планктотрофными личинками. Таким образом, лецитотрофный образ жизни влияет только на структуру личиночной нервной системы, и не отражается на развитии взрослой. Эти две нервные системы обладают различной адаптивной пластичностью и, по всей видимости, эволюционировали независимо.

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КЛЮЧЕВЫЕ СЛОВА: личиночная нервная система, вителляррия, пентактула, прямое развитие, нервные стволы, голотурии, иглокожие.

Introduction

Echinoderms encompass a diverse spectrum of marine organisms, showcasing various morphologies, larval forms and developmental strategies. This diversity extends to their nervous system architecture, characterized by the presence of two distinct parts: the adult nervous system exhibiting pentaradial symmetry and the larval nervous system displaying a bilateral structure (Chia *et al.*, 1978; Chee *et al.*, 1999; Beer *et al.*, 2001; Nakano *et al.*, 2006). The structure of these nervous systems raises inquiries regarding their development, mutual influences, and evolutionary origin. Resolving these issues requires distinguishing evolutionarily conservative aspects of neurogenesis in echinoderms from adaptations to developmental strategies. This underscores the necessity for a comprehensive examination of the development of both larval and adult nervous systems in echinoderms exhibiting diverse larval forms and lifestyles.

Echinoderms manifest a broad spectrum of larval forms and developmental strategies (Fig. 1). Their adults can develop either indirectly through a metamorphosis from a planktotrophic larva or directly through a gradual development of a lecithotrophic larva (Raff, 1987; Kaul-Strehlow, Röttinger, 2015). The planktotrophy is considered as ancestral developmental strategy (Raff, Byrne, 2006). The nervous system of the planktotrophic larva typically consists of one or two anterior ganglia that contain serotonergic neurons, as well as a network of neurons and nerve fibers associated with the ciliary bands (Chee, Byrne, 1999; Beer *et al.*, 2001; Lacalli, Kelly, 2002; Byrne *et al.*, 2007). This nervous system plays a role in regulating the feeding and swimming behavior (Wada *et al.*, 1997; Buznikov *et al.*, 2005). During metamorphosis, the larval nervous system undergoes regression, while the adult nervous system emerges within the forming adult rudiment (Chia, Burke, 1978; Nakano *et al.*, 2006). Throughout this transition, larval serotonin-positive structures disappear and no serotonin-positive elements were detected in the

pentaradial nervous system of juvenile and adult echinoderms (Nakano *et al.*, 2006; Díaz-Balzac, García-Arrarás, 2018; Formery *et al.*, 2021).

The alternative direct developmental strategy occurs without drastic metamorphosis and is also found in all classes of echinoderms (McEdward, Miner, 2001) (Fig. 1). Species with such type of development have large eggs with high yolk content and non-feeding larvae. Their lecithotrophic larvae display partial or complete reduction of ciliary bands and undergo non-feeding accelerated development (Emlet, 1994; Wray, 1996). Investigations of the nervous system development in lecithotrophic species could provide valuable insights into echinoderm neurogenesis. Currently, the larval nervous system has been investigated in a few lecithotrophic species among sea urchins, starfish, and ophiuroids (Bisgrove, Raff, 1989; Katow *et al.*, 2009; Elia *et al.*, 2009; Sweet *et al.*, 2019). Although the acquired data have elucidated differences from planktotrophic larvae, they do not provide details about developmental dynamics of specific nerve elements and possible relations between larval and adult nervous systems during direct development.

Holothuroidea is a class of echinoderms with a large number of species exhibiting direct development (Smiley *et al.*, 1991; McEdward, Miner, 2001) and well-studied adult pentaradial nervous system (Díaz-Balzac *et al.*, 2010, 2014, 2016; Mashanov *et al.*, 2006, 2007, 2009, 2015; Hoekstra *et al.*, 2012; Dolmatov *et al.*, 2018). Like other echinoderms, the adult nervous system of holothurians comprises a circumoral nerve ring, five radial nerve cords, peripheral nerves, and a basiepidermal nerve plexus. General principles of the adult nervous system formation have been studied in planktotrophic *Apostichopus japonicus* (Nakano *et al.*, 2006; Dolmatov *et al.*, 2018) and lecithotrophic *Eupentacta fraudatrix* (Mashanov *et al.*, 2007). The study of the larval nervous system in holothurians has been limited to species with planktotrophic larvae, which undergo indirect development through two larval stages known as auricularia and doliolaria (Burke *et*

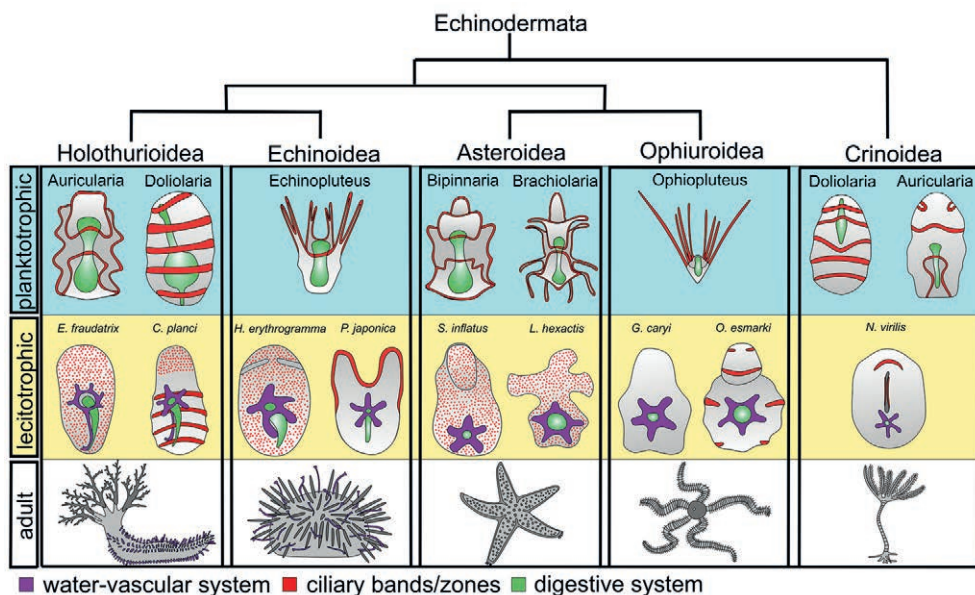


Fig. 1. Diversity of larval forms in echinoderms. Planktotrophic larvae highlighted in blue, lecithotrophic larvae are denoted by a yellow in all echinoderm classes. Lecithotrophic larvae exhibit reduced ciliary bands (cilia location indicates by red) and accelerated development (water-vascular system of the adult rudiment marked by purple). The representations of planktotrophic larvae are adapted from Ivanova-Kazas (1978) and Amemiya *et al.* (2015). The depictions of lecithotrophic larvae are based on the following references: *Eupentacta fraudatrix* (Dolmatov, Yushin, 1993), *Cucumaria planci* (Ivanova-Kazas, 1978), *Heliocidaris erythrogramma* (Emlet, 1995), *Peronella japonica* (Lijima *et al.*, 2009), *Stegnaster inflatus* (Zamora *et al.*, 2018), *Leptasteris hexactis* (Ivanova-Kazas, 1978), *Garganocephalus caryi* (Patent, 1970), *Ophioplocus esmarki* (Sweet *et al.*, 2019), *Notocrinus virilis* (Mortensen, 1920).

Рис. 1. Разнообразие личиночных форм иглокожих. В разных классах иглокожих планктотрофные личинки выделены голубым цветом, а лецитотрофные желтым. Лецитотрофные личинки отличаются редукцией ресничных поясов (расположение ресничек указано красным цветом) и ускоренным развитием (амбулакральная система зачатка взрослой особи обозначена фиолетовым). Изображения планктотрофных личинок сделаны на основе работ Ivanova-Kazas (1978) и Amemiya *et al.* (2015). Схемы лецитотрофных личинок сделаны на основе следующих источников: *Eupentacta fraudatrix* (Dolmatov, Yushin, 1993), *Cucumaria planci* (Ivanova-Kazas, 1978), *Heliocidaris erythrogramma* (Emlet, 1995), *Peronella japonica* (Lijima *et al.*, 2009), *Stegnaster inflatus* (Zamora *et al.*, 2018), *Leptasteris hexactis* (Ivanova-Kazas, 1978), *Garganocephalus caryi* (Patent, 1970), *Ophioplocus esmarki* (Sweet *et al.*, 2019), *Notocrinus virilis* (Mortensen, 1920).

al., 1986; Chen *et al.*, 1995; Byrne *et al.*, 2006; Nakano *et al.*, 2006; Bishop, Burke, 2007; Zheng *et al.*, 2022). The larval nervous system in planktotrophic larvae comprises neurons, distributed along the ciliary bands and within bilateral apical ganglia, and completely disappears during metamorphosis (Byrne *et al.*, 2006; Nakano *et al.*, 2006; Bishop, Burke, 2007). On the other hand, the larval nervous system in the holothurians with lecithotrophic larva remains unstudied. These species undergo accelerated development during which the adult nervous system forms very early (Mashanov *et al.*, 2007),

and develops in parallel with the larval nervous system. However, their spatial relationship and mutual influence have not been investigated, leading to significant gaps in our understanding of neurogenesis in lecithotrophic species.

In this study, we investigate the appearance of specific nerve elements in larvae and settled juvenile of *Eupentacta fraudatrix* Djakonov et Baranova, 1958 (Holothuroidea, Dendrochirotida), a holothurian species with direct developing lecithotrophic larva (Dolmatov, Yushin, 1993) with the emphasis of the relation between larval and adult nervous systems. The

objective of our research is to evaluate how lecithotrophic strategy affects structure of larval and adult nervous systems and their possible interactions. To visualize specific nerve elements, we utilized antibodies targeting specific neuronal markers: acetylated α -tubulin (AcTub) and serotonin (5-HT). The pan-neuronal marker AcTub was used to visualize both larval and adult nerve elements in echinoderms (Carter *et al.*, 2021; Formery *et al.*, 2021; Zeng *et al.*, 2022). 5-HT is widely distributed in the larval nervous system of echinoderms, particularly in the apical ganglia (Bisgrove, Raff, 1989; Chee, Byrne, 1999; Nakajima *et al.*, 2004), but completely absent in the developing nerve cords of the adult nervous system (Nakano *et al.*, 2006; Byrne *et al.*, 2006; Katow *et al.*, 2009; Formery *et al.*, 2021). This combination of antibodies allows the differentiation between larval and adult nervous structures. In our investigation, we identified distinct characteristics of the larval nervous system that we propose are linked to the lecithotrophic lifestyle. Meanwhile, the adult pentaradial nervous system has similar structure to other holothurians, thus remained unaffected by lecithotrophic development. No morphological connections were found between larval nervous elements and rudiment of adult nervous system. We speculate that these distinct nervous systems exhibit disparate adaptive plasticity and likely evolve independently.

Material and methods

Mature individuals of *E. fraudatrix* were collected in Peter the Great Bay (Sea of Japan, Vladivostok) in June 2023. The animals were placed in the aquarium where spontaneous spawning occurred at the beginning of July. Larvae were cultivated in glass vessels with filtered aerated seawater at a temperature of 15–17 °C. Animals were selected for fixation at gastrula, vitellaria and pentactula stages which were determined according to the time after spawning and morphological features according to Dolmatov & Yushin (1993) (Fig. 2).

Fixation was carried out using 4% paraformaldehyde in seawater for 2 hours at room temperature. After triple washing with 0,01M phosphate-buffered saline (PBS) (Sigma P4417, USA), the animals were dehydrated in ascending concentrations of alcohol, transferred to 100% methanol, and stored at –20 °C.

For whole mount immunohistochemical staining, fixed animals were rehydrated in descending concentrations of alcohol and washed in PBS. To permeabilize

the larval membranes, the larvae were incubated in 1% TritonX in PBS for 1–3 days. Then they were washed in PBS with 0.1% TritonX (PBST) and incubated in a blocking solution of 3% goat serum in PBST for 30 minutes. For double immunolabeling of nervous structures, rabbit antibodies against serotonin (5-HT) (Immunostar, 200800, USA) and mouse antibodies against acetylated α -tubulin (AcTub) (Sigma, T-6793, USA), diluted in PBST with 1% goat serum, were used. The negative control samples were incubated in PBST with 1% goat serum. After overnight incubation at 4 °C, the samples were washed three times in PBST. Then, the samples were incubated in diluted in PBST with 1% goat serum in different combinations of following secondary antibodies: goat anti-rabbit antibody, Alexa Fluor™ 488 (Thermo Fisher A-11008, USA), goat anti-rabbit antibody, Alexa Fluor™ 647 (Thermo Fisher A-21244, USA) and goat anti-mouse antibody, Alexa Fluor™ 546 (Thermo Fisher A-21422, USA). After the overnight incubation at 4 °C and washing with PBST, the samples were stained with DAPI (Thermo Fisher D21490, USA). Then the animals were transferred in glycerine for cleaning and mounted on glass slides. Samples were examined under confocal laser scanning microscope (Zeiss LSM 880, Carl Zeiss, Germany), the acquired serial confocal Z-stacks were processed using the ZEN (Carl Zeiss, Germany) and Fiji (Schindelin *et al.*, 2012) software programs. Images of negative control samples are available in Supplementary (Fig. S1 A–C).

Results

LARVAL NERVOUS STRUCTURES. Throughout the development of the larval nervous system, 5-HT-positive elements were observed at all investigated stages, while AcTub expression emerged only from the vitellaria stage onwards. In general, 5-HT-positive elements of the larval nervous system appear during the gastrula stage (Fig. 3A, B), concentrated in a band-like network by the vitellaria stage (Fig. 3C–E), and gradually diminish in settled pentactula (Fig. 3H, I). The surface of the gastrula and vitellaria is evenly covered with cilia, which are visualized by antibodies against AcTub (Fig. 3A–G). Surface cilia do not aggregate into ciliary bands or other ciliated structures and disappear in settled pentactula (Fig. 3I).

First 5-HT-positive cells emerge in the ectoderm of the animal hemisphere at the gastrula stage (Fig. 4A). These cells lack processes (Fig. 4B) and do not form any clusters at the animal pole (Fig. 4C–C’”). No cell bodies were found in the vegetal hemisphere (Fig. 4A, C–C’”). During

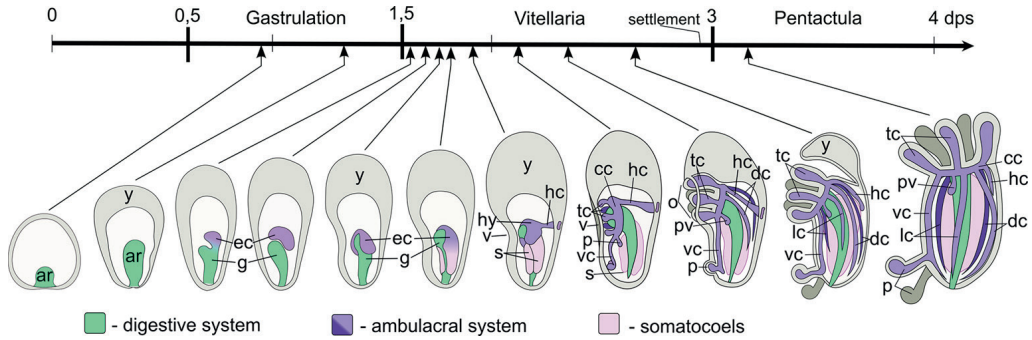


Fig. 2. Development of *Eupentacta fraudatrix*. The free-swimming lecithotrophic larva vitellaria forms at the late gastrula stage when archenteron divides into the gut and the enterocoel. On the ventral side of vitellaria appears the indentation, vestibulum, separating the larva into a preoral lobe, filled with yolk, and a zone of adult rudiment. Enterocoel spreads around the gut and separates into the hydrocoel and somatocoel, which give rise to the channels of the ambulacral system and the coelomic wall, respectively. The ambulacral system comprises five tentacle canals and a circular canal, from which five radial canals extend posteriorly. At the late vitellaria stage, the amount of yolk in the larva decreases, five primary tentacles emerge through the vestibulum opening, and two primary podia appear on the ventral surface. By the pentactula stage, the mouth breaks out at the bottom of vestibulum, pentactula settles and starts to feed using tentacles. The timeline and schematic images of the larvae are adapted from Dolmatov & Yushin (1993).

Abbreviations: ar — archenteron; cc — circular canal; dc — dorso-lateral canal; dps — days post spawning; ec — enterocoel; g — gut; hc — hydropore canal; hy — hydrocoel; lc — ventro-lateral canal, o — vestibulum opening; p — podia; pv — polian vesicle; s — somatocoel; t — tentacle; tc — tentacle canal; v — vestibulum; vc — mid-ventral canal; y — yolk.

Рис. 2. Развитие *Eupentacta fraudatrix*. Свободно плавающая лецитотрофная личинка вителлария формируется на стадии поздней гастролы, когда архентерон разделяется на зачаток кишки и энтероцель. На вентральной поверхности вителларии появляется углубление, вестибурум, разделяющее личинку на предротную лопасть, заполненную желтком, и зону формирующейся взрослой особи. Энтероцель окружает кишку и разделяется на гидроцель и соматоцель, которые дают начало каналам амбулакральной системы и выстилке целома соответственно. Амбулакральная система состоит из пяти каналов щупалец и кругового канала, от которого отходят назад пять радиальных каналов. На стадии поздней вителларии количество желтка в личинке уменьшается, пять первичных щупалец выходят на поверхность через отверстие вестибулула, а на брюшной стороне появляются две первичные ножки. На стадии пентактулы на дне вестибулула прорывается рот, пентактула оседает и начинает питаться с помощью щупалец. Временная шкала и схематические изображения личинок адаптированы из Dolmatov & Yushin (1993).

Обозначения: ar — архентерон; cc — кольцевой канал; dc — дорсолатеральный канал; dps — дни после нереста; ec — энтероцель; g — кишка; hc — канал гидropоры; hy — гидроцель; lc — вентролатеральный канал; o — отверстие вестибулула; p — первичная ножка; pv — полиев пузырь; s — соматоцель; t — щупальце; tc — канал щупальца; v — вестибурум; vc — вентральный канал; y — желток.

the preoral lobe; F–H — maximum-intensity Z-projections of the late vitellaria stage, 5-HT-positive network splits into separate clusters (F, G) and gradually disappears (H); I — maximum-intensity Z-projection of the settled pentactula, remnants of the larval 5-HT-positive structures are indicated by arrowheads.

Abbreviations: ad — adult rudiment; an — animal pole; hc — hydropore canal; nr — circumoral nerve ring; o — oral opening; p — podia; pl — preoral lobe; rc — radial canal; t — tentacle; tc — tentacle canal; veg — vegetal pole; v — vestibulum. Axes directions: a — anterior; p — posterior; v — ventral; d — dorsal; R — right; L — left. Scale bars: 50 μ m.

Рис. 3. Развитие личиночной 5-HT-иммунопозитивной нервной системы *Eupentacta fraudatrix*. A, B — максимальные проекции гастролы, первые 5-HT-иммунопозитивные клетки появляются в анимальном полушарии; C–E — Z-проекции вителларии, 5-HT-иммунопозитивная сеть формируется вокруг основания преоральной лопасти; F–H — Z-проекции поздней вителларии, 5-HT-иммунопозитивная сеть распадается на отдельные кластеры (F, G) и постепенно исчезает (H); I — Z-проекция осевшей пентактулы, остатки 5-HT-иммунопозитивной личиночной нервной системы указаны стрелками.

Сокращения: ad — зачаток взрослой особи; an — анимальный полюс; hc — канал гидropоры; nr — околоротовое нервное кольцо; o — ротовое отверстие; p — первичная ножка; pl — преоральная лопасть; rc — радиальный канал; t — щупальце; tc — канал щупальца; veg — вегетативный полюс; v — вестибурум. Направления осей: a — передний; p — задний; v — вентральный; d — дорсальный; R — правый; L — левый. Масштаб: 50 мкм.

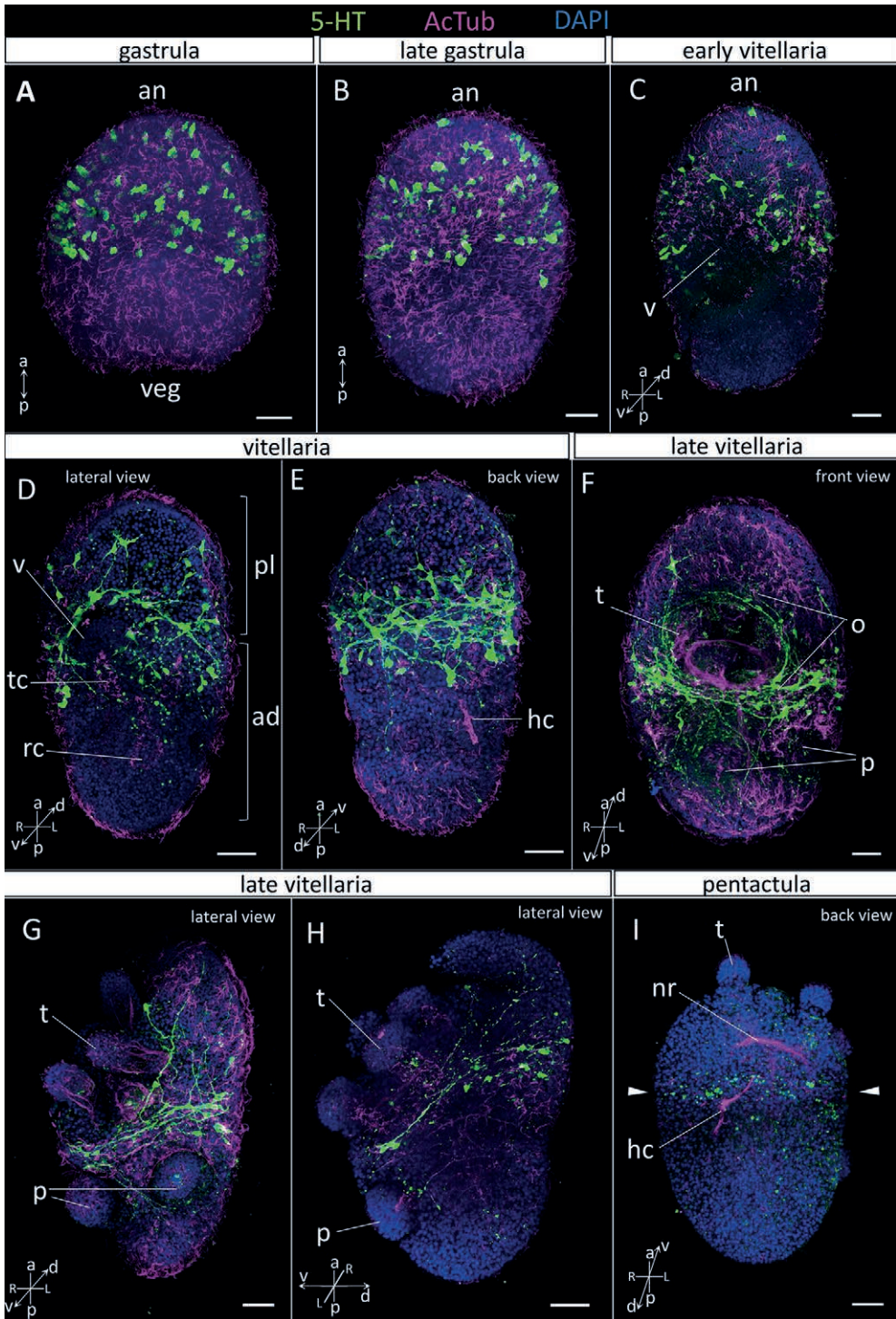


Fig. 3. Development of the larval 5-HT-positive nervous system of *Eupentacta fraudatrix*. A, B — maximum-intensity Z-projections of the gastrula stage, first 5-HT-positive cells appear in the animal hemisphere; C–E — maximum-intensity Z-projections of the vitellaria stage, 5-HT-positive band-like network forms at the base of

the late gastrula stage, 5-HT-positive cells extend processes that combine into a network with the emerging multipolar neurons (Fig. 4D, E, F). From this stage 5-HT-positive structures start to converge in the equatorial area at the level of the base of the preoral lobe (Fig. 4D, F–F’). No difference was observed in ciliary coverage between this area and other regions of the larval surface (Fig. 4D, F).

By the vitellaria stage, 5-HT-positive neurons and their processes form a dense band-like network surrounding the base of preoral lobe (Fig. 5A–C). 5-HT-positive neurons are dispersed throughout this network and do not form distinct ganglia. Thin 5-HT-positive fibers extend to the vegetal half of the larva (arrowheads in Fig. 5C), but no 5-HT-positive cell bodies are present in this region. At this stage, AcTub appear in some 5-HT-positive fibers of larval nervous network (Fig. 5D). Within this network, bipolar 5-HT-positive cells extend to the surface and bear cilia (arrowheads in Fig. 5E), while multipolar 5-HT-positive cells are situated beneath the ectoderm (Fig. 5E, F). All 5-HT-positive structures are exclusively superficially located and are not found in the forming adult rudiment (Fig. 5G). Occasionally, small 5-HT-positive granules were observed near the anterior region of the adult

rudiment in certain optical sections (Fig. 5G). These spherical structures lack nuclei (Fig. 5G’) and are absent in the negative control samples (Supplementary, Fig. S1 C). Projections through this area reveal their location at the base of the vestibulum (Fig. 5H–H’’).

During the late vitellaria stage, the primary tentacles of the adult rudiment start to extend through the larval oral opening (Fig. 6A–A’’). 5-HT-positive larval network breaks into several clusters, positioned below the oral opening and on the larva’s dorsal side (Fig. 6A). 5-HT-positive projections connect these clusters and encircle the oral opening, as well as the base of the primary podia. All 5-HT-positive cells and fibers are located either in the ectoderm or immediately below it (Fig. 6A’), while AcTub is present in the adult nerve structures within the larva (Fig. 6A’), as well as in the larval nervous network on the surface (arrowheads on Fig. 6A’). Some of these fibers contain both 5-HT and AcTub, while others display only AcTub-positive staining (Fig. 6B). Single fibers containing only AcTub penetrate deeper into the body wall (arrowheads in Fig. 6C–C’’).

During settlement, the 5-HT-positive nervous structures begin to degrade. Throughout this process, 5-HT is preserved only in small granules

cells do not aggregate into clusters; D — maximal projection of the late gastrula, 5-HT-positive cells start gathering around the base of the preoral lobe; E — transverse section of the ectoderm, 5-HT-positive cells emanate processes, arrowheads indicate the multipolar neurons; F–F’’ — section through the late gastrula, with a longitudinal projection through the whole larva (F’), showing localization of 5-HT-positive cells only in the animal half, and transverse projection through the base of the preoral lobe (F’’), which demonstrate the uniform distribution of 5-HT-positive cells in this zone, dotted lines indicate the areas of projections.

Abbreviations: ad — adult rudiment; an — animal pole; ar — archenteron; ec — enterocoel; g — gut; pl — preoral lobe; veg — vegetal pole; v — vestibulum. Axes directions: a — anterior; p — posterior; v — ventral; d — dorsal; R — right; L — left. Scale bars: 50 μ m.

Рис. 4. 5-HT-иммунопозитивная личиночная нервная система на стадии гастролы *Eupentacta fraudatrix*. А — Z-проекция гастролы, первые 5-HT-иммунопозитивные клетки распределены по эктодерме анимального конца; В — поперечный срез эктодермы гастролы, 5-HT-иммунопозитивные клетки не имеют отростков; С–С’’ — оптический срез гастролы, с поперечной (С’’) и продольной (С’) проекциями, демонстрирующие наличие 5-HT-иммунопозитивных клеток только в анимальном полушарии, пунктирные линии указывают области гастролы, вошедшие в проекции; С’’’ — Z-проекция вершины гастролы, вид с анимального полюса. 5-HT-иммунопозитивные клетки не образуют скоплений; D — Z-проекция поздней гастролы, 5-HT-иммунопозитивные клетки начинают собираться вокруг основания преоральной лопасти; E — срез стенки гастролы, отростки 5-HT-иммунопозитивных клеток появляются под эктодермой, стрелки указывают на мультиполярные нейроны; F–F’’ — оптический срез поздней гастролы, с продольной проекцией через всю личинку (F’), показывающей локализацию 5-HT-иммунопозитивных клеток в ее анимальной половине, и поперечной проекцией через основание преоральной лопасти (F’’), которая демонстрирует равномерное распределение 5-HT-иммунопозитивных клеток в этой зоне. Пунктирные линии указывают области личинки, вошедшие в проекции.

Сокращения: ad — зачаток взрослой особи; an — анимальный полюс; ar — архентерон; ec — энтероцель; g — кишка; pl — преоральная лопасть; veg — вегетативный полюс; v — вестибулум. Направления осей: а — передний; р — задний; v — вентральный; d — дорсальный; R — правый, L — левый. Масштаб: 50 мкм.

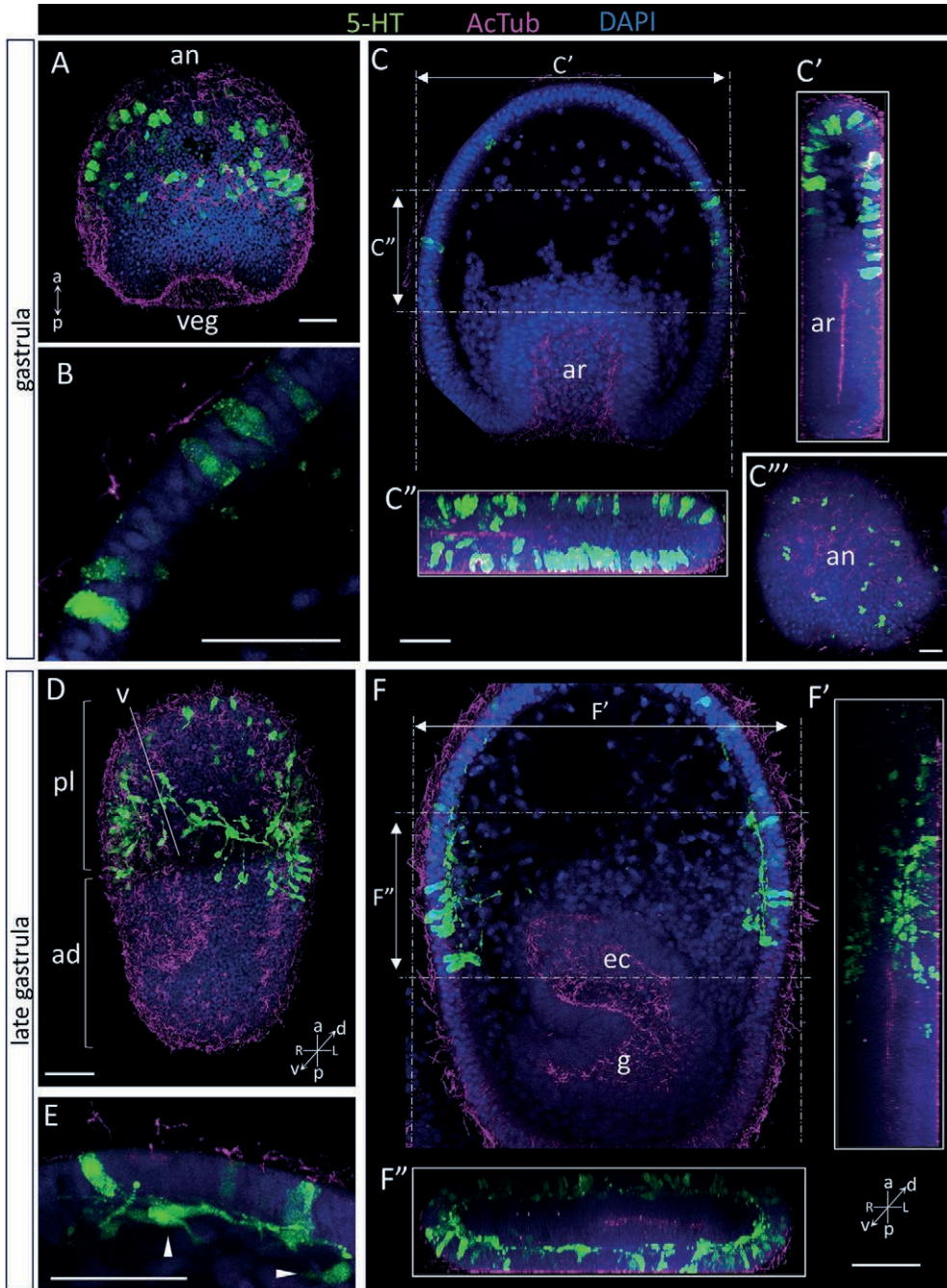


Fig. 4. 5-HT-positive larval elements at the gastrula stage of *Eupentacta fraudatrix*. A — maximum-intensity Z-projection of the gastrula, the first 5-HT-positive cells dispersed throughout the ectoderm of the animal hemisphere; B — section through the ectoderm of the gastrula, 5-HT-positive cells have no processes; C–C'' — section through the gastrula with transverse (C'') and longitudinal (C') projections showing the distribution of 5-HT-positive cells in the animal hemisphere only, dotted lines indicate the areas of projections. C''' — maximum projection of the top of the gastrula, viewed from the animal pole. 5-HT-positive

located in the area previously occupied by the larval nervous system (Fig. 6D, E). At the same time, the larval AcTub-positive network remains in this area (Fig. 6E). In settled juveniles, 5-HT-positive elements are no longer detected, but larval AcTub-positive fibers remain visible on the surface (arrowheads in Fig. 6F).

ADULT NERVOUS SYSTEM. The adult nervous system of *E. fraudatrix* completely lacks 5-HT-positive elements, but is effectively revealed by the antibody against AcTub. This marker also visualizes cilia of luminal epithelium of the water-vascular canals, making it useful for studying their development as well.

In the early vitellaria stage, AcTub is exclusively present in the cilia within the rudiments of the tentacular and radial water-vascular canals, as well as in the hydrophore canal (Fig. 7A). As the mid-ventral and dorso-lateral radial canals extend to the caudal part of the larva, a circumoral nerve ring becomes visible under the base of the tentacles (Fig. 7B, C). Nerve ring consists of a cluster of thin AcTub-positive fibers surrounding the gut and extending into the walls of the tentacles (Fig. 7C). Additionally, a mid-ventral nerve cord becomes apparent along the mid-

ventral water-vascular canal (Fig. 7C, D). This cord comprises numerous fibers originating from the circumoral ring and terminates as a brush of ramified fibers at the base of the primary podia.

During the late vitellaria stage, all five nerve cords become visible. Two dorso-lateral nerve cords emerge alongside the dorso-lateral canals (Fig. 7E). At the level of the ventro-lateral canals, small rudiments of ventro-lateral nerve cords extend from the circumoral nerve ring (Fig. 7E). The mid-ventral nerve cord exhibits increased thickness and extends farther towards the caudal region in comparison to other developing nerve cords.

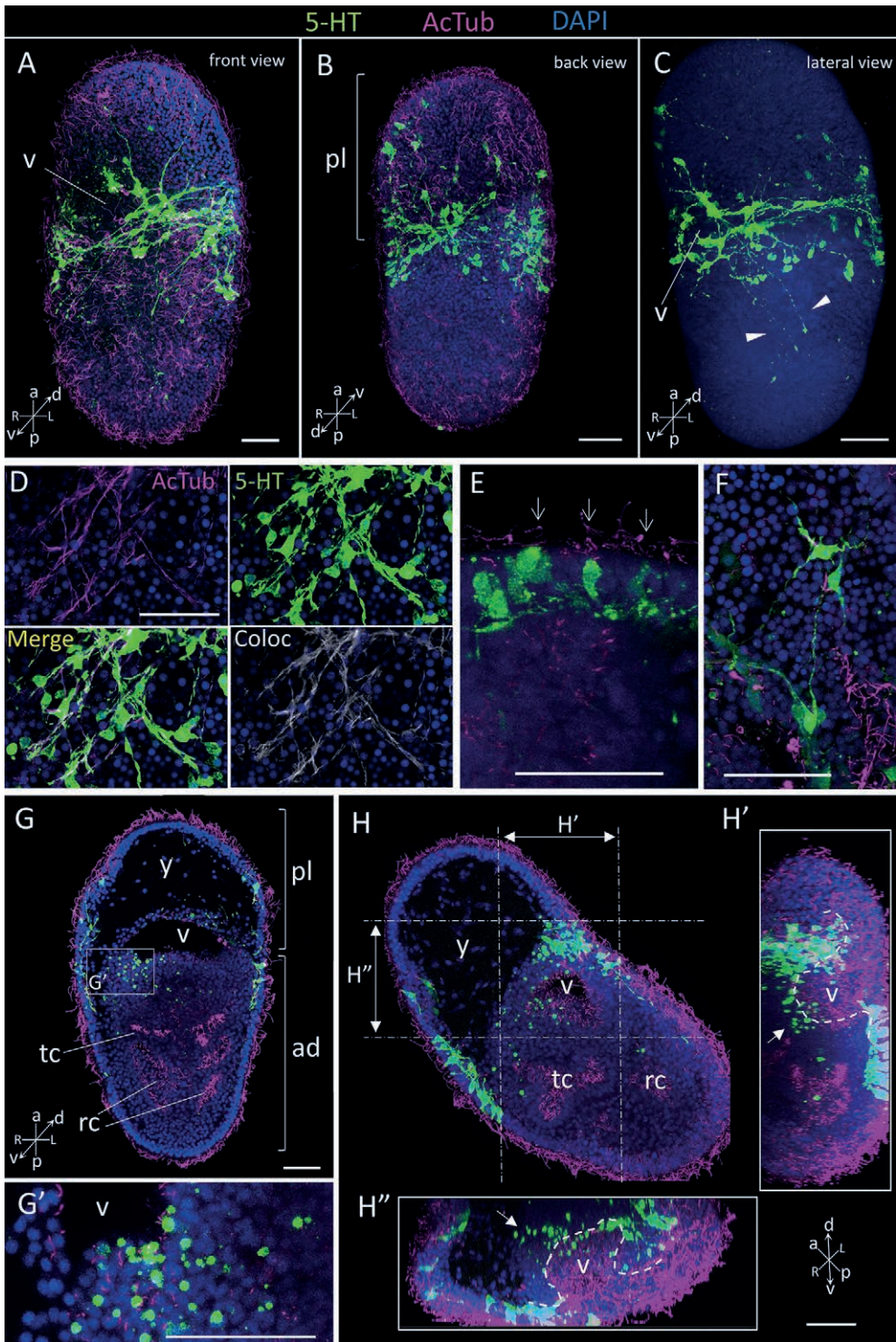
In the settled pentactula, all five radial nerve cords reach the caudal region (Fig. 7F, G). The mid-ventral cord gives some fibers into the primary podia and run towards the anal opening (Fig. 7G, H). Paired dorso-lateral nerve cords joint the mid-ventral nerve cord and their fibers form a caudal nerve ring around the anal opening (Fig. 7H). Later, the fibers of the paired ventro-lateral nerve cords merge into the caudal ring (Fig. 7I). Thus, the juvenile nervous system consists of circumoral and caudal nerve rings, and five radial nerve cords connecting these two rings.

Fig. 5. Larval nervous elements at the vitellaria stage of *Eupentacta fraudatrix*. A–C — maximum-intensity Z-projections of the vitellaria stage, demonstrating 5-HT-positive band-like network at the base of the preoral lobe, arrowheads in C indicate thin nerve endings in the vegetal part of the larva; D — frontal section of the larval nervous network displays both 5-HT- and AcTub-positive nerve elements. Regions containing both markers are depicted in the colocalization channel (Coloc); E — cross-section through the ectoderm, open arrows indicate 5-HT-positive cells with cilia; F — frontal section of preoral lobe with 5-HT-positive multipolar neurons under the ectoderm; G — frontal section through the vitellaria, 5-HT-positive cells are absent inside the adult rudiment. G' — enlarged image of G with 5-HT-positive granular structures; H–H'' — section of the vitellaria, with a longitudinal (H') and transverse (H'') projections through the vestibulum (circled by a dotted curve on projections). Solid arrows indicate 5-HT-positive granular structures in the bottom of vestibulum, dotted lines indicate the areas of projections.

Abbreviations: ad — adult rudiment; pl — preoral lobe; tc — tentacle canal; rc — radial canal; v — vestibulum. Axes directions: a — anterior; p — posterior; v — ventral; d — dorsal; R — right; L — left. Scale bars: 50 μ m.

Рис. 5. Элементы личиночной нервной системы у вителлярии *Eupentacta fraudatrix*. А–С — Z-проекции вителлярии, демонстрирующие 5-HT-иммунопозитивную нервную сеть, вокруг основания преоральной лопасти, стрелки на С указывают на тонкие нервные окончания в нижней половине личинки; D — фронтальный срез личиночной нервной сети демонстрирует как 5-HT-, так и AcTub-иммунопозитивные нервные элементы. Области, содержащие оба маркера, отображены в колокализационном канале (Coloc); E — поперечный срез эктодермы, стрелки указывают на 5-HT-иммунопозитивные клетки с ресничкам; F — фронтальный срез преоральной лопасти с мультиполярными нейронами под эктодермой; G — фронтальный срез вителлярии, 5-HT-позитивные клетки отсутствуют внутри зачатка взрослой особи; G' — увеличенное изображение G с 5-HT-иммунопозитивными гранулярными структурами. H–H'' — срез вителлярии с продольной (H') и поперечной (H'') проекциями на уровне вестибулума (обведен пунктирными кривыми на проекциях). Стрелки указывают на 5-HT-иммунопозитивные структуры на дне вестибулума, пунктирные линии обозначают области, вошедшие в проекции.

Сокращения: ad — зачаток взрослой особи; pl — преоральная лопасть; tc — канал щупальца; rc — радиальный канал; v — вестибулум. Направление осей: а — передний; р — задний; v — вентральный; d — дорсальный; R — правый; L — левый. Масштаб: 50 мкм.



Among these radial nerve cords, the mid-ventral cord remains notably larger than the paired ventro-lateral and dorso-lateral cords (Fig. 7I).

Discussion

Features of larval nervous system development

We have provided the first description of 5-HT- and AcTub-positive nerve elements in a holothurian species with lecithotrophic larva. Our findings unveil significant differences in the structure of the 5-HT-positive nervous system between *E. fraudatrix* and planktotrophic larvae of holothurians.

Unique distribution of first 5-HT-positive cells. The initial localization of first 5-HT-positive cells varies in different echinoderms classes. In planktotrophic larvae of sea urchins and holothurians, 5-HT-positive cells emerge directly at the sites where ganglia and nerve tracts will form, specifically at the apical pole and along the ciliary bands (Nakajima *et al.*, 2004; Garner *et al.*, 2016; Nakano *et al.*, 2006; Bishop, Burke, 2007; Zeng *et al.*, 2022). In sea stars, 5-HT-positive cells are evenly scattered throughout gastrula ectoderm and then migrate towards the ciliary bands (Chee, Byrne, 1999;

Nakajima *et al.*, 2004; Yankura *et al.*, 2013). In *E. fraudatrix*, the first 5-HT-positive cells appear exclusively in the animal hemisphere of the gastrula and then begin to cluster around the base of the preoral lobe (Fig. 8A). It is unclear whether these cells migrate in this region or are displaced due to larval growth. Therefore, the distribution pattern of early 5-HT-positive elements in *E. fraudatrix* differs from that observed in all described echinoderms.

Absence of a distinct apical organ at the animal pole. In planktotrophic echinoderm larvae, the most prominent larval nervous structure — the apical organ — consists of sensory 5-HT-positive neurons which are typically concentrated at the anterior pole (Chia, Burke 1978; Byrne *et al.*, 2007). The structure of the apical organ can vary: in sea urchins, it is a distinct ganglion with a noticeable neuropil (Beer *et al.*, 2001; Nakajima *et al.*, 2004), while in sea stars and holothurians, it consists of multiple clusters associated with the lateral loops of the preoral ciliary band (Lacalli, Kelly, 2002; Nakano *et al.*, 2006; Byrne *et al.*, 2006; Bishop, Burke, 2007). The majority of 5-HT-positive neurons in the planktotrophic holothurian larva *auricularia* are located in the apical ganglia, where it comprises 6–22 cells (Byrne *et al.*, 2006). On the contrary, at the apical pole of *E. fraudatrix*, there are no distinct clusters of 5-HT-positive neurons; instead, they

larval nervous network; F — surface projection of pentactula, arrowheads indicate AcTub-positive fibers in the region where 5-HT-positive nervous system was located.

Abbreviations: c — cilia; dn — dorso-lateral nerve cord; nr — circumoral nerve ring; o — oral opening; p — podia; bnp — basiepidermal nerve plexus; tn — tentacle nerve; vn — mid-ventral nerve cord. Axes directions: a — anterior; p — posterior; v — ventral; d — dorsal; R — right; L — left. Scale bars: 50 μ m.

Рис. 6. Постепенное исчезновение 5-HT-иммунопозитивных нервных элементов в течение позднего развития *Eupentacta fraudatrix*. А — срез через поверхность поздней вителлярии, 5-HT-иммунопозитивная нервная сеть разбивается на несколько кластеров; А' — срез через середину поздней вителлярии, 5-HT-иммунопозитивные структуры расположены поверхностно, в то время как AcTub присутствует в нервных стволах внутри зачатка взрослой особи. А'' — срез поверхности поздней вителлярии, AcTub выявляется в волокнах личиночной нервной системы (указан стрелками); В — увеличенное изображение кластера нейронов из А, несеротонергические AcTub-иммунопозитивные волокна появляются в личиночной нервной системе, зоны содержащие оба маркера представлены в отдельном канале (Coloc); С–С'' — увеличенное изображение из А', стрелки показывают тонкие AcTub-иммунопозитивные волокна, идущие из базиепидермального сплетения в нижележащие ткани; D — исчезновение 5-HT-иммунопозитивной нервной системы на стадии поздней вителлярии; E — увеличенное изображение из D, остатки 5-HT-иммунопозитивных элементов расположены рядом с AcTub-иммунопозитивными волокнами, которые сохраняют морфологию нервной сети личинки; F — поверхностная проекция пентактулы, стрелки указывают на AcTub-иммунопозитивные волокна в районе, где располагалась 5-HT-иммунопозитивная нервная система.

Сокращения: с — реснички; dn — дорсалатеральный нервный ствол; nr — околоротовое нервное кольцо; o — ротовое отверстие; p — первичная ножка; bnp — базиепидермальное нервное сплетение; tn — нерв щупальца; vn — вентральный нервный ствол. Направления осей: а — передний; p — задний; v — вентральный; d — дорсальный; R — правый; L — левый. Масштаб: 50 мкм.

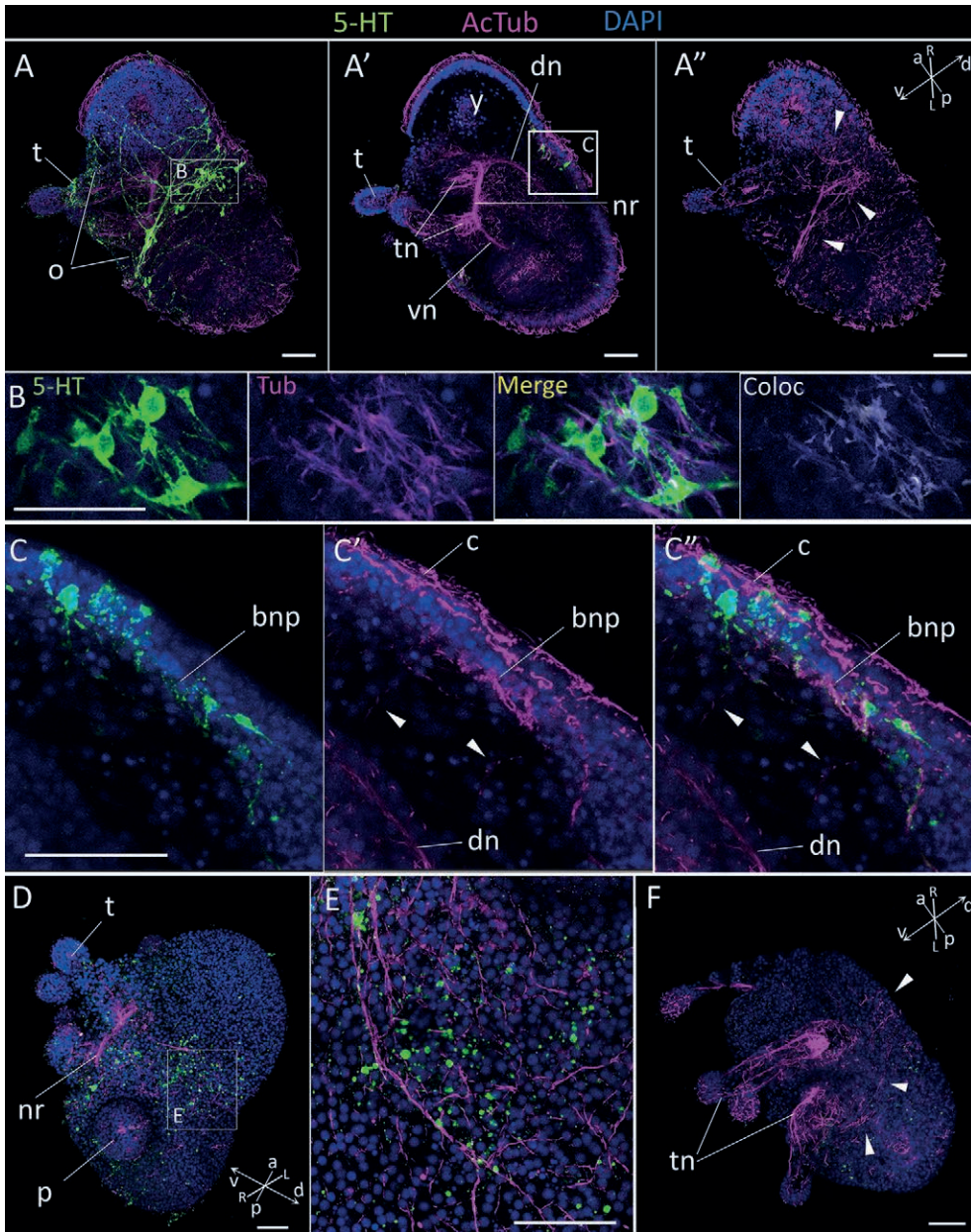


Fig. 6. Gradual disappearance of 5-HT-positive elements in the course of late development of *Eupentacta fraudatrix*. A — surface section of the late vitellaria, larval 5-HT-positive band-like network splits into separate clusters. A' — mid-section of the late vitellaria, 5-HT-positive structures are located superficially, while AcTub is present in nerve cords inside the adult rudiment. A'' — surface section of the late vitellaria, AcTub-positive fibers in the larval nervous system (arrowheads); B — enlarged image of cell cluster from A, AcTub-positive non-serotonergic fibers appear in the larval nervous system, colocalization channel (Coloc) displays the areas containing both markers; C–C'' — enlarged image from A', arrowheads indicate thin AcTub-positive fibers extending from the basiepidermal nerve plexus into the underlying tissues; D — disappearance of 5-HT-positive elements at the late vitellaria stage; E — enlarged image from D, the remnants of 5-HT-positive elements are located alongside AcTub-positive fibers, which retain the morphology of the

are abundant in the nervous network at the base of the preoral lobe (Fig. 8A, B). Consequently, without specific markers, it becomes challenging to determine whether the apical organ is a component of this band-like network, is entirely absent, or if this entire network is an enlarged and displaced apical ganglion.

Absence of the nerve cords, associated with ciliary bands. In planktotrophic larvae of holothurians, the larval nervous elements are organized into nerve cords, which are closely associated with the ciliary bands (Burke *et al.*, 1986; Chen *et al.*, 1995; Nakano *et al.*, 2006; Byrne *et al.*, 2006; Bishop, Burke, 2007; Zheng *et al.*, 2022). The architecture of the larval nervous system of *E. fraudatrix* vitellaria does not resemble the nerve cords of planktotrophic auricularia and doliolaria (Fig. 8B). Furthermore, there are no ciliary bands on the surface, and the whole larval body is uniformly covered with cilia. In echinoderm larvae the reduction of ciliary bands is associated with the evolutionary shift to non-feeding development (Emler, 1994; Wray, 1996). In lecithotrophic holothurian larvae, the extent of this reduction can vary; some larvae are uniformly ciliated, while others

develop 2–5 ciliary bands (Dolmatov, Yushin, 1993; McEdward, Miner, 2001). Information regarding the structure of the nervous system in these species is lacking. Thus, it is unclear how variations in ciliary bands numbers influence the structure of the larval nervous system and what relationships exist between ciliary cells and larval neurogenesis.

Similarity of larval nervous systems in lecithotrophic larvae across different classes of echinoderms. 5-HT-positive nervous system of *E. fraudatrix* show significant similarities to lecithotrophic larvae of other echinoderm classes. For example, in sea urchins that undergo direct development, such as *Heliocidaris erythrogramma* and *Holopneustes purpureescens*, the first 5-HT-positive neurons emerge at the animal pole, similar to urchins with planktotrophic larva (Bisgrove, Raff, 1989; Katow *et al.*, 2009). However, during development, 5-HT-positive neurons disappear at the animal pole and form a network that encircles the larva at the level of the vestibulum. The structure of this network is very similar to what we found in *E. fraudatrix* (Fig. 8B). In the vitellaria of direct-developing ophiuroid *Ophioplocus esmarki* larval nervous

nerve cords reach the posterior region, note the absence of 5-HT-positive elements; G — maximum-intensity Z-projection of pentactula, mid-ventral and dorso-lateral nerve cords connect in the caudal region (arrow); H, I — maximum-intensity Z-projection of settled one month pentactula, the mid-ventral nerve cord is thicker than paired ventro-lateral and dorso-lateral cords, caudal nerve ring appear near anal opening. 5-HT-positive elements are absent.

Abbreviations: dc — dorso-lateral canal; dn — dorso-lateral nerve cord; hc — hydropore canal; lc — ventro-lateral canal; ln — ventro-lateral nerve cord; nr — circumoral nerve ring; p — podia; pc — podia canal; pn — podia nerve; tc — tentacle canal; tn — tentacle nerve; vc — mid-ventral canal; vn — mid-ventral nerve cord; y — yolk. Axes directions: a — anterior; p — posterior; v — ventral; d — dorsal, R — right; L — left. Scale bars: 50 μ m.

Рис. 7. Формирование взрослой нервной системы *Eupentacta fraudatrix*. Стрелки указывают на расположенные исключительно поверхностно 5-HT-иммунопозитивные элементы. А — медиальный срез ранней вителлярии, AcTub выявляется только в ресничках внутри каналов амбулакральной системы (обведена пунктирной линией); В — медиальный срез вителлярии, AcTub-иммунопозитивные нервные волокна появляются в околоротовом нервном кольце и нервах щупалец, амбулакральная система обведена пунктирной линией; С, D — медиальные срезы вителлярии, AcTub-иммунопозитивные волокна появляются в вентральном нервном стволе, формирующемся вдоль вентрального амбулакрального канала; Е — медиальный срез поздней вителлярии, AcTub-иммунопозитивные волокна появляются в зачатках дорсо-латеральных и вентро-латеральных нервных стволов. F — Z-проекция ранней пентакуты, все AcTub-иммунопозитивные радиальные нервные стволы достигают каудального района; G — Z-проекция пентакуты, волокна вентрального и дорсо-латерального нервных стволов контактируют в каудальном районе (указано стрелкой); H, I — максимальная проекция одномесечной пентакуты, вентральный нервный ствол толще, чем парные вентро-латеральные и дорсо-латеральные стволы, каудальное нервное кольцо появляется около анального отверстия. 5-HT-иммунопозитивные элементы полностью отсутствуют.

Сокращения: dc — дорсолатеральный канал; dn — дорсолатеральный нервный ствол; hc — канал гидropоры; lc — вентролатеральный канал; ln — вентролатеральный нервный ствол; nr — околоротовое нервное кольцо; p — первичная ножка; pc — канал первичной ножки; pn — нерв первичной ножки; tc — канал щупальца; tn — нерв щупальца; vc — вентральный канал; vn — вентральный нервный ствол; y — желток. Направления осей: а — передний; p — задний; v — вентральный; d — дорсальный; R — правый; L — левый. Масштаб: 50 мкм.

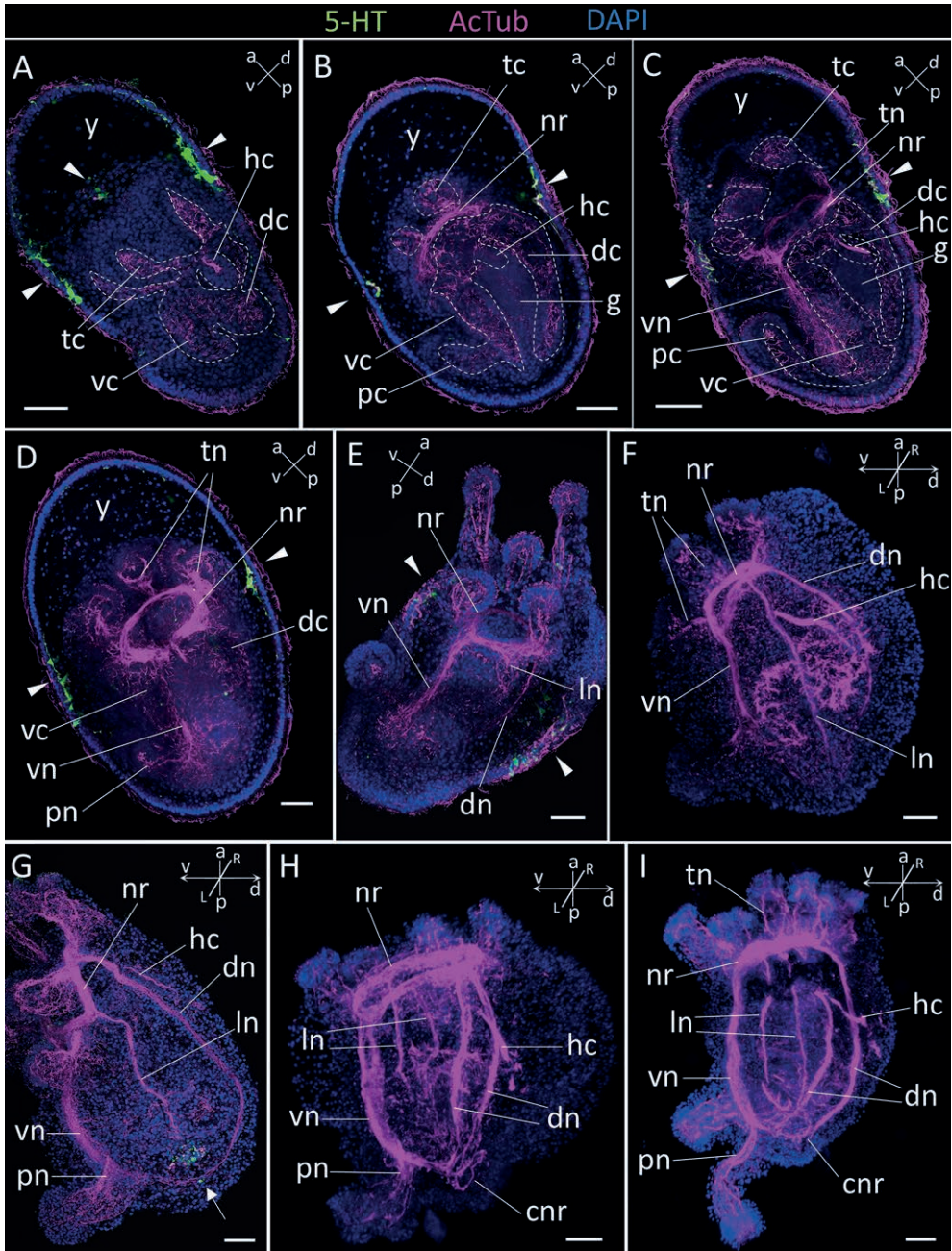


Fig. 7. Formation of the adult nervous system of *Eupentacta fraudatrix*. Arrowheads indicate an exceptionally superficial arrangement of 5-HT-positive elements. A — mid-section of the early vitellaria, AcTub is present in cilia within the water-vascular system canals only (outlined by dotted line); B — mid-section of the vitellaria, AcTub-positive fibers form the circumoral nerve ring and the nerves of the tentacles, canals of water-vascular system outlined by dotted line; C, D — mid-sections of the vitellaria, AcTub-positive fibers appear in the mid-ventral nerve cord, which forms along the mid-ventral water-vascular canal; E — mid-section of late the vitellaria stage, AcTub-positive fibers mark the rudiments of dorso-lateral and ventro-lateral nerve cords; F — maximum-intensity Z-projection of the early pentactula stage, all AcTub-positive radial

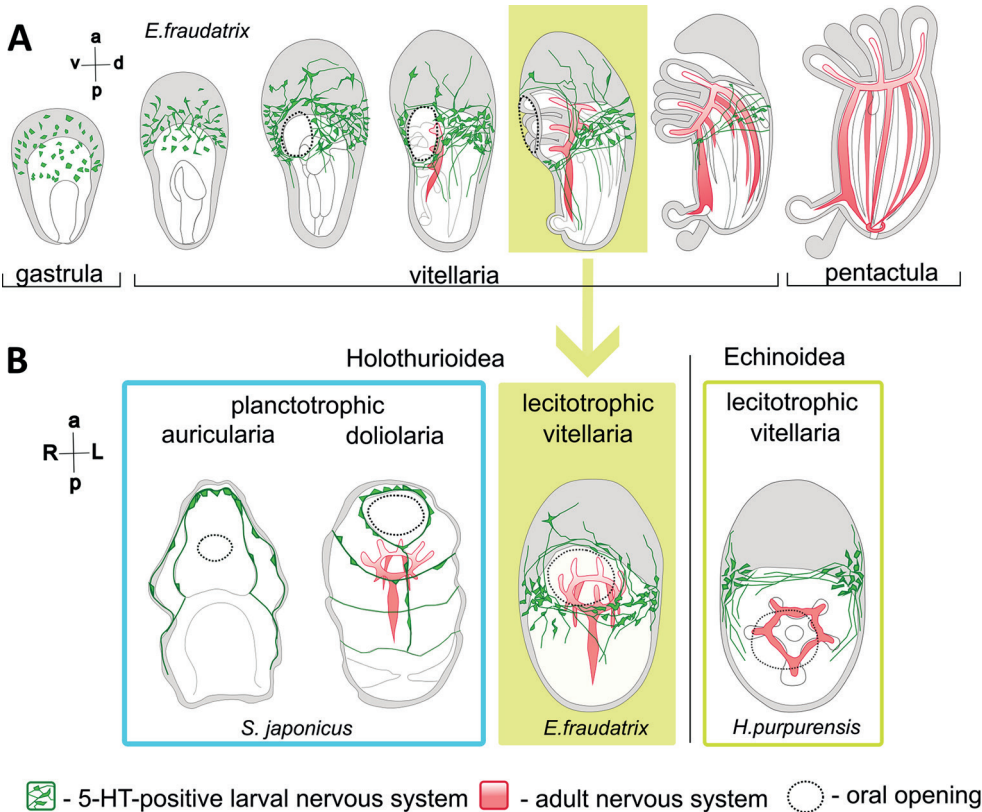


Fig. 8. Nervous system in lecithotrophic larva of *Eupentacta fraudatrix*. A — 5-HT-positive larval and AcTub-positive adult nervous system at different stages of *E. fraudatrix* development. During the second half of development, both nervous systems are present in the larva simultaneously, but they do not form connections; B — a comparison of the nervous system in planktotrophic and lecithotrophic larvae. The adult nervous system in holothurians remains unchanged across larvae with different lifestyles (doliolaria of *S. japonicus* and vitellaria of *E. fraudatrix*). While 5-HT-positive larval nervous system in vitellaria of *E. fraudatrix* exhibits significant differences with planktotrophic larvae and shows greater resemblance to the lecithotrophic larvae of other classes of echinoderms (vitellaria of *H. purpurensis*). The schemes of *Stichopus japonicus* and *Holopneustes purpurensis* are based on Nakano *et al.* (2006) and Katow *et al.* (2009).

Axes directions: a — anterior; p — posterior; v — ventral; d — dorsal; R — right; L — left.

Рис. 8. Нервная система лекцитотрофной личинки *Eupentacta fraudatrix*. А — 5-НТ-иммунопозитивная личиночная и AcTub-иммунопозитивная взрослая нервная система на разных стадиях развития *E. fraudatrix*. Во второй половине развития обе нервные системы одновременно присутствуют в личинке, но они не образуют связей; В — сравнение нервной системы планктотрофных и лекцитотрофных личинок. Развитие взрослой нервной системы голотурий остается неизменным у личинок с разным образом жизни (долиолярии *S. japonicus* и вителляррии *E. fraudatrix*). В то время как 5-НТ-позитивная личиночная нервная система *E. fraudatrix* значительно отличается от планктотрофных личинок и проявляет большее сходство с лекцитотрофными личинками других классов иглокожих (вителляррией *H. purpurensis*). Схемы нервных систем *Stichopus japonicus* и *Holopneustes purpurensis* сделаны на основе работ Nakano *et al.* (2006) и Katow *et al.* (2009).

Направления осей: а — передний; р — задний; в — вентральный; d — дорсальный; R — правый; L — левый.

system also occupies the region surrounding the base of preoral lobe (Sweet *et al.*, 2019). Similarly, the nervous elements in lecithotrophic larvae of sea stars *Meridastrea calcar* and

Parvulastra exigua is concentrated in the attachment complex located in the preoral region (Elia *et al.*, 2009). Thus, these species share many common features with *E. fraudatrix*, such as

the localization of the larval nervous system at the base of the preoral region and the absence of neural cords associated with ciliary bands. Additionally, there is no distinct apical ganglion at the animal pole. Therefore, it appears that the structure of the larval nervous system is more affected by the larval lifestyle than by phylogenetic position. Our results are insufficient to determine whether these shared features evolved independently in different classes of echinoderms. On the one hand, phylogenetic studies suggest that lecithotrophic development arose independently in each class (McEdward, Janies, 1997; Raff, Byrne, 2006), so the modification of the larval serotonergic nervous system may be a consequence of convergent evolution. On the other hand, the apical organ of primary ciliated larvae is the most evolutionarily conserved nervous structure among Bilateria (Nielsen, 2015; Arendt *et al.*, 2016). Some authors have suggested a homology between the preoral ganglion of direct-developing sea urchins and the apical ganglion of planktotrophic plutei (Katow *et al.*, 2009). However, comparative gene expression studies have not been conducted to confirm it. Thus, further research is needed to elucidate the influence of lecithotrophy on the formation of the apical ganglion and to fully understand the evolution of the serotonergic nervous system in echinoderm larvae.

Features of adult nervous system development

Although the development of the adult nervous system in *E. fraudatrix* shows no significant differences from that of holothurians with planktotrophic larvae (Nakano *et al.*, 2006; Dolmatov *et al.*, 2018; Zheng *et al.*, 2022), we have identified unique features that set apart the adult neurogenesis of holothurians from other echinoderm species.

Early establishment of the adult nervous system. The first structures of the adult nervous system in *E. fraudatrix*, circumoral nerve ring and mid-ventral nerve cord, were detected using antibodies targeting AcTub in the middle of the vitellaria stage. However, Mashanov *et al.* (2007) demonstrated, through electron microscopy, the presence of the precursor of the circumoral nerve ring in *E. fraudatrix* significantly earlier than what can be visualized using antibodies targeting

AcTub. According to Dolmatov *et al.* (2018), during the early stages of holothurian development, the adult neural structures lack nerve fibers and comprised only of neuroblasts. Acetylation of tubulin in nerve fibers is necessary for the mechanical stability, restriction of branching and transport of neuronal vesicles, and occurs during late neuronal differentiation (Moutin *et al.*, 2021). Therefore, AcTub was not detected in the early rudiments of the adult nervous system of *E. fraudatrix*. However, subsequent maturation of the adult nervous structures delineated by AcTub staining is consistent with findings from electron microscopic studies (Mashanov *et al.*, 2007). In planktotrophic holothurian larvae, the circumoral nerve ring and mid-ventral nerve cord are detected at doliolaria stage, which is equivalent to the vitellaria stage in *E. fraudatrix* (Nakano *et al.*, 2006; Dolmatov *et al.*, 2018; Zheng *et al.*, 2022) (Fig. 8B). It seems that early establishment of the adult nervous system is a characteristic feature for all holothurians irrespective of the larval lifestyle. In planktotrophic and lecithotrophic sea stars and sea urchins, the elements of the adult nervous system were initially observed in the podia feet and subsequently appear in the circumoral nerve ring and radial nerve cords (Elia *et al.*, 2009; Katow *et al.*, 2009). In holothurians, the rudiments of circumoral nerve ring and ventral nerve cord are formed earlier, before the emergence of the podia feet (Nakano *et al.*, 2006; Mashanov *et al.*, 2007; Zheng *et al.*, 2022; this study). These findings underscore the diverse mechanisms underlying nervous system development across holothurians and other echinoderm species.

Asymmetrical development of radial nerve cords. The mid-ventral nerve cord of *E. fraudatrix* differs from the other radial cords by its earlier formation, thickness and accelerated development. Only several months after settlement, all radial nerve cords in *E. fraudatrix* reach the same size (Mashanov *et al.*, 2007). In juvenile holothurians with planktotrophic larvae, the mid-ventral nerve cord also predominates the other radial cords (Nakano *et al.*, 2006; Dolmatov *et al.*, 2018; Formery *et al.*, 2021; Zheng *et al.*, 2022). To the contrary, in juvenile sea stars, brittle stars, and sea urchins, the radial nerve cords appear simultaneously and have similar thickness (Elia *et al.*, 2009; Sweet *et al.*, 2019; Formery *et al.*, 2021). In juvenile crinoids, the

radial nerve cords are also equal in size (Nakano *et al.*, 2009; Omori *et al.*, 2020). These data suggest that the initial bilateral symmetry observed during the formation of the adult nervous system in holothurians is unique. While some fossil echinoderms demonstrated bilateral adult forms (Smith, 2005), discerning whether the asymmetric development of radial nerve cords in holothurians is an ancestral trait or a secondarily acquired feature remains challenging.

Caudal nerve ring connects the radial nerve cords. For the first time we revealed caudal ring that connect radial nerve cords near the anal opening in *E. fraudatrix* juveniles. Previous studies have described the central nervous system in holothurians as open in the caudal region (Mashanov *et al.*, 2006, 2009; Hoekstra *et al.*, 2012). However, these studies were conducted on large adult specimens, which do not allow for detailed whole-mount investigations. Similar ring-like nerve structures have been observed on aboral side of the other echinoderms. For instance, sea urchins possess an aboral nervous ring around the anus that regulates gonad contraction (Yoshida *et al.*, 1987). Crinoids have a large aboral nervous system (Hyman 1955; Bohn, Heinzeller, 1999), which appears after the oral one in development (Nakano *et al.*, 2009). While aboral nervous structures found in all classes of echinoderms, their homology has a long story of debate (Hyman, 1955).

Adult and larval nervous system probably develop independently

The regions involved in the formation of larval and adult nervous systems are significantly distinct, and larval nervous elements were not detected in the central adult nervous system of *E. fraudatrix*. However, the potential existence of these structures in its peripheral regions cannot be discounted.

Larval and adult nervous systems have no morphological connections. Throughout an extended period of development (vitellaria stage), both the larval and adult nervous systems coexist in *E. fraudatrix* larval body. However, we did not find any morphological connections between these two nervous systems. It is possible that the markers we used do not identify all larval nervous structures, and expanding the panel of markers will provide new data. However,

at the moment, research on other echinoderms using the pan-neuronal marker synaptotagmin also demonstrates that the larval nervous system of most echinoderms lacks anatomical connections with the forming adult nervous system (Chia, Burke, 1978; Nakano *et al.*, 2006; Díaz-Balzac, García-Arrarás, 2018; Formery *et al.*, 2021). As an example of a possible exception, 5-HT-positive larval fibers have been described at the tops of the primary podia of the adult rudiment during development of sea urchin with lecithotrophic larva (Katow *et al.*, 2009). This innervation disappears by the end of the larval development, whereas non-serotonergic fibers persist in the podia. Since 5-HT-positive fibers are absent in the forming adult nervous cords, this transient innervation is attributed to the larval nervous system (Katow *et al.*, 2009). In *E. fraudatrix* we did not detect any 5-HT-positive structures in the primary podia of the adult rudiment. Meanwhile, during late development, these regions are actively innervated by AcTub-positive fibers extending from the mid-ventral radial cord, thus indicating their association with the adult nervous system.

5-HT-positive elements disappear in settled juveniles. The adult nervous system of *E. fraudatrix* exhibits a distinction from the larval nervous system due to the absence of 5-HT-positive structures. The disappearance of 5-HT-positive cells and fibers during echinoderm development has been noted by various researchers (Nakano *et al.*, 2006; Byrne *et al.*, 2006; Katow *et al.*, 2009; Formery *et al.*, 2021). Thus, this phenomenon occurs in both planktotrophic and lecithotrophic species and consider to be a possible consequence of the unique evolution of echinoderms pentaradial nervous system. However, there are several inconsistencies regarding the presence of 5-HT-positive cells in the adult echinoderms. While 5-HT has not been detected in the nerve cords of adult echinoderms (Squires *et al.*, 2010; Díaz-Balzac, García-Arrarás, 2018; Formery *et al.*, 2021), there are few reports of 5-HT-positive structures in their peripheral tissues (Carnevali *et al.*, 1996; Inoue *et al.*, 2002; Sugni *et al.*, 2004). Moreover, pharmacological experiments have elucidated the presence of 5-HT receptors and metabolic enzymes in adult echinoderms, along with the influence of 5-HT on muscle contraction and tissue regeneration (Inoue *et al.*, 2002; Sugni *et al.*, 2004; Squires *et al.*,

2010). Hence, it is plausible that 5-HT-positive neurons might exist in the adult peripheral tissues and their presence and role in adult echinoderms remains understudied.

Basiepidermal nerve plexus as independent peripheral part of the adult nervous system. While 5-HT-positive cells and fibers disappear upon settlement of *E. fraudatrix*, the AcTub-positive basiepidermal nerve plexus persists in juveniles. It is possible that at least some of that non-serotonergic nerve fibers presented in the larval nervous system became integrated into the basiepidermal nerve plexus in adult. In species like *Holothuria glaberrima* and *Holothuria mexicana*, the peripheral plexus of adults contains catecholaminergic neurons and fibers (García-Arrarás *et al.*, 2001; Díaz-Balzac *et al.*, 2010). It is quite possible that these nervous structures are descendants of the larval nervous system and persists in adults. In juvenile sea urchins, the basiepidermal nerve plexus consists of neurites without a clear hierarchy and operates relatively independently from the central nervous system (Formery *et al.*, 2021). The authors of the aforementioned study propose that ancestral echinoderms possessed two independent parts of the adult nervous systems: the basiepidermal nerve plexus for local sensitivity and the central nervous system for motor outputs generation and overall coordination (Formery *et al.*, 2021). In such a case the autonomy of the basiepidermal nerve plexus may be attributed to its independent origin as part of the remaining larval nervous system.

Conclusion

Our investigation provides a detailed analysis of 5-HT- and AcTub-positive nerve elements in lecithotrophic holothurian larvae, ranging from the swimming gastrula stage to settled juveniles. Notable differences in the structural organization of the larval nervous system are observed compared to planktotrophic larvae of other holothurian species. These include a distinct distribution pattern of larval 5-HT-positive cells and the absence of a well-defined apical organ and nerve cords associated with locomotory cilia. However, despite these differences, the development of the adult nervous system in vitellaria resembles that of planktotrophic doliolaria larvae. Throughout ontogenesis, ele-

ments of the larval and adult nervous systems are situated in separate regions of the body and remain disconnected. Our findings suggest that larval and adult holothurian nervous systems likely develop independently, exhibiting differing adaptive plasticity, and may be influenced by distinct evolutionary pressures. Obtained data underscores the potential for species with direct development to serve as convenient models for investigating adult neurogenesis in echinoderms and emergence of neurogenic zones.

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

Supplementary data.

Suppl. Fig. S1. Images of control samples, incubated without first antibodies of *Eupentacta fraudatrix*. A — maximum-intensity Z-projection of pentactula. B, C — cross sections of vitellaria. Note the absence of specific immunopositive signal in all channels. The areas where 5-HT-positive non-cellular structures were detected are delineated by dotted lines. Whole mount labelling with goat anti-rabbit antibody Alexa Fluor™ 488 (a-rab Al-488), goat anti-rabbit antibody Alexa Fluor™ 647 (a-rab Al-647) and goat anti-mouse antibody Alexa Fluor™ 546 (a-m Al-546), nuclear staining by DAPI. Scale bars: 50 µm.

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