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Neural Projections in Planarian Brain Revealed by Fluorescent Dye Tracing

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ABSTRACT—The planarian brain has an inverted-U shaped structure with functional regionalization. To investigate how each region in the brain connects to each other, we traced neural projections by microinjection of fluorescence dye tracers. We found that external light and olfactory/taste signals received in the head region are conveyed in the main lobes (sponge region) of the brain. Chemosensory neurons distributed in the lateral branches project to the peripheral region of the sponge and visual neurons project to the medial region of the sponge. Parts of the sensory neurons project directly to the corresponding sensory neurons on the opposite side of the brain. However, all of the dye labeled brain neurons in the left and right lobes connect to each other via commissural neurons in the central region of the sponge. In addition to these observations, we detected regional differences in the planarian visual neurons. Posterior visual neurons have ipsilateral projection, but anterior visual neurons project to the contralateral side of the brain. A pair of longitudinal ventral nerve cords (VNC) connect to the brain on the ventral side, suggesting that they transmit signals which are integrated and processed in the brain. We also detected the direct connection of neurons in the brain and those of the pharynx, even though most pharynx neurons connect to VNC neurons. Here, we report for the first time on neural connections in the planarian central nervous system after overcoming technical difficulties specific to flatworms.

Key words: planarian, brain, DiI, neural projection, confocal microscopy

INTRODUCTION

The freshwater planarian is an excellent model system when considering the evolution and origin of the central nervous system (CNS) (Halton and Gustafsson, 1996; Mineta *et al*., 2003). Our previous studies demonstrated that the planarian brain is more organized than previously expected. The planarian brain has an inverted-U shaped structure with functional regionalization (Agata *et al*., 1998; Nakazawa *et al*., 2003). Classical morphological studies by Golgi and Lucifer Yellow staining also show that flatworms have diversified neurons, such as monopolar, bipolar and multipolar neurons (Keenan *et al*., 1981; Koopowitz, 1986).

The planarian CNS consists of a brain in the head region and a pair of longitudinal ventral nerve cords (VNC), which are distinct structures that connect to the brain on the ventral side (Agata *et al*., 1998; Cebrià *et al*., 2002a). The planarian brain consists of two lobes with nine pairs of lateral branches and a pair of eyes located on the dorsal side of the brain (Agata *et al*., 1998). Each lobe is connected to each other in its anterior portion by a single commissure, and each lobe contains axon bundles of brain neurons in the central sponge region (Tazaki *et al*., 1999). Lateral branches are composed of chemosensory neurons whose processes reach to the head surface and form sensory organs. Notably, the 6th to 9th branches cluster more closely and form auricles on the surface of the head which may function as taste organs (Pigon, 1974).

When observed at the molecular level, the planarian brain can be divided into at least three regions by differential expression of three homeobox genes (Umesono *et al*., 1997, 1999). Interestingly, their expression domains correspond to discrete functional and structural domains. Expression of *DjotxA* overlaps with both visual neurons and visual

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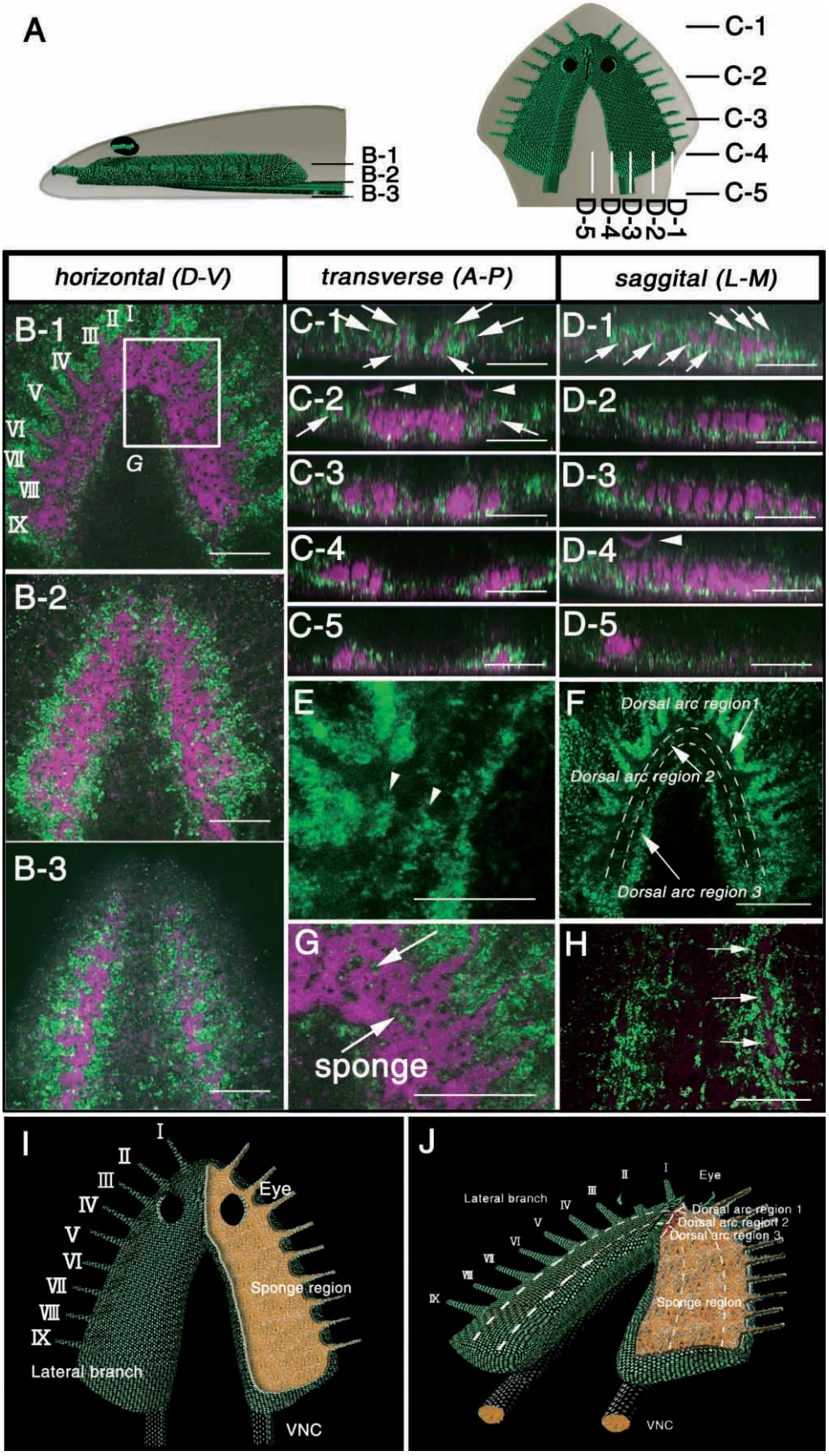
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D-1
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D-3
D-4
D-5
E
F
Dorsal arc region 1
Dorsal arc region 2
Dorsal arc region 3
G
sponge
H
I
Eye
Sponge region
Lateral branch
VNC
J
Lateral branch
Eye
Dorsal arc region 1
Dorsal arc region 2
Dorsal arc region 3
Sponge region
VNC

centers, located in the dorsal-medial region of the brain. *Djotp* is specifically expressed in the lateral branch region and *DjotxB* is expressed in the main lobes located between the *DjotxA* and *Djotp*-positive regions (Umesono *et al*., 1997, 1999). It has also been demonstrated that each of these three domains can be further divided into more diverse regions by various gene expression patterns (Cebrià *et al*., 2002b; Mineta *et al*., 2003; Nakazawa *et al*., 2003).

To investigate neural connections between these domains we have attempted to trace neural projections by fluorescent dye injection (Vercello *et al*., 2000). However, mucus secretion by the planarian hinders the dye injection process, leading to extensive diffusion of fluorescent dye on the surface of the body. In addition, the planarian brain is indistinguishable by light microscopy due to its non-transparent body, in contrast to other experimental animals. The success rate for this type of injection was less than 5% prior to this study, and because of these obstacles little is known of nerve projections in the planarian. In terms of reproducibility, when posterior visual neurons were labeled with dye, all 26 obtained samples showed both contralateral and ipsilateral projections.

We managed to overcome these problems by repeated DiI injections and extensive feedback training. At the beginning of this study we tried to construct a three dimensional image of the planarian brain using a general neuronal marker, PC2 (Agata *et al*., 1998), and then performed repeated injections. When we obtained successful results by these injections, we reconstructed our three dimensional image and incorporated the spatial information to accurately perform the next set of injections. Using this feedback technique the success rate of dye injections in the planarian was improved to more than 30%. The current report presents the results obtained by this approach for the last five years which clearly indicate how each domain interconnects in the planarian brain.

MATERIAL AND METHODS

Animals

A clonal strain of the Planarian (*Dugesia japonica*) originated from the Iruma River, Gifu prefecture, Japan. All planarians used were starved for at least one week and ranged in size from 4–7 mm.

Whole mount *in situ* hybridization

Animals were fixed by modified relaxant solution (Kato *et al*., 1999) for 24 h at 4°C. Bleaching and rehydration was followed by hybridization, as previously described (Umesono *et al*., 1997). During hybridization, DIG-labeled probe was used and then carried in fluorescent detection (TSA kit, NEN life science Product).

Tyramide signal amplification indirect

The following steps were subsequently performed at RT. After probe wash, samples were incubated in TNT wash buffer (0.1 M Tris-HCl at pH 7.5 containing 0.15 M NaCl and 0.05% Tween 20) three times for 15 min. Blocking by TNB blocking buffer (0.1 M Tris-HCl at pH 7.5, 0.15 M NaCl, 0.5% Blocking reagent (supplied in kit) for 30 min) was carried out and incubated by anti-DIG horseradish peroxidase (Boehringer Mannheim) for 2 h at 1:250 in TNB. After washing with TNT three times for 1 h, samples were incubated with tyramide-biotin at 1:50 for 20 min. Following TNT washing three times for 1 h samples were then incubated with streptavidin conjugated FITC in TNB for 30 min at 1:500. Finally, they were washed three times for 1 h with TNT.

Whole mount immunostaining

The samples above then underwent immunostaining and blocking (PBS containing 0.1% Triton-X-100 and 10% goat serum for 2 h at RT). Anti-PC2 antibody was used as primary antibody at 1:2000. Finally, the signal was detected by anti-mouse Alexa-594 antibody (Molecular Probes).

DiI injection

As a neuronal tracer, DiI or DiD (Molecular Probes D-282, D-307) was dissolved in dimetyl-sulfoxide (DMSO) at 10 μg/μl-40 μg/μl. Glass micropipet was made by puller (Narishige) and microinjection was performed by manipulation. Under microscopic observation, planarians were placed on ice and after amputation by well-sharpened tungsten needle at the injection site, DiI/DiD solution was microinjected. Samples were kept under water for 3 h-72 h at 14°C. Primary fixation was 2%HCl, 4%PFA in 5/8 Holtfreter and secondary fixation was performed by 4%PFA in 5/8 Holtfreter overnight at 4°C. Hoechst 33258 (Sigma) was used for cell nuclei staining.

Confocal laser scanning microscopy

The preparations were examined as whole mounts by LSM-510 confocal laser scanning microscopy (Carl Zeiss). Laser power was settled with UV 60% 351/364nm, Argon 60% 458/488nm, HeNe1 100% 543nm, HeNe2 100% 633nm and the appropriate filter set was selected according to fluorescent markers. 40X 1.20-numerical aperture C-apochromat with water immersion lens (Carl Zeiss), 20X 0.5-numerical aperture Plan NEO Fluar (Carl Zeiss) were mainly used in DiI tracing and brain structure analysis. Crosstalk by multicolor staining was virtually protected with

Fig. 1. Brain structure revealed by confocal laser scanning microscopy (CLSM). (A) Schematic image of head and location of the figure as follows: Left side shows the location of horizontal views and right side shows the location of transverse and sagittal views. (B, C, D) CLSM images by PC2 RNA probe (green) and anti-PC2 antibody (red). (B-1, 2, 3) Horizontal views. (B-1) Nine pairs of lateral branches extended to the head surface. (B-2) The brain appears more dorsally than VNC. (B-3) Ventral side image shows that ventral nerve cords are also in the head region. (C-1, 2, 3, 4, 5) Transverse views. (C-1) Lateral branches (arrows) are round in the most anterior part. (C-2) Eyes (auto-fluorescence background) are located on the connective region between the two halves of the brain. (C-3) Connective region is separated into two halves. (C-4) Each half of the brain extends to the more outer side in the posterior region of the brain. (C-5) Only VNC is located on the ventral side. (D-1, 2, 3, 4, 5) Sagittal views. (D-1) Lateral branches (arrows) are lined with a round shape and the density of lateral branches is higher in the posterior side than in the anterior side. (D-2, 3, 4) The brain has a flat shape. (D-5) Anterior portion is the connective part between left and right in midline. (E) Magnification image of the dorsal side with PC2 RNA probe (green). Arrows show arc like PC2 RNA probe positive cells. (F) Horizontal image of the dorsal side with PC2 RNA probe (green). The dorsal side of the brain shows two rows of cells (dots). We named these dorsal arc region 1, 2, 3 from outside (arrows). (G) Magnification view of B-1. The sponge region has a few PC2 RNA positive cells (arrows). (H) Horizontal image of VNC with PC2 RNA probe (green) and PC2 immunostaining (red). Arrows indicate regularity of ganglion knot. (I) (J) Diagram of planarian brain structure showing main components (lateral branches, eye, dorsal arc region 1, 2, 3, VNC). Scale bar is 100 μm.

multitracking mode.

RESULTS

Three dimensional analysis of the planarian brain

The planarian brain is indiscernible by light microscopy, although its appealing eyes are easily identifiable. To accurately inject fluorescent dye into the intended position of the planarian brain, the three dimensional structure of the brain was analyzed by confocal laser scanning microscopy (Fig. 1). When nerve fibers were stained with fluorescent labeled anti-PC2 antibody (red) and their cell bodies with PC2 RNA probe (green), we found that the cell bodies of brain neurons were located in the outer layer of the brain, and that the inner region of the brain was composed of bundles of nerve fibers (Fig. 1 B1-3, C1-5, D1-5, G). In this study planarians with an average size of 6 mm in length, 0.15 mm in thickness and 0.5 mm in width were used. The brain is located in the anterior-ventral side of the animal with an average length of 500 μm, thickness of 50 μm and width of 450 μm.

A pair of eyes is located on the dorsal side of the brain (Fig. 1C-2, D-4 arrowheads). Visual neurons are easily recognizable by the distinct white area of the eye found in the dorsal side of the head region at a depth of 30 μm from the dorsal epidermis.

Nine pairs of segmented conical structures, known as lateral branches, were observed in the lateral side of the brain. The structures were composed of axon bundles surrounded by cell bodies similar to the main lobes (Fig. 1B-1, C-1, D-1 arrows). The length of each lateral branch from the 1st to 5th is 90 μm and that of the 6th to 9th is 70 μm. Although these lateral branches are not clearly distinguishable from the ventral side of the body, we can speculate on the position of each branch by its relative position to the eye, which is located on the dorsal side of the 3rd branch. Lateral branches were stained by injecting dye at a depth of 30 μm from the ventral epidermis.

We named the axon bundles of the main lobes the sponge region. The left and right lobes of the brain are connected to each other in the anterior portion of the inverted U-shaped sponge region (Fig. 1B-1, C-2, D-5). Confocal microscopic observations clearly indicated three different layers in the dorsal sponge region, named dorsal arc region 1, 2 and 3 in a lateral to medial direction (Fig. 1F arrows): Two arc like cell rows by PC2 RNA probe (Fig. 1 E arrows and F dots; outside and inside arc) define dorsal arc region 1 which indicates the more outer side than outside arc, dorsal arc region 2 between outside and inside arc and dorsal arc region 3 of the more inner area than inside arc. VNC connect to the medial/ventral region of the brain and ganglion knots exist from the head to the trunk region (Fig. 1B-3, C-5, H arrows). We injected the fluorescent dye from the ventral epidermis at a depth of 40–50 μm to the axon bundles of the sponge.

The three dimensional structure of the planarian brain was reconstructed using confocal images based on the results obtained by PC2 RNA probe and anti PC2 antibody staining (Fig. 1I, J). We performed subsequent dye injection based on the three dimensional image obtained by this analysis.

Projection of visual neurons

Planarian eyes are composed of only two cell types; black pigment cells and visual neurons. Visual neurons have a bipolar morphology with one side showing microvilli morphology that form rhabdomeres, and the other side projecting to the dorsal-medial region of the brain with long axons (Sakai *et al*., 2000). When left and right visual neurons were stained with DiI and DiD respectively (Fig. 2A), each neuron projected to both the ipsilateral and contralateral sides of the brain to form the chiasma (Fig. 2B). We found that each visual neuron projected to the opposite side of the brain, but did not form an axon bundle (Fig. 2H arrow). Interestingly, we also found that some visual axons projected to the visual neurons in the other eye, which previously could not be distinguished by monoclonal antibody staining (Sakai *et al*., 2000; Fig. 2C, D arrow). In conclusion, planarian visual axons project in three directions: to the ipsilateral side of the brain, the contralateral side of the brain and to the opposite eye. It was confirmed that visual axons project along dorsal arc region 3, forming a tubular like structure surrounded by what are believed to be specialized neurons expressing *DjotxA* (Fig. 2E, F, G, arrow).

Regional differences in visual neurons

To investigate whether any differences exist among visual neurons concerned with projection patterning, we performed DiI injection into visual neurons located in different positions along the anterior-posterior axis. Repeated injections to various positions of the eyes indicated a possibility in which the anterior visual neurons project to only the contralateral side, although posterior visual neurons project to both contralateral and ipsilateral sides. This observation was confirmed by injecting DiI into the chiasma region (Fig. 3A). Only the visual neurons with rhabdomeres penetrating into the anterior portion of the eye cup were always stained (Fig. 3B, C arrowheads). However, in some animals the cell bodies of these neurons were located in the central portion of the eye (Fig. 3C). This may be one of the reasons why we could not obtain distinct regional differences when we injected directly into the eye. Injection of DiI into one side of dorsal arc region 3 showed that only anterior visual neurons were stained in the eye located in the opposite side to the injected site (Fig. 3F). Conversely, only posterior visual neurons were stained in the eye located on the same side of the injected site (Fig. 3G). The results are summarized in Fig. 3D and H.

Projection of lateral branch neurons

The nine pairs of lateral branches consisting of chemosensory neurons can be classified into two groups. The 1st to 5th branches reach the head surface separately,

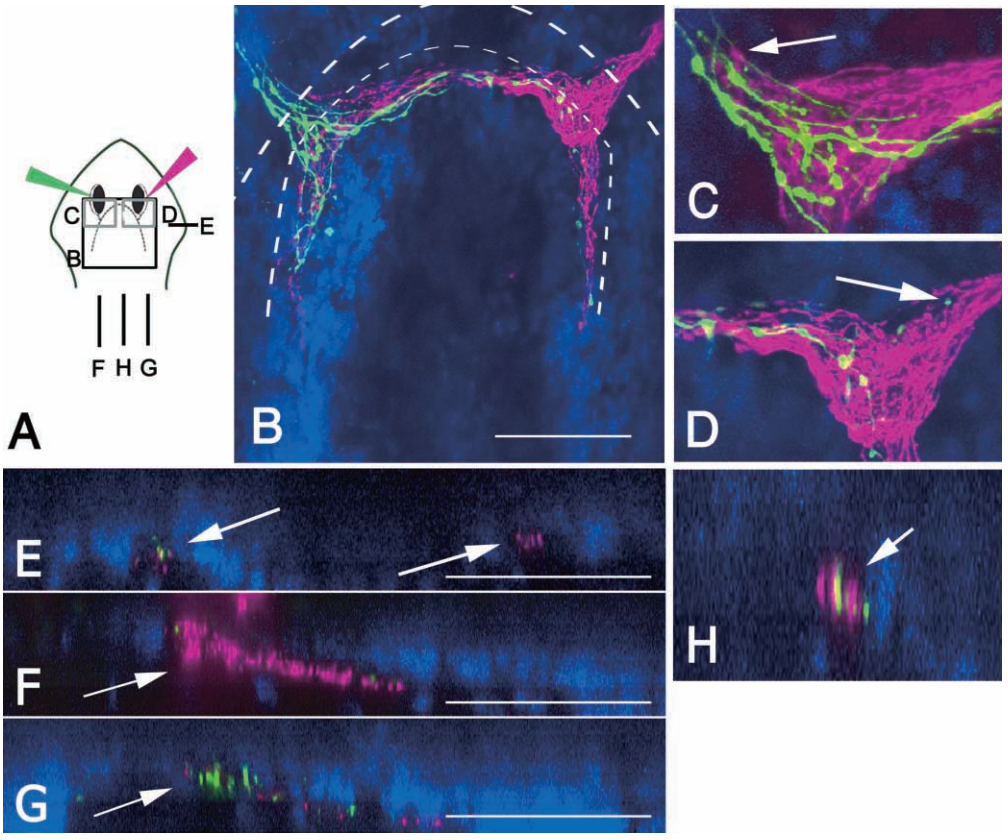

Fig. 2. The projection of visual neurons by DiI/DiD tracing. (A) Schematic drawing of injection site in both sides by DiI (red) and DiD (green) and location indicated as follows. (B) The projection of visual neurons of both sides labeled by DiI and DiD. Both visual neurons showed ipsilateral and contralateral projections. (C) Magnified view of left side. (D) Magnified view of right side. These show that some of the contralateral projections are also extended directly to the opposite eye (arrow). (E) Transverse image of visual axons in the medial region of brain. Arrows show that visual axons are located in the dorsal side of the brain and formed a compartment. (F) (G) Sagittal image of left and right sides. Visual axons are located in the dorsal side and form a compartment. (H) Sagittal image of visual axons in midline. Visual axons from both sides are mixed together (arrow). Scale bar is 100 μm.

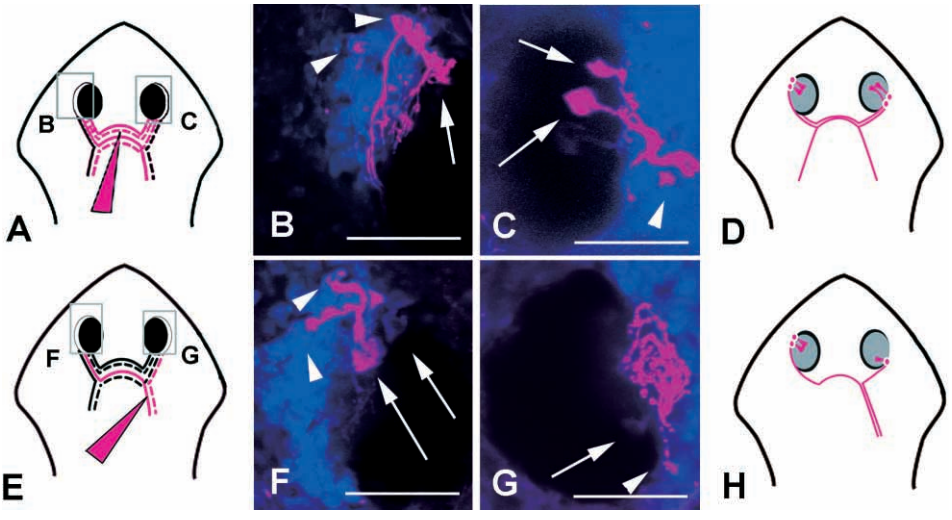

Fig. 3. Regional differences in visual neurons. (A) Schematic drawing of DiI injection site (I). Dye labeled contralateral visual neurons shown in red. (B) Left eye. Cell bodies (arrowheads) are on the anterior side as well as the rhabdomere (arrows). (C) Right eye. Rhabdomeres (arrows) are located anteriorly but cell bodies (arrowheads) are central. (D) Summary of contralateral visual neurons. (E) Schematic drawing of DiI injection site (II). Red shows the dye labeled ipsilateral (injection side) and contralateral visual neurons (opposite to injection side). (F) Left eye. Rhabdomeres (arrows) and cell body (arrow head) are found on the anterior side. (G) Right eye. Cell body (arrowhead) and rhabdomere (arrowhead) are both located posteriorly. (H) Summary of visual neurons in unilateral side of visual center. Scale bar is 50 μm.

whilst the 6th to the 9th branches form clusters on the head surface (Agata *et al.*, 1998). To investigate previously unknown projection patterns of lateral branch neurons, we injected DiI or DiD into each lateral branch. When we inject dye into the proximal or distal portion of each lateral branch, bipolar neurons located in the middle portion of the lateral branch were stained (Fig. 4A inset; 4B, C arrow) and nerve fibers that extended to the head surface were seen to penetrate into the epidermal layer with a dendrite-like structure (Fig. 4D). However, when DiI was injected to the distal region of the individual lateral branches, they projected to the stump region of each lateral branch in a radial pattern, where they corresponded to the periphery of the sponge region (Fig. 4E, F, G, H, I, J, K, L, M, N, O, P). Higher magnification view of the projection of the 1st branch clearly revealed that part of the 1st branch neurons project on to the other side of the 1st branch through the most peripheral region of the sponge (Fig. 4F arrow). Such projection to the opposite side of the branch was observed from the 1st to 5th branches suggesting that nerve fibers of branches project to the opposite side of each corresponding branch like visual axons. However, such neurons were not detected in the 6th to 9th branches. To investigate to what extent the target regions in the stump overlap with each other, we conducted double staining of the neighboring lateral branches using both DiI and DiD (Fig. 5A). The results clearly indicated that

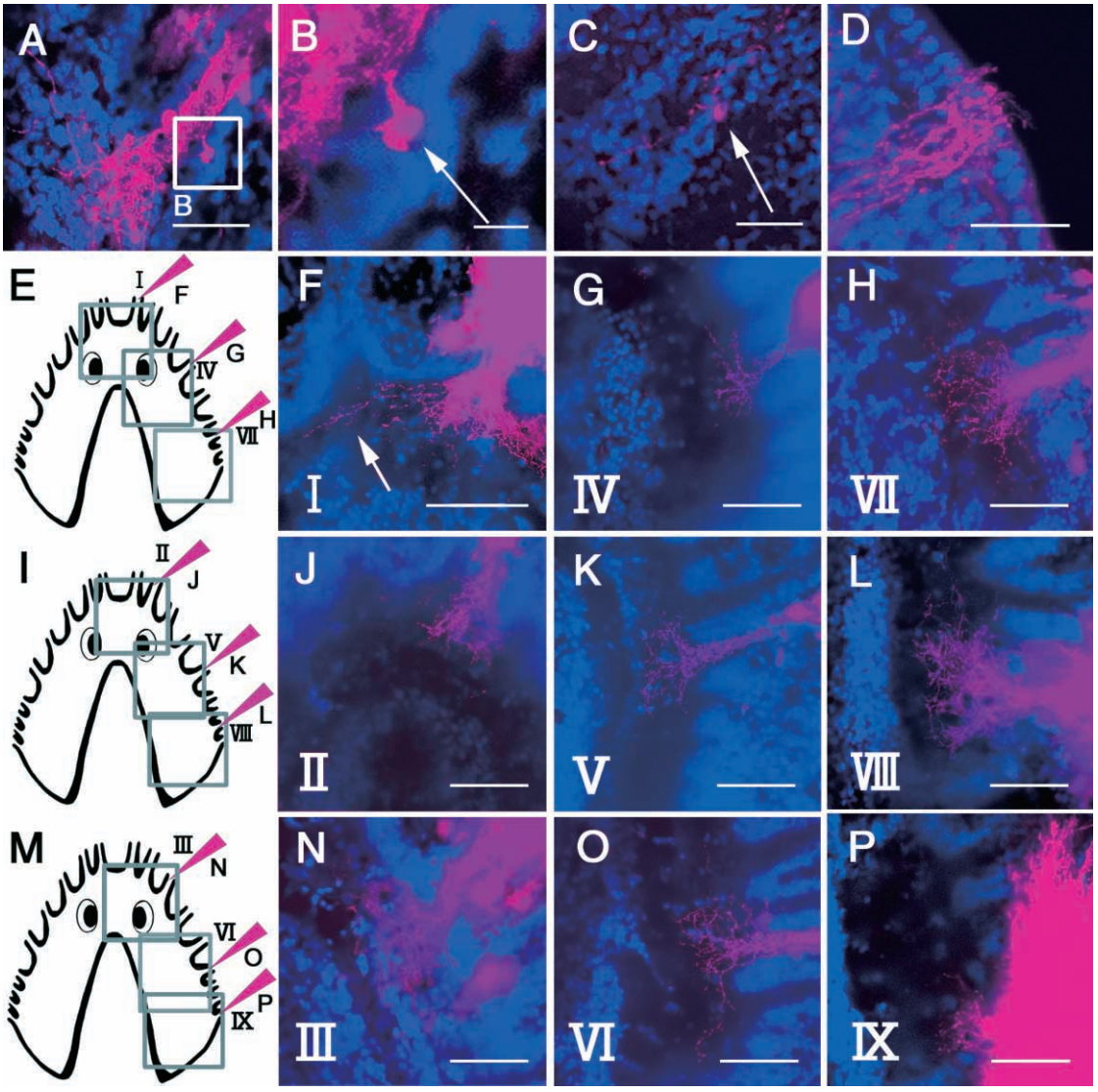

Fig. 4. Projection of lateral branches by neural tracer DiI. (A) Image of the proximal side of lateral branch. (B) (C) Morphology of lateral branch neuron. Lateral branch neuron is bipolar (arrow). (D) Image of the distal side of lateral branch. Their dendrite-like processes penetrate the epidermis. (E) Schematic drawing of injection sites in lateral branches (I). (F) 1st lateral branch. (G) 4th lateral branch. (H) 7th lateral branch. (I) Schematic drawing of injection sites in lateral branches (II). (J) 2nd lateral branch. (K) 5th lateral branch. (L) 8th lateral branch. (M) Schematic drawing of injection sites in lateral branches (III). (N) 3rd lateral branch. (O) 6th lateral branch. (P) 9th lateral branch. When the distal region of each lateral branch was labeled, the labeled fibers projected into the stump region of each lateral branch. Arrow in (F) shows the contralateral projection to the opposite brain half in 1st lateral branch. Scale bar is 50 μm.

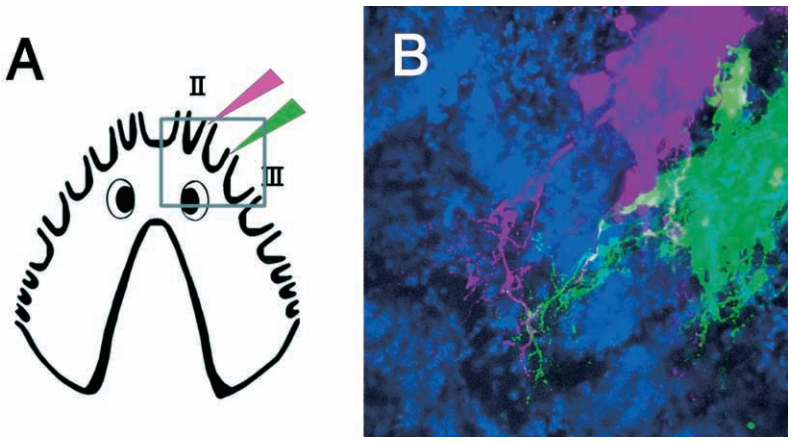

Fig. 5. The relation of nerve projections between neighboring lateral branches. (A) Schematic drawing of injection site. (B) Injection of side by side branches showing overlapped patterning in each stump region.

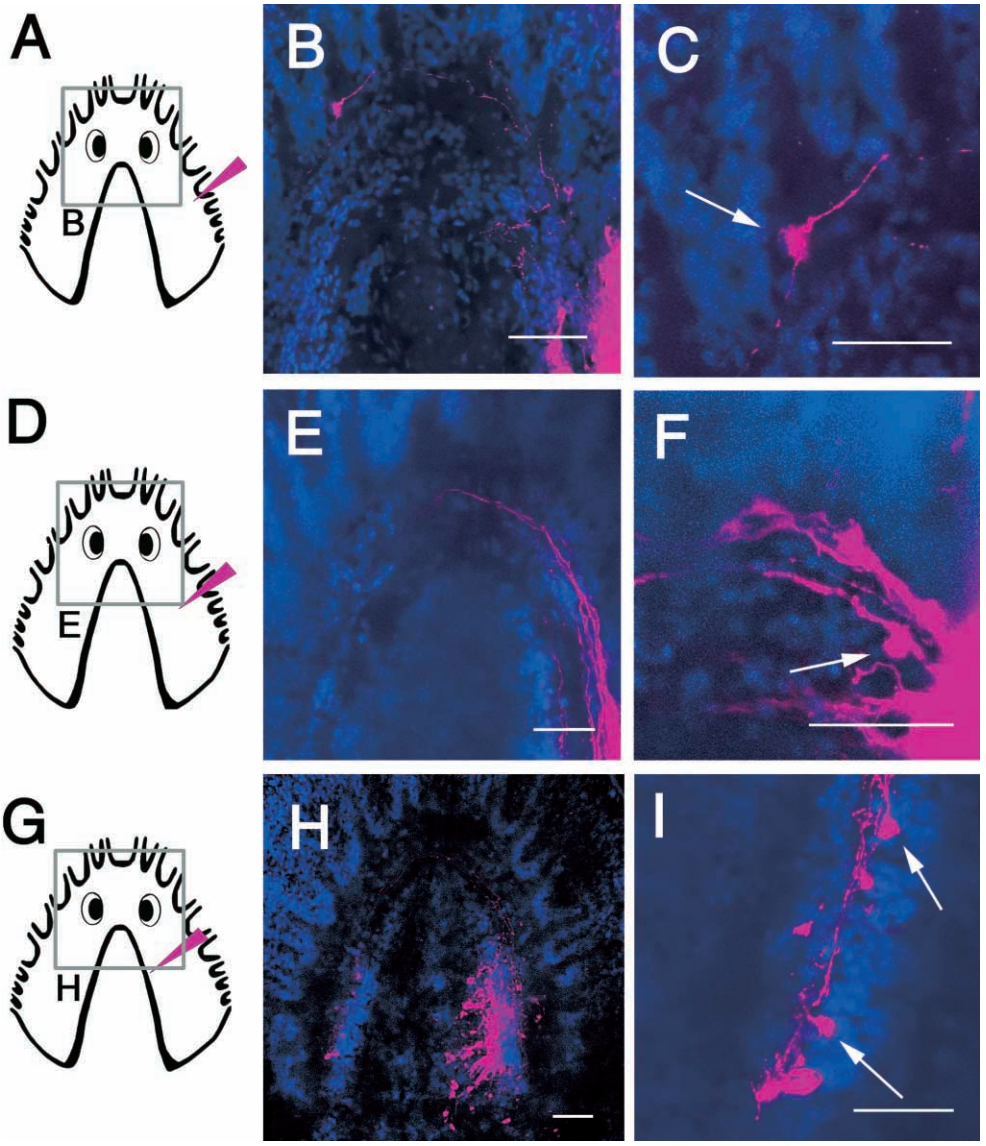

Fig. 6. The projection of commissural neurons in dorsal arc regions. (A) Image of DiI injection into sponge of brain (I). (B) Image of the nerve projection in dorsal arc region 1. (C) The morphology shows bipolarity (arrow). (D) Schematic drawing of injection into sponge of brain (II). (E) Image of nerve projection in dorsal arc region 2. (F) There is bipolarity in dorsal arc region 2. (G) Schematic drawing of injection into sponge of brain (III). (H) Image of nerve projection in dorsal arc region 3. (I) This morphology also shows bipolarity in dorsal arc region 3. Scale bar is 50 μm.

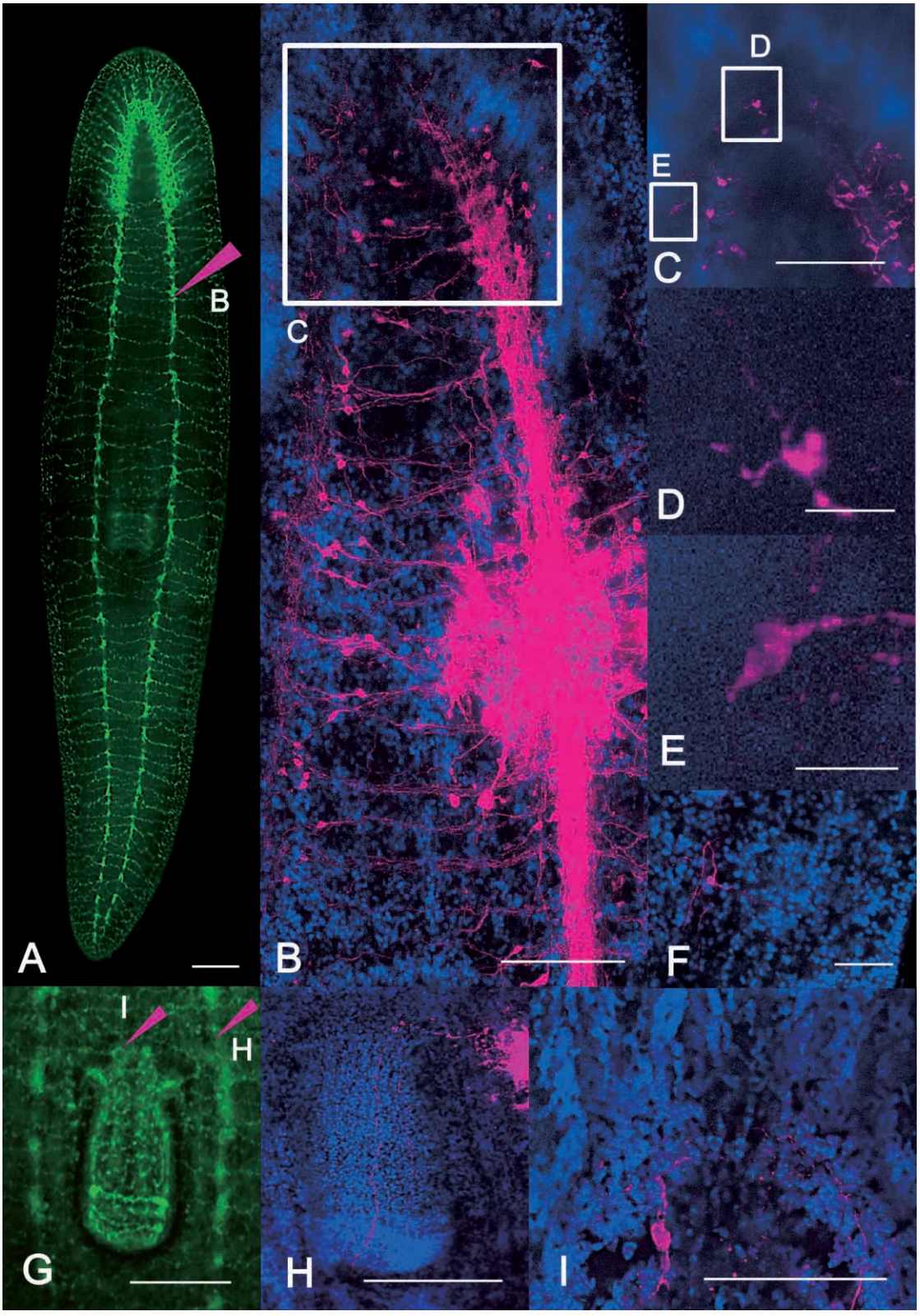

Fig. 7. The projection of ventral nerve cords and pharynx nerves. (A) Image of DiI injection in the trunk region. (B) Image of head to trunk region by DiI injection. Nerves form bundles from the VNC to the head and some cell bodies are located in the VNC commissure and head region. (C) Image of the dorsal side of brain. (D) The multipolar neurons of the brain project their fibers into ventral nerve cords. (E) Bipolarity in the dorsal side of brain. (F) The multipolar neuron extends branches into the lateral side. (G) Location of injection site by DiI. (H) When VNC of right side was labeled by DiI, nerve fibers reached the chip of pharynx. (I) Nerve fibers from the root of the pharynx were extended to dorsal arc region2. Scale bar is 50 μm (D, E, F) and 100 μm (A, B, C, G, H, I).

part of the nerve fibers overlap with each other in the stump region of the lateral branches (Fig. 5B).

Commissural neurons in the sponge region

Ladder like commissural neurons are always drawn in the head region in addition to the trunk region in text books. However, in our observations, right and left lobes are not connected to each other by ladder like commissural neurons. When we stained the sponge region of the right lobe, an arc like projection was traced. Ladder like staining patterns were not observed (Fig. 6B, E, and H). These results clearly demonstrate that left-right lobes are connected to each other by the anterior portion of the two lobes, forming arc like commissural connections. Ladder like commissural connections in the head region are the commissural connection between a pair of VNC attached on the ventral side of the brain (Fig. 7A, B).

We have indicated that there are three arc structures in the dorsal sponge region, and that a part of the lateral branch axons project to the opposite side of the lateral branches via the dorsal arc region 1, and that visual axons contralaterally project via the dorsal arc region 3. When the dye was injected into dorsal arc region 2 located between dorsal arc region 1 and 3, it was clearly indicated that the axon bundle of dorsal arc region 2 forms commissural neurons between two lobes (Fig. 6E).

Commissural neurons in dorsal arc region 3 were found in the medial/posterior region of the brain and their nerve fibers formed an arc shape commissure (Fig. 6H). Commissural neurons in dorsal arc regions 1, 2 and 3 were located in the dorsal side of the brain and displayed bipolarity (Fig. 6C, F, I). These results suggest that most of neurons consisting of the sponge have bipolar morphology.

VNC projection to the brain

To investigate whether there are any direct connections between the brain and VNC, VNC located alongside the pharynx was labeled by DiI (Fig. 7A, B). A pair of longitudinal ventral nerve cords, ladder like commissural nerves and lateral axon extensions to the muscle were strongly stained (Fig. 7B, F), suggesting that most of VNC neurons forms neural networks independently of the brain. However, some neurons with bipolar or multipolar morphology in the ventral region of the brain could be stained (Fig. 7C, D, E), indicating that although the majority of VNC neurons connect to the ventral side of the brain (Fig. 7B), some VNC connect directly to the brain neurons (Fig. 7C).

Relationship between pharynx nerves and the brain

The pharynx has well organized nerve networks in both basal and distal portions. To understand the relationship between pharynx nerves and the brain, DiI was injected into the VNC close to the pharynx or the basal region of the pharynx (Fig. 7G, H, I). We detected neural connection between VNC and the nerve ring in the distal portion of a pharynx (Fig. 7H). Interestingly, some fibers directly connecting between the basal region of the pharynx and dorsal arc region 2 in the brain can be detected (Fig. 7I).

DISCUSSION

Integration of sensory signals into the main brain lobes

Although previous immunostaining and gene expression analysis revealed the gross structure of neural networks and domain structure of the brain in *Dugesia japonica* (Agata *et al*., 1998; Tazaki *et al*., 1999; Sakai *et al*., 2000), the connection between each domain remains unclear. Here, we directly investigate the neural connection by dye tracing (Fig. 8A, B). The results clearly indicated that a variety of sensory neurons distributed in the head region project to the main lobes of the brain. Chemosensory neurons located in the lateral branch regions and mechanosensory neurons distributed in the head peripheral regions projected to the peripheral region of the main lobes (dorsal arc region 1). Visual neurons project to the dorsal-medial region of the brain (dorsal arc region 3). Various external signals may be integrated into the brain main lobes and then signals integrated in the main lobes may be transmitted to the entire region of the body via VNC to control movement of the animals.

Connection between left and right sides of the brain

Although we have already shown that planarian visual axons form a chiasma (Sakai *et al*., 2000), we did not detect any visual axons projecting to the eye of the other side by immunological staining. DiI tracing experiments clearly demonstrated that a part of the visual neurons directly connect to the visual neurons of the other side. Similar direct connection between left and right sensory neurons was also detected in the 1^{st} to 5^{th} branches. These connections may also have a role to enhance contrast of the signals between left and right sides for chemo-attraction. However, such neurons can not be detected in the 6^{th} to 9^{th} branches which form auricles, suggesting that left and right auricles communicate with each other through the commissural neurons located in dorsal arc region 2, since a part of the axons derived from the auricles seem to project to dorsal arc region 2 (Fig. 4H, L).

To investigate molecular mechanisms controlling left-right neural connections we are conducting RNA interference approaches. We have already obtained netrin, DCC, slit and Robo homologues by the planarian EST project (Cebrià *et al*., 2002a; Mineta *et al*., 2003). We are especially interested in function of the netrin homologues. We have already shown that visual axons project to the visual neurons of the other side at the early step of eye regeneration and then start to project to the dorsal/medial region of the brain when netrin expression is activated in this region (Inoue *et al*., 2004). From these observations we speculated that chiasma may be formed by at least two steps, netrin-independent and netrin-dependent steps.

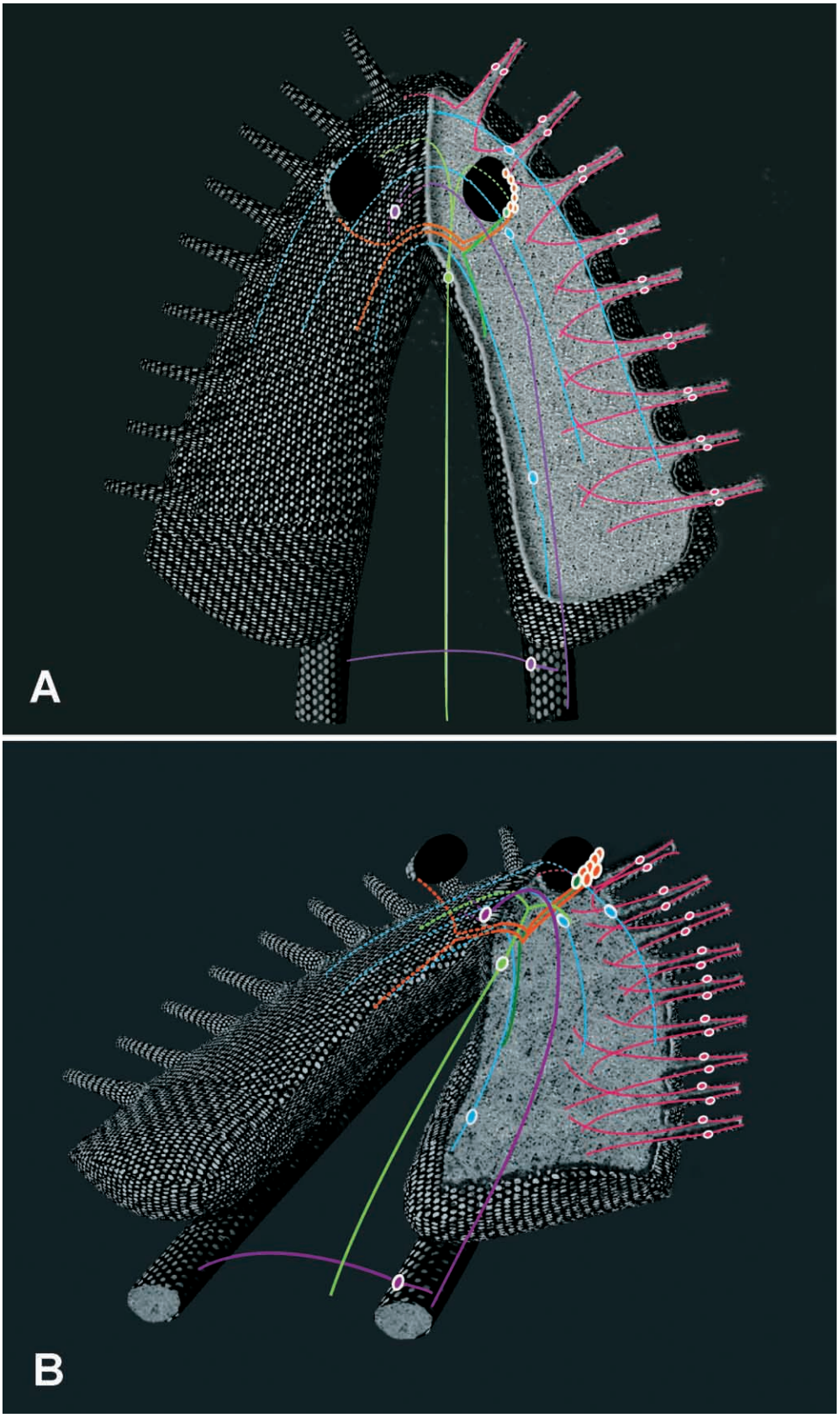

Fig. 8. Neural networks in planarian CNS. (A) (B) Summary of neural networks in planarian CNS. Visual neurons project in three directions. 1. Ipsilateral side (green). 2. Contralateral side: the medial region (orange). 3. Contralateral side: to the opposite eye (orange). Lateral branch neurons project to their stump region (pink). Commissural neurons connect the left and right areas through dorsal arc regions (blue). Pharynx nerves connect with dorsal arc region 2 (light green). Brain and VNC have multipolar neuron (purple).

Regional differences in visual neurons

It is of great interest to know whether planarian has regional differences in the eye. Recently we found genes whose expression was restricted in the anterior visual neurons, suggesting that planarian eyes may have regional differences along anterior-posterior axis (unpublished data). To investigate regional differences in the eye we directly injected dye into the anterior or posterior visual neurons. However, we could not get distinct regional differences by this analysis, since it is difficult to restrict the injection site into a precise position. When we changed injection sites from cell bodies to the visual axons, we detected a difference between anterior and posterior regions in the eye (Fig. 3D, H). Posterior visual neurons have ipsilateral projection, but anterior visual neurons project to the contralateral side of the brain, demonstrating that the planarian has already developed regional differences in the eye. Following illumination of rhabdomeres lying along the outer posterior edge and the outer ventral edge of the eye-cup, the planarian turns towards the light. Conversely, illuminating the rhabdomeres of the center, anterior and vertical edges of the pigment-cup resulted in the planarian turning away from the light (Taliaferro, 1920). When we consider these descriptions together with DiI tracing results, we can consider that rhabdomeres lying along the outer posterior edge and outer ventral edge of the eye-cup (which show mainly ipsilateral projections) affect the brain and VNC on the illuminating side. Rhabdomeres of the center, anterior and vertical edges of the pigment-cup (which show mainly contralateral projections) influence the opposite brain and VNC on the illuminating side. And we speculate that the direct connection of visual axons between left and right visual neurons may have an inhibitory role in enhancing the difference of signal strength between the left and right sides. Now, we are conducting RNA interference approach using genes which are specifically expressed in the anterior visual neurons to investigate the biological meaning of regional differences in the planarian visual system.

Chemosensory system in planarian

In insects, chemosensory signals are transmitted to the brain via specific interneurons which form glomeruli structure (Gao *et al*., 2000). However, we could not detect such neurons in the planarian. Chemosensory neurons located in the lateral branches directly projected to the brain main lobes. A part of the nerves of neighboring lateral branches overlapped with each other in the periphery of the main lobes. We have not observed functional or molecular differences among nine pairs of the lateral branches so far, even though we have already analyzed expression patterns of more than a thousand genes. From these observations we speculate that glomeruli structure observed in other animals may have been acquired in the process of evolution.

The pharynx also has chemosensory neurons which form nerve rings in the tip of the pharynx. Wulzen (1917) indicated that the pharynx, even though freed from the rest of the body, can orient itself towards the source of the diffused juice and moves forward by a very wormlike series of extensions, contractions and wriggling until the mouth comes into direct contact with the meat. While in contact with food, a freed pharynx carries out coordinated movements for food ingestion. From these observations, the pharynx can be seen to contain an autonomic neural machinery. However, we detected bipolar neurons in the brain directly connected between lateral branch neurons and the basal region of the pharynx (Fig. 7I), suggesting that some of the pharynx function is controlled by the brain.

In conclusion we can understand the overall connection among different portions of the nervous system by fluorescence dye tracing. To investigate the molecular mechanisms involved in neural circuit formation we are planning to conduct RNA interference experiments combined with dye tracing and behavior analysis.

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