DIVISION OF CELL PROLIFERATION (ADJUNCT)

Professor: *Motoya Katsuki*
Research Associate: *Kei Ito*
JST Research Staff: *Takeshi Awasaki (~ Sep. 30, 2000 CREST, Oct. 1, 2000~ PRESTO)*
Postdoctoral Fellow: *Ryuichi Okada*
Graduate Student: *Yusuke Uchiyama (Kyoto University)*
Nobuaki Tanaka (Graduate Univ. Advanced Studies)
Hideo Otsuna (Nagoya University)

The aim of this adjunct division, started in June 1998, is to understand the basic rules by which elaborate neural circuits develop and function. With less than 10^5 neurones, and subject to powerful molecular and genetic techniques, the brain of the fruit fly *Drosophila melanogaster* is a good model system for investigating the whole of an easily accessible nervous system that shares certain of the architectural and functional features of the more complex vertebrate brains. Third year of the five-year term, we continued a large-scale screening to find strains useful for this purpose.

I. Comprehensive identification of cells in the adult brain

A comprehensive and detailed anatomical knowledge of the brain is a prerequisite for 1) analysing the phenotypes of nervous system-related mutants, 2) identifying the cells that express cloned genes, 3) understanding the way information is processed in the brain, and 4) devising computer models that simulate brain functions. In spite of the hundred years of efforts using Golgi and other anatomical techniques, however, the circuit structure of higher order associative regions of the brain is still essentially unresolved. Moreover, traditional neuroanatomy tends to focus only on the mature adult brain, leaving the developmental processes largely uninvestigated. Since many nervous system-related mutants show structural defects, however, understanding the role of the responsible genes requires detailed basic knowledge about when and how the brain structure is formed. Thus, "developmental neuro-anatomy" becomes all the more important in the age of molecular cloning.

The GAL4 enhancer-trap system, which is widely used for mutagenesis and gene cloning of *Drosophila*, is also a powerful tool for obtaining a vast array of transformant strains that label specific subsets of brain cells. We screen such lines from a stock of 4500 GAL4 strains made by the "NP consortium", a joint venture of eight Japanese *Drosophila* laboratories organised by us. Our screening consists of two stages. In the first step, all the lines are crossed with the flies carrying the UAS-*GFP* transgene, which fluoresces only in the cells where GAL4 expression is active. The patterns of the GAL4-expressing cells are recorded from freshly dissected, unfixed adult brain tissue using a high-speed confocal microscope. Photographs of between 20 and 100 optical sections are taken for each line. As of December 2000, ca. 108,000 photographs depicting 3,400 of the total 4,500 strains have been accumulated in a computer database. In the second step, useful lines are selected from the database, and fixed and clealised brain specimens at various developmental stages are subjected to confocal serial sectioning with a conventional confocal microscope and to three-dimensional reconstruction with a UNIX workstation.

Although the long-term aim of this project is to identify as many neurones and glial cells as possible to get the comprehensive overview of the fly brain structure, at the initial stage a few brain regions are chosen for intensive study. The first target is to identify projection interneurons that connect lower-level sensory neuropile and higher-order associative regions. These fibres convey olfactory, gustatory, auditory and visual sensory information.

For visual pathways, we identified in total 30 types – among which 22 were novel – of projection neurones (in total ca. 500 cells) that connect lower-level sensory neuropile in the optic lobe to the higher order regions in the central brain (Fig. 1). For olfactory pathways, we identified 12 types of projection neurones (in total ca. 75 cells), that connect glomeruli in the first-order sensory neuropile of olfaction (olfactory lobe, antennal lobe) to the second-order processing cites (mushroom body and lateral horn). Further completion of such connection map would give us important insights about how sensory information is conveyed and integrated before association with signals of other sensory modalities.

II. Mapping of neurotransmitters and receptors in the adult brain

To understand the neural network of the brain, it is also important to reveal what kind of synapses is used in each identified neurone. For this purpose, information about the types of neurotransmitters and receptors is indispensable. Previously, such information is obtained by staining brain tissue with various antibodies. This approach, however, has the limitation due to the availability of good andibodies. Antibodies raised against transmitters or receptors of certain animal species do not always label corresponding molecule in other species.

Taking the advantage of the completion of *Drosophila* genome project, we thus employed a novel approach. Using homology and other information, we first search genes that code receptors or enzymes associated to transmitter synthesis. Cells that express those genes are labelled by using in-situ RNA hybridisation. This technique can visualise only the cell bodies of the labelled cells. We then screen GAL4 enhancer-trap strains that label cells in the corresponding area of the cortex, and perform double labelling to certify the colocalisation of GAL4

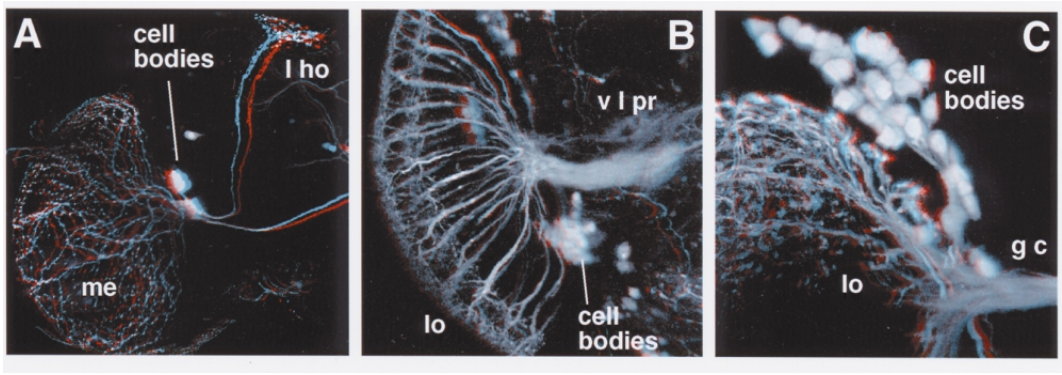

Fig. 1 Neurones that connect optic lobe and the central brain Three-dimensional reconstruction of about 200 confocal sections. Red-Cyan stereograph, posterior view of the whole-mount brain. To get 3-D image, use red filter for the left eye and green (or blue) filter for the right eye. A: Neurones that connect the outer layer of medulla (me) to lateral horn (l ho). A subset of fibres project to contralateral medulla. B: Columnar array of neurones that connect lobula (lo) and ventrolateral protocerebrum (v l pr). Note that arborisation occurs only at defined layers in the outer region of the lobula neuropile. C: Another type of array neurons that connect both sides of lobula via great commissure (g c). Arborisation occurs only in the inner region of lobula.

expression and in-situ label. This way, we can map the position and morphology of cells that use certain transmitters and receptors. First year of this project, we established a reliable and efficient protocol for in-situ staining of the dissected whole brain, and successfully visualised the expression pattern of three receptors and three enzymes associated with transmitter synthesis.

III. Analysis of the cell lineage-dependent modular structures in the brain

Combining the flippase-FRT recombination induction system and GAL4-UAS expression activation system, we have previously developed a novel technique – the " FRT-GAL4 system "– with which one can label a small number of neural stem cells at any desired developmental stage and reveal the projection patterns of their progeny at a later period.

The central brain of *Drosophila melanogaster* is produced by an average of 85 stem cells (neuroblasts) per hemisphere. We found that the majority of clones keep their cell bodies in tightly packed clusters. In 30 out of the 32 clone types identified so far, the neurites fasciculate to form a single bundle that runs from a cell body cluster, and innervate a limited number of neuropile regions in a stereotypic manner, forming clearly defined units of neural circuits. These suggest that in many cases the progeny of a single stem cell forms a lineage-dependent circuit structure unit, which we named a "clonal unit."

The clustering of clonal cell bodies and the fasciculation of neurites are already apparent in the developing larval brain. In larvae, glial cells form the border of clonal cell body clusters.

IV. Development of an improved red fluorescent protein (RFP) reporter system suitable for double labelling and birth order analysis of neural fibres

The green fluorescent protein (GFP) and its enhanced mutants are widely used to monitor protein localisation and gene expression of various organisms. Recently, red fluorescent protein, such as DsRed, was isolated from corals. Its significantly red-shifted emission and absorption maxima provide good complementation to GFP. Its use, however, is still restricted by its rather low quantum yield and parasitic green fluorescence peak that cross-talks with GFP emission. To overcome this problem, we developed a new variant of DsRed (DsRed S203Y), which is significantly brighter than the wild-type and free from green fluorescence peak, making it an ideal reporter for double labelling with GFP.

DsRed requires significantly longer fluorescence maturation time than GFP. By co-expressing GFP and DsRed under the control of the same promoter, time after the onset of gene expression can be monitored by its colour change from green (GFP only) to yellow (green by GFP + red by mature DsRed) over time. By using a GAL4 enhancer-trap strain that drives expression shortly after cells are born, it is possible to study birth order of the labelled cells. Though birth order analysis has been possible by pulse-chase labelling of DNA-replicating cells with ^{3}H-thymidine or bromodeoxyuridine (BrdU), the label was limited to cell bodies. Since GFP and DsRed spread even into fine cellular-protrusions, the GFP/DsRed system makes it possible to reveal birth order of fibre processes. We applied this to the developing mushroom body neurons of *Drosophila* brain and visualised, for the first time, the formation order of fibres within a neural fiber bundle (Fig. 2).

(This work is performed under collaboration with Drs. Vladislav V. Verkhusha, Hiroki Oda and Shoichiro Tsukita of ERATO Tsukita Cell Axis Project, Kyoto.)

V. Contribution to the science community

As a joint venture with German and US research groups, we maintain *Flybrain*, a web-based image database of the *Drosophila* nervous system (http://flybrain.nibb.ac.jp). Over 2000 images has

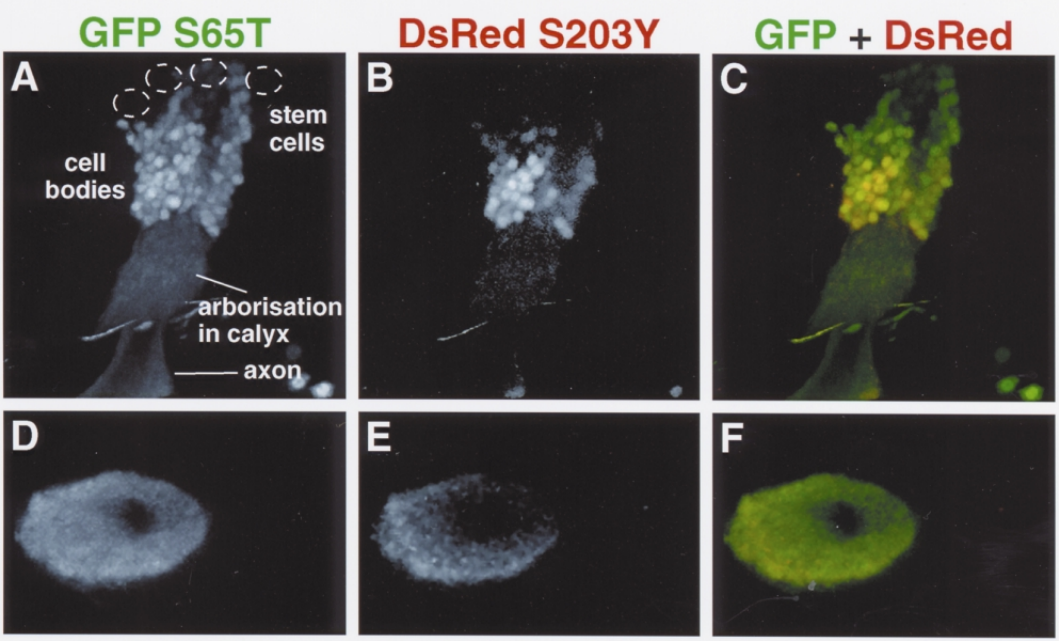

Fig. 2 Birth order analysis of neural fibres in the mushroom body Late third-instar larval brain of the GAL4 enhancer-trap strain *201y* expressing UAS-GFP S65T and UAS-DsRed S203Y. A-C: Reconstruction of horizontal optical sections. D-F: Vertical section of the axon bundle of pedunculus. GFP labels a majority of cell bodies in the larval mushroom body (A). Arborisation and axons are also labelled. Accordingly, most fibres are labelled in the section of the axon bundle (D). DsRed, on the other hand, labells only cell bodies that are far from the stem cells, which are older than those near the stem cells (B). In the section, Only fibres near the periphery of the bundle are labelled (E). Comparison of GFP and DsRed labelling (C, F) reveals that older fibres run in the periphery. Thus, it is likely that newly elongating fibres run into the core, rather than along the outer surface, of the fibre bundle.

already been stored and served worldwide. Another database maintained here is *Jfly*, which is intended to help the exchange of information among Japanese-speaking *Drosophila* researchers (http://jfly.nibb.ac.jp). Archives of research-related discussions, images, movies and experimental protocols, as well as meetings and job announcements, are provided.

Publication List:

Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Ito, K., Hama, C. (2000) The *Drosophilla* Trio plays an essential role in patterning of axons by regulating their directional extention . *Neuron.***26:** 119-131

Awasaki, T. and Kimura, K. (2001) Multiple function of *poxn* gene in larval PNS development and in adult appendage formation of *Drosophila*, *Dev. Genes. Evol.* **211:** 20-29

.