DIVISION OF BEHAVIORAL NEUROBIOLOGY

Professor
HIGASHIJIMA, Shin-ichi

Assistant Professor:	*KIMURA, Yukiko*
	TANIMOTO, Masashi
Technical Staff:	*TAKEUCHI, Yasushi*
JSPS Postdoctoral Fellow:	*SUZUKI, Daichi*
SOKENDAI Graduate Student:	*SUGIOKA, Takumi*
	KAWANO, Kohei
	SHIMIZU, Sayaka
Visiting Graduate Student:	*UEMURA, Yuto*
Visiting Undergraduate:	*KATAYAMA, Taisei*
Technical Assistant:	*TERASAWA, Yoko*
	WATAKABE, Ikuko
	ITO, Hiroko
	TAKEUCHI, Yoshiko
	SETO, Kumiko

The vertebrate central nervous system (CNS) contains many different types of neurons that form at distinct characteristic positions, and develop specific axonal connections and functions. This complexity has made it difficult to perform detailed functional analysis of neuronal circuits. In particular, it has been very difficult to reproducibly identify cell types. However, molecular genetic studies conducted over the past 15 years have strongly suggested that the expression of transcription factors in the developing CNS helps determine the morphological and functional properties of neurons. This has opened up the possibility that researchers can use these transcription factors as markers to identify cell types in the CNS. Transgenic animals that express fluorescent protein in specific subsets of neurons are particularly powerful tools in studying the functions of the corresponding neurons in the neuronal circuits.

To fully exploit the methodology described above, we use larval zebrafish as experimental animals. The biggest advantage of doing so is that larval zebrafish are almost completely transparent. This allows us to utilize many optical techniques, including morphological/functional imaging and optogenetics. We can also perform targeted in vivo electrophysiological recording with relative ease using this transparent model. An additional advantage of zebrafish is that their CNS is much simpler than that of mammals. This enables us to perform detailed functional analysis of neuronal circuits at a single cell resolution. Our hope is to reveal the operational principles of vertebrate CNS by using this simple system.

We have been focusing on studying neuronal circuits that control locomotion. Much of the control of locomotor movements is accomplished by neuronal circuitry located in the spinal cord. Therefore, the focus of our studies has been spinal neuronal circuits in larval zebrafish.

In addition to zebrafish, we have also started to use medaka as experimental animals. Medaka have many advantages that are similar to those of zebrafish. Because NIBB is the main hub of the Medaka National Bioresource Project, we are ideally located in regards to experiments using medaka. To begin with, we explored whether knock-in fish could be efficiently generated using the CRISPR/Cas9 technique.

I. Generation of Transgenic zebrafish

We have been generating transgenic zebrafish that express fluorescent proteins (GFP or RFP), Gal4, or Cre in specific classes of neurons in the CNS by using gene promoters/enhancers of genes and are known to be expressed in subsets of neurons. Most of the genes we used are transcription factors expressed in subsets of neurons in the developing CNS. We also used genes whose expressions are tightly related to neurotransmitter properties of neurons (*i.e.*, vesicular glutamate transporter).

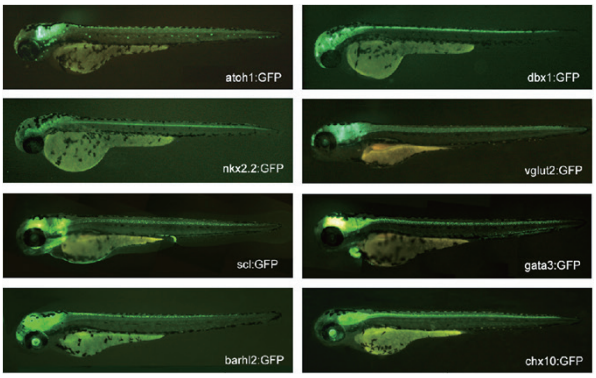

Figure 1. Examples of transgenic fish expressing GFP in specific classes of neurons.

In our early studies, we used a BAC-based transgenic technique for the generation of transgenic fish. By 2014, we succeeded in establishing a reliable knock-in method by utilizing the CRISPR-Cas9 system. The method we have developed is highly efficient, so much so that nearly one-third of the animals we raise become transgenic founders. Thus far, we have established more than 20 knock-in transgenic fish. Thus, this method greatly facilitates our functional analysis on neuronal circuits.

II. Neuronal circuits that control rhythmic pectoral fin movements

Limbed vertebrates exhibit coordinated rhythmic movements of the forelimbs and hindlimbs during locomotion. Neuronal circuits that control rhythmic limb movements in mammals have been investigated for decades, but our knowledge of them is still limited because of the complexity of their limbs. In this case, rhythmic movement of pectoral fins during swimming in larval zebrafish is an attractive model (Figure 2). The pectoral fins of larval zebrafish show left and right alternated rhythmic movements, and they are actuated only by two types of muscles: the abductor (Ab) and the adductor (Ad) (Figures 3). Due to the simplicity of pectoral fins, we expect that we will be able to characterize neuronal

circuits that control rhythmic pectoral fins in more detail.

We performed electrophysiological recordings of Ab motoneurons (MNs) and Ad MNs during fictive swimming. Both Ab MNs and Ad MNs show rhythmic spiking activities (Figure 4). Activities of Ab MNs and Ad MNs on the same side alternated. Voltage clamp recordings showed that both Ab MNs and Ad MNs received alternating excitatory and inhibitory inputs during swimming cycles. Excitation mainly occurred in their preferential firing phase, and inhibition mainly occurred for the rest of the period. To obtain insights into the source of these inputs, we are now investigating the timing of spiking activities in possible premotor interneurons.

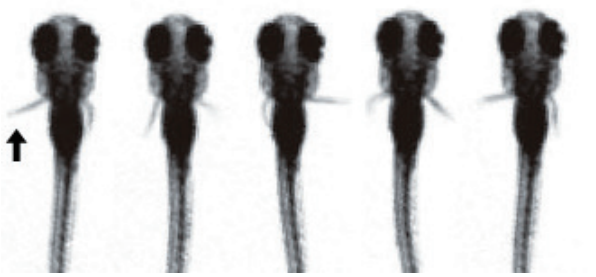

Figure 2. Rhythmic movements of the pectoral fin (arrow) during swimming in larval zebrafish.

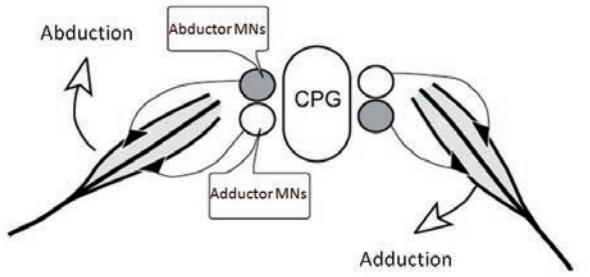

Figure 3. Schematic of rhythmic movements of pectoral fins during swimming. CPG, Central Pattern Generator.

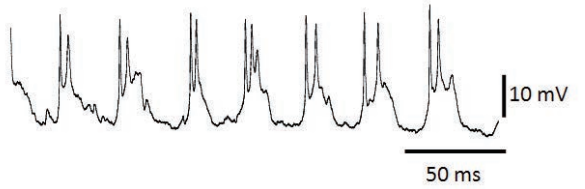

Figure 4. Rhythmic firings of an abductor motoneuron during swimming.

III. *In vivo* functional imaging analysis of the vestibular sensory organ

Maintaining head and body orientation relative to the Earth's vertical gravity axis is vital for survival. Vestibular organs in the inner ear play a crucial role for this task. Sensory hair cells in the otolith organs receive linear acceleration, *e.g.*, head tilt, translation and vibration. Direction of the acceleration is detected by the polarized arrangement of hair bundles in the hair cells (Figure 5). Each otolith organ contains hair cells with different but topographically organized hair-bundle polarity that reverses at a line of polarity reversal (LPR). Although morphofunctional specialization of the vestibular hair cells has been widely studied, the direction- and modality-selective responses to the head motion have not been systematically studied *in vivo*, therefore how the head motion signals are processed in the vestibular system remains unclear.

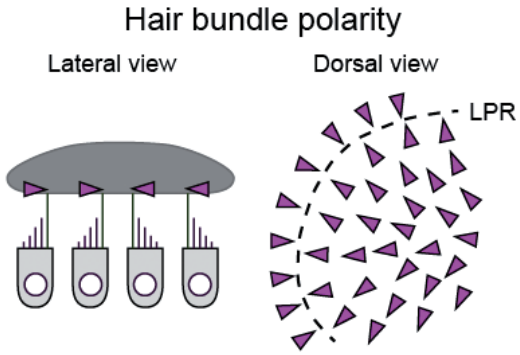

Figure 5. Hair bundle polarity (arrowheads) in the utricle.

To visualize hair cell responses to the head motion, we built a microscope in which an objective lens can tilt with a small sample 360 degree by a motorized stage during Ca^{2+} imaging (Figure 6). A spinning-disk confocal scanner and an image splitting optics formed green and red fluorescent images on a digital camera. This ratiometric imaging setup reduced artifacts derived from non-uniformity of the excitation light and optical distortion during the optics motion. With this tiltable objective microscope, we imaged neural activity in all the utricular hair cell at the single-cell level during pitch or roll tilt/vibration in 5-day-old transgenic zebrafish larvae expressing Ca^{2+} indicator, jGCaMP7f, and red fluorescent protein, tdTomato, in the hair cells.

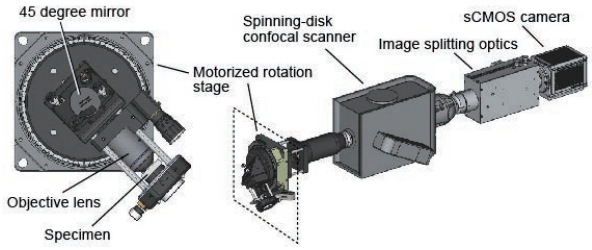

Figure 6. Tiltable objective microscope.

Consistent with the morphological hair-bundle polarity, hair cells medial to the LPR are activated by the lateral-down roll tilts, whereas those lateral to the LPR are activated by the medial-down tilts (Figure 7). In response to the nose-down pitch tilts, hair cells medial to the LPR in the rostral utricle and those lateral to the LPR in the caudal utricle are activated, whereas the rest of the hair cells are activated by the tail-down tilts. Interestingly, hair cells in the medial utricle exhibited larger responses to the head tilt compared to the lateral hair cells. In contrast to the responses to the head tilt, the vibratory stimulus in the pitch or roll axis activated the hair cells only in the rostral and lateral utricle near the LPR.

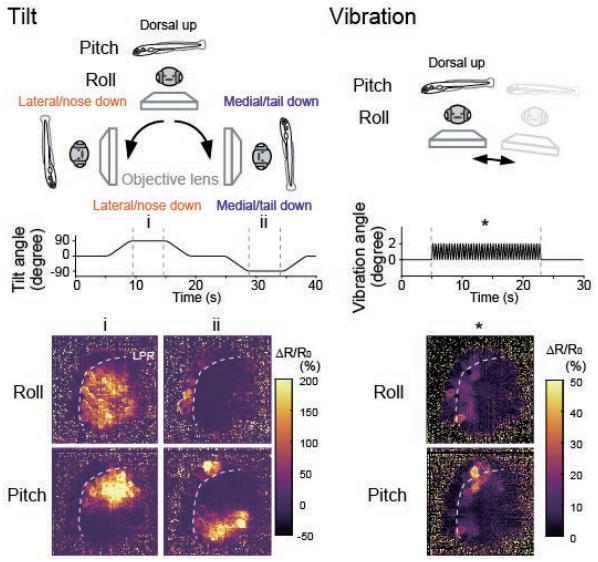

Figure 7. Hair cell responses to head tilt or vibration in the utricle

Together, the tiltable objective microscope visualized, for the first time to our knowledge, the topographically organized response selectivity for the stimulus direction and modality in the vestibular periphery. The imaging strategy we have established here is applicable to the central nervous system, and thus it will provide deeper understanding of the vestibular processing in the brain.

IV. Functional diversity of glycinergic commissural inhibitory neurons in larval zebrafish

Coordinated movements of the left and right side of the body is critical for any types of locomotion including walking, flying and swimming. The coordination is mainly mediated by commissural neurons located in the spinal cord. In fish swimming, it is known that commissural inhibitory neurons in the spinal cord ensure left-right alternating movements. The developmental origin of these commissural inhibitory neurons, however, has been elusive. We investigated anatomy and function of two commissural inhibitory neuron types, dI6*dmrt3a* and V0d, derived from the pd6 and p0 progenitor domains, respectively. We found that both of these commissural neuron types have monosynaptic, inhibitory connections to neuronal populations active during swimming, supported their role in providing inhibition to the contralateral side. V0d neurons tend to fire during faster and stronger movements, while dI6*dmrt3a* neurons tend to fire more consistently during swimming. Ablation of dI6*dmrt3a* neurons leads to an impairment of left-right alternating activity through abnormal co-activation of motor neurons on both sides of the spinal cord. Our results suggest that dI6*dmrt3a* and V0d commissural inhibitory neurons synergistically provide inhibition to the opposite side across different swimming behaviors (Figure 8).

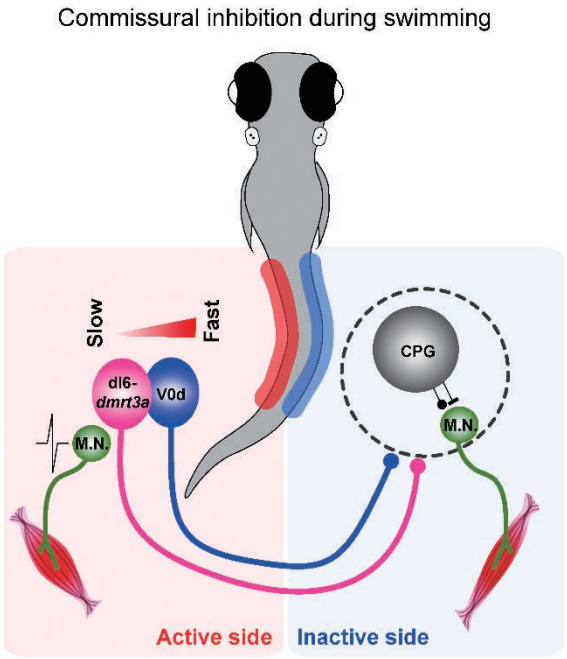

Figure 8. Summary of the proposed role of dI6*dmrt3a* and V0d neurons during swimming. CPG, central pattern generator

Publication List:

〔Original Papers〕

- Liu, Z., Kimura, Y., Higashijima, S., Hildebrand, D.G.C., Morgan, J.L., and Bagnall, M.W. (2020). Central vestibular tuning arises from patterned convergence of otolith afferents. Neuron *108*, 748+. DOI: 10.1016/j.neuron.2020.08.019
- Mizoguchi, T., Fukada, M., Iihama, M., Song X., Fukagawa, S., Kuwabara, S., Omaru, S., Higashijima, S., and Itoh, M. (2020). Transient activation of the Notch-her15.1 axis plays an important role in the maturation of V2b interneurons. Development *147*. DOI: 10.1242/dev.191312
- Uemura, Y., Kato, K., Kawakami, K., Kimura Y., Oda, Y., and Higashijima, S. (2020). Neuronal circuits that control rhythmic pectoral fin movements in zebrafish. J. Neurosci. *40*, 6678–6690. DOI: 10.1523/JNEUROSCI.1484-20.2020