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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 was very difficult to reproducibly identify cell types during investigation. For the past 15 years, however, molecular genetic studies 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 to study 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 this system 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 recordings with relative ease in this transparent model. An additional advantage of zebrafish is that their CNS is much simpler than mammals. This enables us to perform detailed functional analysis of neuronal circuits at a single cell resolution. Our hope is to reveal 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.

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 CNS by using promoter/enhancer of genes that 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 those genes whose expressions are tightly related to neurotransmitter properties of neurons (i,e, vesicular glutamate transporter).

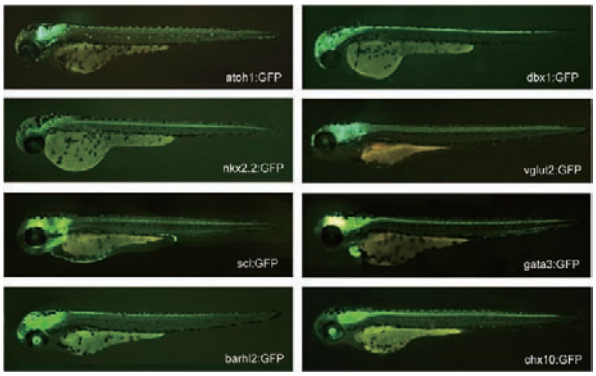

Figure 1. Examples of transgenic fish expressing GFP in specific classes of neurons.

In our early studies, we used a BAC-based transgenic technique for the generation of transgenic fish. In 2014, we succeeded in establishing a reliable knock-in method by utilizing the CRISPR-Cas9 system. The method we developed is highly efficient, such that nearly one-third of the raised animals become transgenic founders. Thus far, we have established more than 20 knock-in transgenic fish. The 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 forelimbs and hindlimbs during locomotion. Neuronal circuits that control rhythmic limb movements in mammals have been investigated for decades, but our knowledge is still limited because of the complexity of their limb. Rhythmic movements of pectoral fins during swimming in larval zebrafish is an attractive model (Figure 2). Pectoral fins of larval zebrafish show left and right alternated rhythmic movements, and they are actuated only by two types of muscles, i.e., abductor (Ab) and adductor (Ad) (Figures 3). Due to the simplicity of pectoral fins, we expect that we well be able to characterize neuronal circuits that control rhythmic pectoral fin more deeply.

We performed electrophysiological recordings of Ab motoneurons (MNs) and Ad MNs during fictive swimming. Both Ab MNs and Ad MNs show rhythmic spiking activitie (Figure 4). Activities of Ab MNs and Ad MNs on the same side essentially alternated. Voltage clamp recordings showed that both Ab MNs and Ad MNs received alternating excitatory and inhibitory inputs in a swimming cycle. Excitations 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 timings of spiking activities in possible premotor interneurons.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2016. The former title is indicated by an asterisk (*).

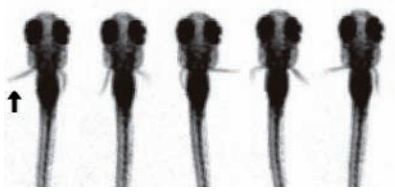

Figure 2. Rhythmic movements of the pectoral fin (arrow) during swimming in larval zebrafish.

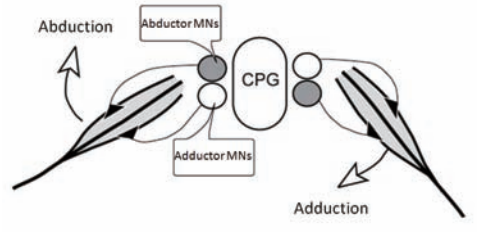

Figure 3. Schematic of rhythmic movements of pectoral fins during swimming. CPG, Central Pattern Generator.

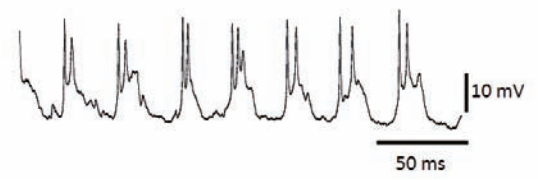

Figure 4. Rhythmic firings of an abductor motoneuron during swimming.

III. Functional analysis of En1-positive neurons for axial movements during swimming.

Inhibitions play important roles for shaping motor outputs during locomotion. In the spinal cord of larval zebrafish, there are mainly two types of inhibitory neuron: commissural inhibitory neurons and ipsilaterally-projecting inhibitory neurons. The role of the former (commissural inhibitory neurons) is easy to understand: they are likely to play important roles for ensuring antagonistic movements of the left and the right side of body. The role of the latter (ipsilaterally-projecting inhibitory neurons) is less clear.

En1-positive neurons constitute major components of ipsilaterally-projecting inhibitory neurons in the spinal cord. To investigate the function of En1-positive neurons, we have genetically-ablated En1-positive neurons by using the Cre-loxP system (Figure 5A). In the resultant larvae, the cycle period for the rhythmic bending of the body was prolonged (Figure 5B), indicating that swimming speed was slowed down. The results show that En1-positive neurons play an important role for controlling locomotion speed.

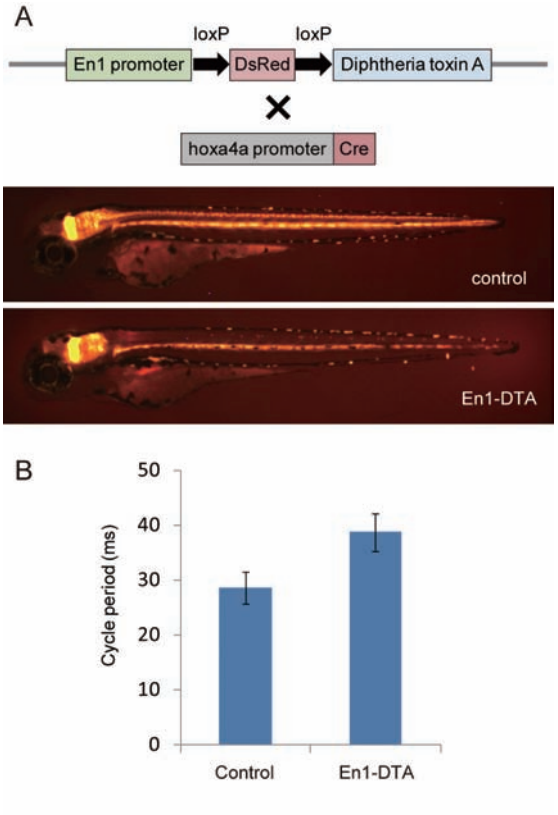

Figure 5. Spinal En1 neurons play an important role for controlling swimming speed. A, Ablation of spinal En1 neurons by using the Cre-loxP system. DTA, diphtheria-toxin A. B, Cycle period of the motor bursts during regular swimming in control and En1-DTA larvae.

Publication List:

〔Original Papers〕

- Chou, M.-Y., Amo, R., Kinoshita, M., Cherng, B.-W., Shimazaki, H., Agetsuma, M., Shiraki, T., Aoki, T., Yamazaki, M., Higashijima, S., and Okamoto, H. (2016). Social conflict resolution regulated by two dorsal habenular subregions in zebrafish. Science *352*, 87-90.
- Ota, S., Taimatsu, K., Yanagi, K., Namiki, T., Ohga, R., Higashijima, S., and Kawahara, A. (2016). Functional visualization and disruption of targeted gene using CRISPR/Cas9-mediated eGFP reporter integration in zebrafish. Sci. Rep. *6*, 34991.