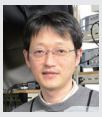
DIVISION OF BEHAVIORAL NEUROBIOLOGY



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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 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 system.

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).

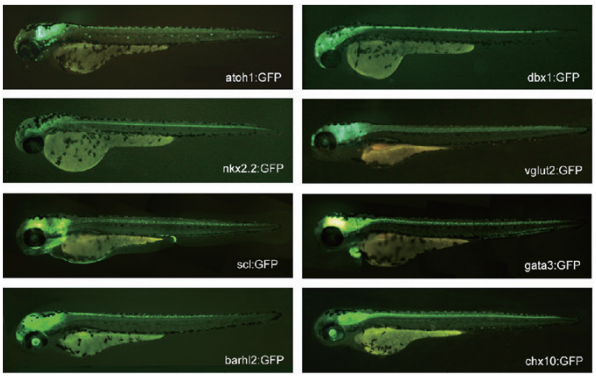

Figure 1. Examples of transgenic fish expressing GFP in specific classes of neurons.

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.

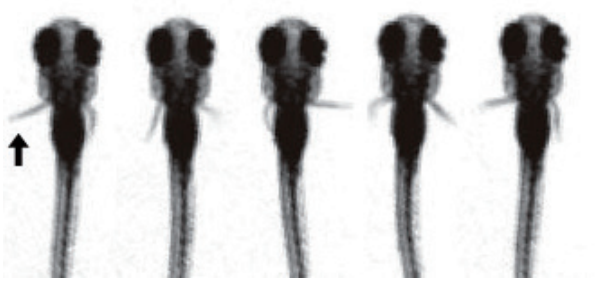

Figure 2. Rhythmic movements of the pectoral fin (arrow) during swimming in larval zebrafish.

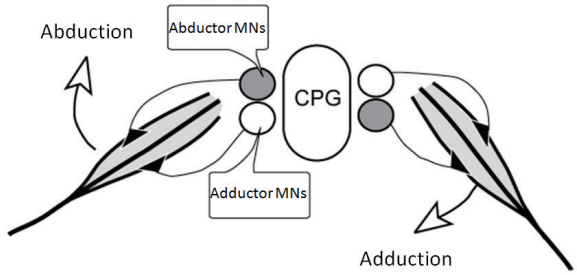

Figure 3. Schematic of rhythmic movements of pectoral fins during swimming. CPG, Central Pattern Generator.

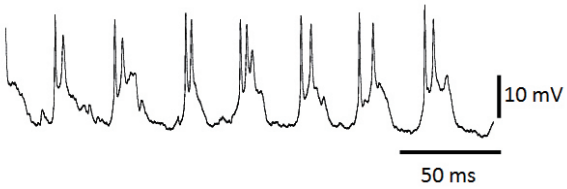

Figure 4. Rhythmic firings of an abductor motoneuron during swimming.

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

Inhibition plays an important role in shaping motor outputs during locomotion. In the spinal cord of larval zebrafish, there are mainly two types of inhibitory neurons: 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 an important role in ensuring antagonistic movements of the left and 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 had slowed down. The results show that En1-positive neurons play an important role for controlling locomotion speed.

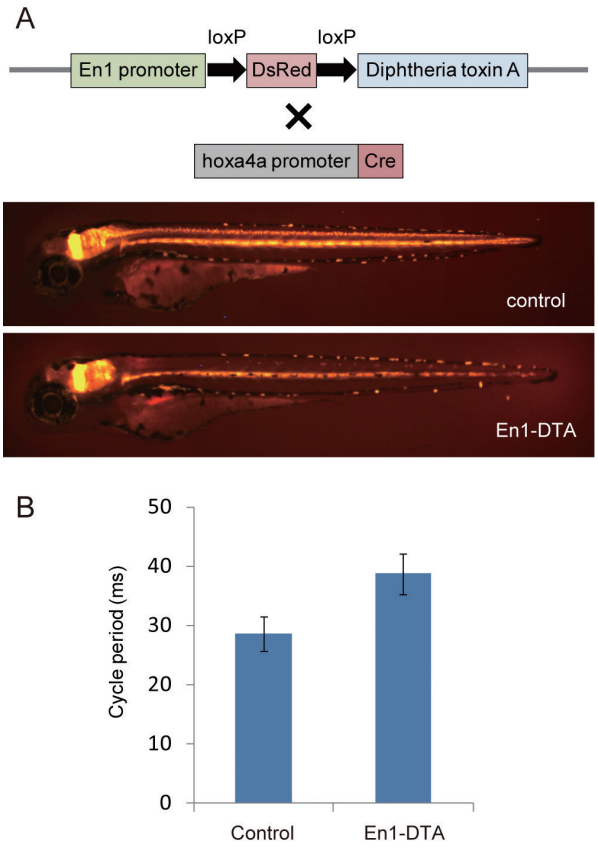

Figure 5. Spinal En1 neurons play an important role for controlling swimming speed. A, Ablation of spinal En1 neurons by using the Cre-loxP system. DTA, diphtheria-toxin A. B, Cycle period of the motor bursts during regular swimming in control and En1-DTA larvae.

IV. Behavioral role of the reciprocal inhibition between a pair of Mauthner cells during fast escapes in zebrafish

Vertebrates possess a bilateral CNS consisting of left and right sides of the brain and spinal cord. Whereas the bilateral CNS works symmetrically in some behaviors, in many cases it works asymmetrically between the left and right sides, such as when animals swim, walk, run, or perform lateralized movements. The reciprocal inhibition between the left and right sides is believed to play a key role in the asymmetrical activation of the bilateral CNS. However, despite the importance of reciprocal inhibition in the control of vertebrate behaviors, the identification of reciprocal inhibition circuits at the individual cell level and the contribution of each neuron to the asymmetric activity is still quite limited, largely because multiple circuits may participate in shaping motor output, making it difficult to identify circuit neurons, and to evaluate how each neuron contributes to producing these movements.

Teleost fish's fast escape response to sudden stimuli provides a typical lateralized behavioral model for investigating network organization and the function of reciprocal inhibition because the principal circuits from sensory inputs to motor outputs are simple and identifiable. The behavior is also basic enough that it is possible to evaluate the contribution of the key neurons. A pair of giant retic-

ulospinal neurons in the hindbrain, Mauthner (M) cells, are known to trigger the escape response, especially in response to sound/vibration stimuli. When one of the paired M-cell fires, it directly or indirectly activates the spinal motoneurons of the contralateral trunk muscles. Thus, the single spiking of an M-cell induces the contraction of trunk muscle exclusively along the axis on the contralateral side. However, sound/vibration stimuli may activate both M-cells because it is received by both ears directly as well as through the swim-bladder. Nevertheless, fish exhibit a C-bend and consistently escape in one direction. For that to occur, it has been believed that the reciprocal inhibition between the two M-cells play a critical role. It is known that there is a reciprocal inhibition between M-cells mediated by cranial relay neurons (CRNs) that receive excitatory inputs from M-axons and excite glycinergic interneurons contacting the contralateral M-cell. The role of the reciprocal inhibition for M-cell excitability and escape behavior, however, is unclear because the number and location of CRNs that mediate the reciprocal inhibition is unknown.

In the present study, we identified two paired CRNs in a GFP-expressing transgenic line of zebrafish larvae and examined the effects of their elimination on M-cell firing and sound/vibration-evoked escape behavior. We firstly demonstrated that two paired CRNs located in the posterior hindbrain largely mediate the reciprocal inhibition of M-cells, and secondly showed that the reciprocal inhibition plays a critical role in preventing bilateral firing of M-cells, thereby allowing for full flexion of the C-bend during escape.

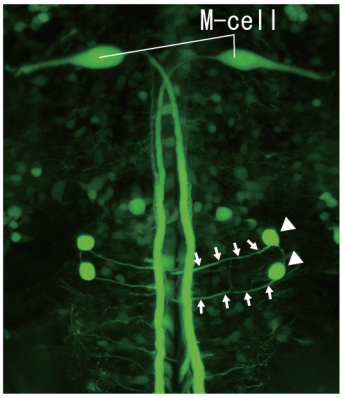

Figure 6. The Tol-056 enhancer trap line labels CRN neurons. In the enhancer trap line, GFP expression is present in a pair of M-cells in the hindbrain. In addition, GFP is expressed in a pair of two relatively large neurons (12-13 μm in diameter) in the caudal hindbrain (arrowheads). Prominent commissural axons arise from these two cells (arrows). These neurons are CRN neurons.

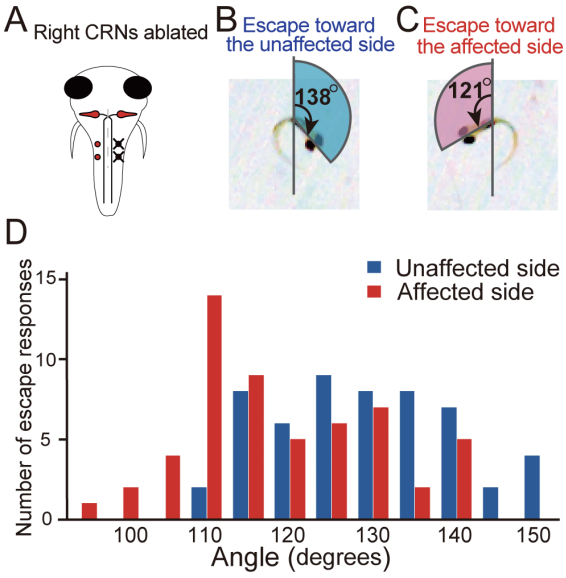

Figure 7. Behavioral experiments in the animals in which CRN neurons are unilaterally ablated. A, Schematic showing that CRN neurons on the right side are ablated. B, C, Examples of escape turns in an animal in which CRN neurons on the right side were ablated. (A) shows an example of a right turn (unaffected side), while (B) shows an example of a left turn (affected side). D, Histograms of the maximum bend angles collected from all the trials. Escapes turns toward the ablated side are categorized as “unaffected side” (blue) and those toward the opposite side are categorized as “affected side” (red).

Publication List:

〔Original Paper〕

- Watakabe, I., Hashimoto, H., Kimura, Y., Yokoi, S., Naruse, K., and Higashijima, S. (2018). Highly efficient generation of knock-in transgenic medaka by CRISPR/Cas9-mediated genome engineering. Zool. Lett. *4*, 3.

〔Original paper (E-publication ahead of print)〕

- Shimazaki, T., Tanimoto, M., Oda, Y., and Higashijima, S. Behavioral role of the reciprocal inhibition between a pair of Mauthner cells during fast escapes in zebrafish. J. Neurosci. 2018 Dec 23.