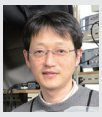
DIVISION OF BEHAVIORAL NEUROBIOLOGY



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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 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).

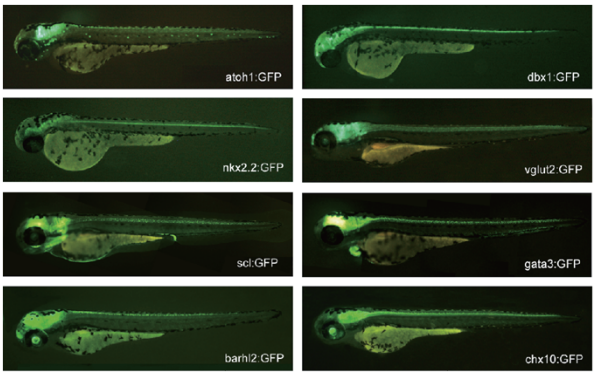

Figure 1. Examples of transgenic fish expressing GFP in specific classes of neurons.

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.

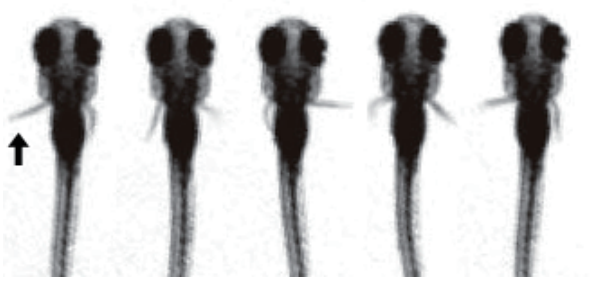

Figure 2. Rhythmic movements of the pectoral fin (arrow) during swimming in larval zebrafish.

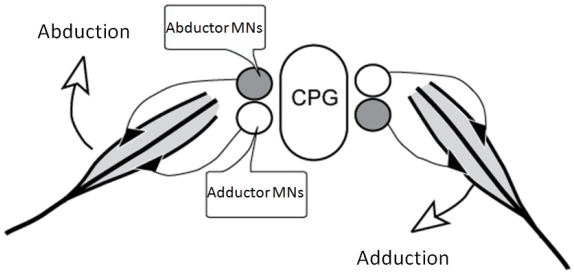

Figure 3. Schematic of rhythmic movements of pectoral fins during swimming. CPG, Central Pattern Generator.

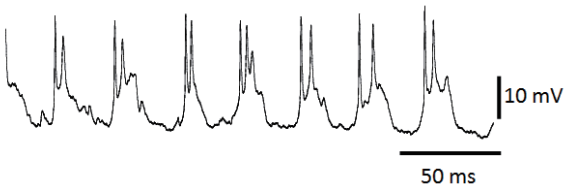

Figure 4. Rhythmic firings of an abductor motoneuron during swimming.

III. Functional analysis of V1 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 (see Section IV). The role of the latter (ipsilaterally-projecting inhibitory neurons) is less clear.

V1 neurons are one class of neurons that are defined by the expression of a transcription factor En1 and constitute major components of ipsilaterally-projecting inhibitory neurons in the spinal cord. To investigate the function of V1 neurons, we genetically ablated spinal V1 neurons in larval zebrafish using diphtheria toxin A (En1-DTA fish, Figure 5). From the analysis of the En1-DTA fish, we revealed two roles of V1 neurons.

First, we found that the swimming frequency was declined in the En1-DTA fish (Figure 6). The results show that V1

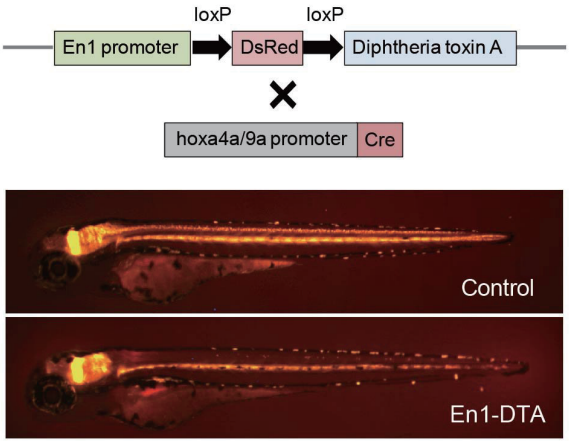

Figure 5. Ablation of spinal V1 neurons by using the Cre-loxP system. DTA, diphtheria-toxin A.

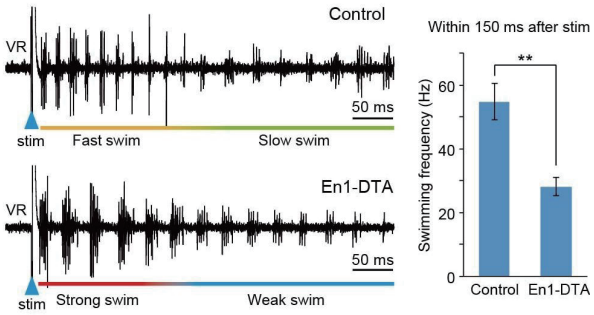

Figure 6. Ventral root recordings of fictive swimming elicited by electrical stimulations (stim) in a control and an En1-DTA fish (left). Swimming frequency of control and En1-DTA fish during the initial phase of swimming elicited by electrical stimulation (right).

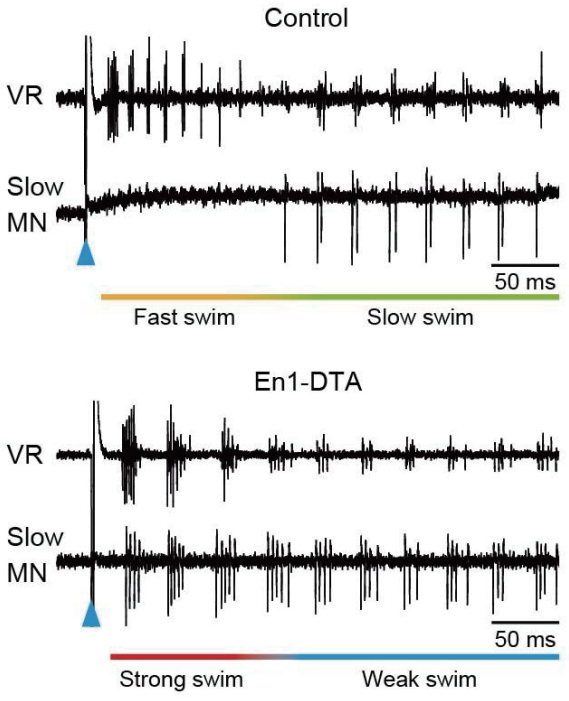

Figure 7. Activity of slow type motoneurons (MNs) in control and En1-DTA fish during fictive swimming.

neurons play an important role for controlling locomotion speed.

Second, we showed that V1 neurons play an important role in the selection of active sets of neurons. Slow-type motor neurons, which project slow muscle, are known to be inactive during strong/fast movements in larval zebrafish. In the En1-DTA fish, however, we found that slow-type motor neurons were vigorously active during strong movements (Figure 7). We investigated the mechanism underlying this phenotype. In the wild type, slow-type MNs were found to receive strong inhibitory inputs. In the En1-DTA fish, these strong inhibitory inputs were found to be absent. The results indicate that V1 neurons are the source of the strong inhibition onto slow-type MNs and that the inhibition play a crucial role in suppressing spiking activities of slow-type MNs during strong/fast swimming.

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).

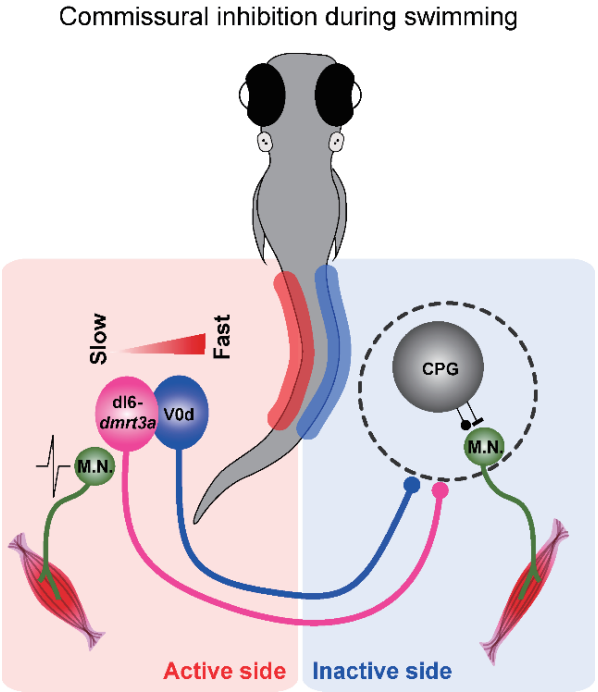

Figure 8. Summary of the proposed role of dI6*dmrt3a* and V0d neurons during swimming. CPG, central pattern generator

Publication List:

〔Original Papers〕

- Callahan, R.A., Roberts, R., Sengupta, M., Kimura, Y., Higashijima, S.-I., and Bagnall, M.W. (2019). Spinal V2b neurons reveal a role for ipsilateral inhibition in speed control. eLife *8*, e47837. doi: 10.7554/eLife.47837
- Frank, T., Mönig, N.R., Satou, C., Higashijima, S.-I., and Friedrich, R.W. (2019). Associative conditioning remaps odor representations and modifies inhibition in a higher olfactory brain area. Nat. Neurosc. *22*, 1844-1856. doi: 10.1038/s41593-019-0495-z
- Kimura, Y., and Higashijima, S.-I. (2019). Regulation of locomotor speed and selection of active sets of neurons by V1 neurons. Nat. Commun. *10*, 2268. doi: 10.1038/s41467-019-09871-x
- Satou, C., Sugioka, T., Uemura, Y., Shimazaki, T., Zmarz, P., Kimura, Y., and Higashijima, S.-I. (2020). Functional diversity of glycinergic commissural inhibitory neurons in larval zebrafish. Cell Rep. *30*, 3036-3050. doi: 10.1016/j.celrep.2020.02.015
- Shimazaki, T., Tanimoto, M., Oda, Y., and Higashijima, S. (2019). Behavioral role of the reciprocal inhibition between a pair of Mauthner cells during fast escapes in zebrafish. J. Neurosci. *39*, 1182-1194. doi: 10.1523/JNEUROSCI.1964-18.2018