PERSPECTIVE

Function of pioneer neurons specified by the basic helix-loop-helix transcription factor atonal in neural development

Basic helix-loop-helix (bHLH) transcription factors regulate the differentiation of various tissues in a vast diversity of species. The bHLH protein Atonal was first identified as a proneural gene involved in the formation of mechanosensory cells and photoreceptor cells in *Drosophila* (Jarman et al., 1993, 1994). Atonal is expressed in sensory organ precursors and is required and sufficient for the development of chordotonal organs (Jarman et al., 1993). Moreover, Atonal expression is observed in the developing eye and is essential for the differentiation of R8 photoreceptors, which are the first photoreceptors that appear during development. Atonal is not involved in the formation of other photoreceptors (R1–R7) directly. However, R8 photoreceptors recruit other photoreceptors from the surrounding cells (Jarman et al., 1994).

The roles of Atonal as a proneural gene are conserved throughout evolution. In vertebrates, Atonal orthologs are classified into three gene families: neurogenin genes (*Neurog1*, *Neurog2*, and *Neurog3*), neurogenic differentiation genes (*NeuroD1*, *NeuroD2*, *NeuroD4*, *NeuroD6*, *Atoh1*, and *Atoh7*), and Olig genes (*Olig1*, *Olig2*, and *Olig3*) (Huang et al., 2014). For example, ATOH7, one of the most closely related human orthologs of Atonal is expressed in the progenitors of retinal ganglion cells (RGCs) and is essential for RGC differentiation and retinal development. *Atoh7* mutant mice lack optic nerves and have a reduction in the numbers of RGCs, which affects the development of their retinal vasculature (Huang et al., 2014). Highlighting the importance of ATOH7 function in retinal development, mutations of *ATOH7* have been identified in populations with congenital diseases of the optic nerve and retinal vasculature, such as non-syndromic congenital retinal nonattachment (NCRNA) in the Iranian Kurdish population (Ghiasvand et al., 2011). Individuals with NCRNA lack optic nerves, are totally blind, and have no perception of light.

In addition, Atonal-related genes regulate the differentiation of olfactory receptor neurons (ORNs). In *Drosophila*, *Atonal* is a proneural gene that acts on a subset of ORNs (Gupta and Rodrigues, 1997). In mammals, a number of bHLH transcription factors, such as Mammalian achaete-scute homologue 1 (Mash1), Neurogenin1, and NeuroD, are required for the differentiation of olfactory sensory neurons (OSNs) (Cau et al., 2002). It is suggested that ORNs specified by Atonal pioneer antennal lobe development in *Drosophila*. However, the detailed mechanism underlying this process has not yet been fully understood.

We recently reported the hierarchical axon targeting of ORNs specified by the bHLH transcription factors Atonal and Amos (Okumura et al., 2016). In *Drosophila*, ORNs whose cell bodies are located at the sensilla on the surface of antennae and maxillary palps detect odors and send olfactory information to the antennal lobe in the brain (**Figure 1A**). There are four types of sensilla: basiconic, trichoid, coeloconic, and intermediate sensilla. While Atonal specifies coeloconic sensilla on the antenna and basiconic sensilla on the maxillary palp, Amos specifies basiconic and trichoid sensilla on the antenna. There are about 200 Atonal ORNs (ORNs specified by Atonal) and 1,000 Amos ORNs (ORNs specified by Amos) on each side. The axons of ORNs that express the same olfactory receptors innervate a single glomerulus out of approximately 50 glomeruli in the antennal lobes. The axons of Atonal ORNs and Amos ORNs target the posterior and anterior parts of the antennal lobe, respectively. To understand the function of Atonal ORNs in the regulation of antennal lobe development, we eliminated Atonal ORNs using *atonal* mutants or a genetic cell ablation system that induces cell death specifically in Atonal ORNs. Even though the axons of Atonal ORNs innervate only the posterior part of the antennal lobe, the whole antennal lobe structure, including the glomeruli targeted by Amos ORN axons, was disorganized. In these animals, the glomeruli were barely distinguishable because of their fuzzy glomerular borders (**Figure 1B**). We also observed axon targeting of Amos ORNs when we ablated Atonal ORNs. Loss of Atonal ORNs led to the axon mistargeting of Amos ORNs within the antennal lobe, the loss of axon commissure formation, and axon accumulation outside of the antennal lobe (**Figure 1B**). In contrast, the ablation of Amos ORNs did not affect the axon targeting of Atonal ORNs, although the numbers of Amos ORNs were much larger than those of Atonal ORNs. These results suggest that Atonal ORNs are necessary for the formation of the whole antennal lobe structure and the correct targeting of Amos ORNs, and that Amos ORNs are not essential for the axon targeting of Atonal ORNs.

As Atonal ORNs control the development of the whole antennal lobe and the axon targeting of Amos ORNs, we hypothesized that the innervation of the antennal lobe by Atonal ORNs occurs earlier than innervation by Amos ORNs. To examine this hypothesis, we investigated the developmental timing of Atonal ORNs and Amos ORNs by labeling both types of ORNs simultaneously. As expected, the axons of Atonal ORNs arrive at the antennal lobe and form axon commissures earlier than those of Amos ORNs.

To reveal the underlying molecular mechanism of the hierarchical axon targeting of Atonal and Amos ORNs, we focused on N-cadherin, a classical cadherin that is highly expressed in the antennal lobe during the pupal and adult stages. We knocked down the expression of *N-cadherin* in Atonal ORNs specifically and examined the axon targeting of Amos ORNs. Knockdown of *N-cadherin* in Atonal ORNs caused the axon mistargeting of Atonal ORNs, the disorganization of the entire antennal structure, the mislocalization of Brp (presynaptic marker), and the defasciculation of Amos ORN axons. These results suggest that N-cadherin expression in Atonal ORNs is required for antennal lobe formation and the axon fasciculation of Amos ORNs.

We have thus demonstrated the hierarchical axon targeting of Atonal ORNs and Amos ORNs, and have determined that N-cadherin is involved in this process. How do Atonal ORNs affect the axon targeting of Amos ORNs? One possibility is that the axons of Atonal ORNs and those of Amos ORNs interact directly. Even though the targeting glomeruli of Atonal ORNs and Amos ORNs are separated within the antennal lobe at the adult stage, the ORN axons elongate within a relatively broad region during development. Therefore, Atonal ORNs and Amos ORNs might interact with each other directly via membrane proteins, such as N-cadherin. Another possibility is that the axons of Atonal ORNs extend to the antennal lobe earlier to express secreted molecules or guidance molecules, which are then used for the axon targeting of Amos ORNs. So far, no such guidance molecules expressed by Atonal ORNs have been identified. However, in the mouse olfactory system, the late-arriving OSNs express an axon guidance receptor, Nrp2, and early-arriving OSNs secrete Sema3F, which is a repulsive ligand of Nrp2 that is required for the correct projection of the late-arriving OSNs (Takeuchi et al., 2010). Thus, Atonal ORNs might support the axon targeting of Amos ORNs by secreting guidance molecules. The genetic system we have established will help us to understand the underlying molecular mechanism of the hierarchical axon targeting of Atonal and Amos ORNs.

Several lines of evidence support the idea that cells specified

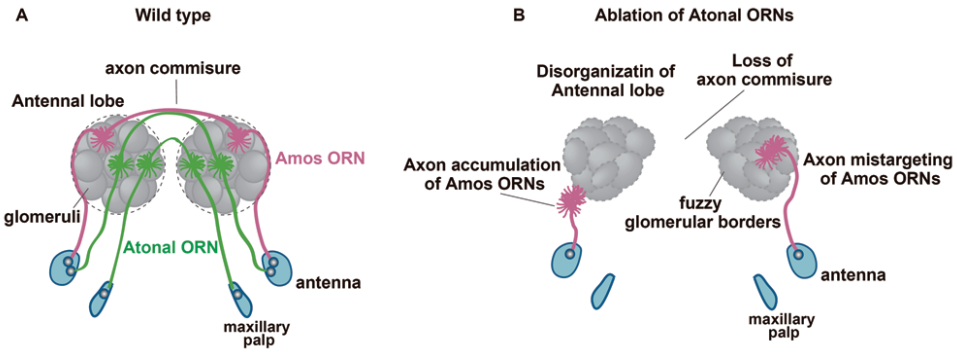

Figure 1 Atonal olfactory receptor neurons (ORNs) are essential for the whole antennal lobe structure and the axon targeting of Amos ORNs.
(A) The schematic image of *Drosophila* ORNs. The cell bodies of Atonal ORNs (green) are located on the antenna and maxillary palp and elongate their axons to the posterior glomeruli the antennal lobe in the brain. Each grey circle represents individual glomeruli in the antennal lobe. The cell bodies of Amos ORNs (magenta) are on the antenna and the axons of Amos ORNs target the anterior glomeruli. Both the axons of Atonal and Amos ORNs extend to the contralateral antennal lobe to form axon commissure. (B) The ablation of Atonal ORNs affects the whole antennal lobe structure and the axon targeting of Amos ORNs. The whole antennal lobe structure was disrupted with fuzzy glomerular borders and the Amos ORNs target their axons to the incorrect glomeruli or the axons accumulate outside of the antennal lobe. The loss of axon commissure in Amos ORNs is also observed.

by Atonal are pioneering cells during development and are involved in the development of other cells. As we have shown, Atonal ORNs are the early-arriving ORNs and affect the development of the late-arriving Amos ORNs. In *Drosophila* eye development, Atonal is expressed in the progenitors of R8, which is the first photoreceptor generated in each ommatidium and is the photoreceptor that recruits the other photoreceptors (Jarman et al., 1994). The axons of R8 extend to the target region and other photoreceptors follow R8. In mammals, ATOH7 is essential for the differentiation of RGCs, which are the earliest generated cells in retinal development and are important for retinal vessel formation (Huang et al., 2014). These results suggest that Atonal is expressed at an earlier stage during development and affects the development of late-born cells.

Since Atonal acts as a proneural gene and also affects the development of other cells, it may be a suitable therapeutic target in the injured brain or in neurodegenerative disease. Indeed recent studies suggested the function of Atonal-related genes in neuronal regeneration. Fish have the ability to regenerate damaged retina by the reactivation of quiescent radial glia called Müller glia (MG) cells and the formation of neurogenic clusters. In medaka fish, the expression of a single factor Atoh7 in MG cells triggers the cell cycle re-entry of MG cells *via* activation of Notch signaling and formation of neurogenic clusters that differentiate into various retinal neurons *in vivo* in the absence of any injuries (Lust et al., 2016). Another potential target is Atonal-related transcription factor NeuroD1, which is important for embryonic brain development and neuronal differentiation during adult neurogenesis in the hippocampus. After brain injury and in neurodegenerative disease, activated glial cells proliferate and become hypertrophic in the injured region. The expression of NeuroD1 in reactive glial cells of the cortex of stab-injured or Alzheimer's disease model mice (5xFAD transgenic mice) can reprogram glial cells into functional neurons *in vivo* (Guo et al., 2014). Therefore Atonal and its related genes are promising gene targets for regeneration. Further studies to reveal how Atonal regulates target gene expression in a context dependent manner and how cells specified by Atonal have an influence on the development of late-born cells by using the genetic system we have established in *Drosophila* or using loss-of-function/gain-of-function animals in vertebrate may provide insights into the molecular mechanisms of neural development and neural regeneration.

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