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Generation of late-born neurons in the ventral spinal cord requires the coordination of retinoic acid and Notch signaling

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ABSTRACT

Neural progenitor cells generate various types of neurons and glia in a tightly regulated manner. During primary neurogenesis, retinoic acid (RA) acts earlier than Notch signaling and regulates differentiation and proliferation by upregulating proneural and neurogenic genes in the neural plate. However, the relationship between Notch signaling and the retinoid pathway during late neurogenesis remains unclear. We investigated the role of Mindbomb (Mib)-mediated Notch signaling in the differentiation of neural progenitors during late neurogenesis by overexpressing Mib and administering RA to *Tg[hsp70-Mib:EGFP]*. The majority of cells in the p3 domain differentiated into GABAergic Kolmer–Agduhr (KA) cells in *Tg[hsp70-mib:EGFP]* embryos heat-shocked during late neurogenesis, whereas these phenotypes were suppressed by exogenous RA. Our observations suggest that Mib-mediated Notch signaling plays a critical role in the temporal differentiation of neural progenitors, and that the generation of late-born KA″ cells is regulated by the interplay between Mib and RA.

1. Introduction

Different progenitors generate distinct subtypes of neurons, which are located topologically along the dorsoventral axis of the spinal cord [1]. This sophisticated structure raises the question of how neuronal identities can be obtained by combining intrinsic factors with extrinsic signals. The gene encoding *retinaldehyde dehydrogenase 2* (*raldh2*), an enzyme capable of synthesizing retinoic acid (RA), is expressed in the ventral spinal cord [2]. RA is a well-known signaling molecule that specifies the identity of a later-born subset of motor neurons along the dorsoventral axis [2]. Although cross-talk between many signaling pathways such as retinoids, sonic hedgehog, and Notches is required for neurogenesis as well as the pathway [1–3,6], it remains unclear when or where the signaling pathways are required for the generation of a specific neuronal cell type in the ventral spinal cord. In zebrafish embryos, distinct neurons such as motor neurons, GABAergic Kolmer–Agduhr (KA) cells, and Rohon–Beard sensory neurons (RB) emerge in a spatiotemporal order from neural precursors [7]. In this study, we investigated how Mindbomb (Mib)-mediated Notch signaling and the retinoid pathway is involved in the differentiation of GABAergic KA cells in the most ventral spinal cord. Our observations may have identified a critical role for Mib-mediated Notch signaling and the retinoid pathway for the differentiation of proliferating neural precursors in a discrete compartment of the neural tube during vertebrate development.

2. Materials and methods

2.1. Fish lines and mutants

Zebrafish were maintained as described in Yeo and Chitnis [3]. For experiments, AB wild-type, *mib* mutants [4] and *Tg[hsp70-Mib:GFP]* were used.

2.2. Generation of Tg[hsp70-mib:EGFP] zebrafish

The plasmid for the *Tg[hsp70-mib:EGFP]* zebrafish was constructed in the following manner. The *EcoR I–BamH I* fragment of the zebrafish *mib* [4] was amplified by PCR and sequenced following

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0304-3940/

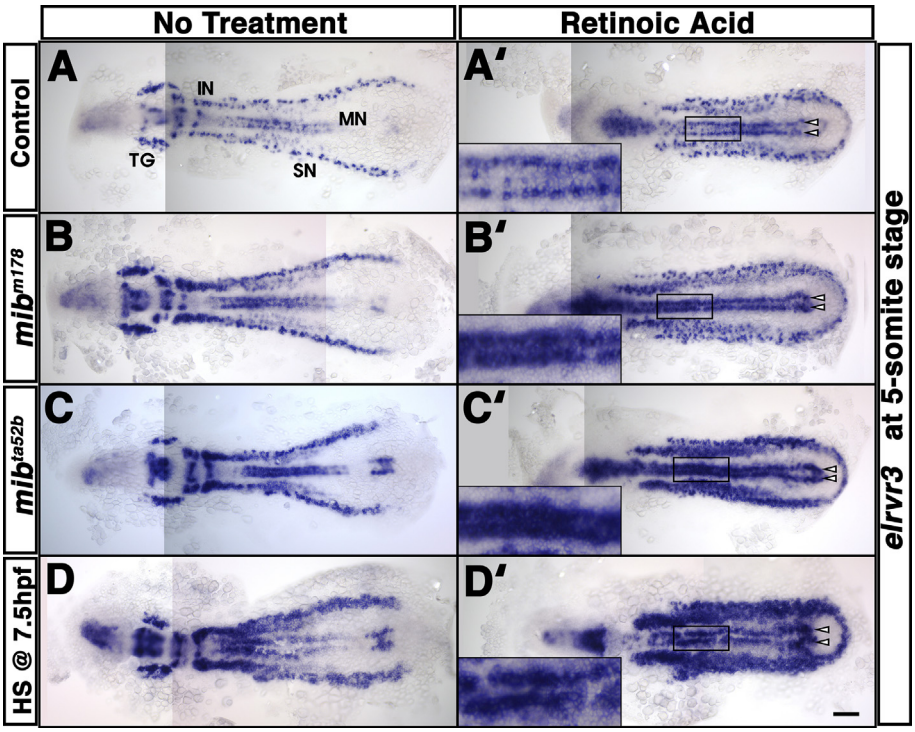

Fig. 1. Effects of retinoic acid (RA) on primary neurogenesis. Dorsal views. Anterior to the left. Expression of *elavr3* at the 5-somite stage following no treatment of RA (A–D) or RA treatment (A′–D′) in control embryos (A and A′), *mibm*^{178} mutant (B, B′), *mib*^{ta52b} mutant (C, C′) and homozygotic *Tg[hsp-mib:EGFP]* embryo (D and D′). The insets show the high magnification views of squares in the MN domain (A′–D′). The density of the sensory neuron is higher in Mib:GFP-overexpressed embryos than in *mib* mutants. Open arrowheads indicate the motor neuron domain (A′–D′). The density of the spinal motor neuron in *mib* mutants is much higher than that in Mib:GFP-overexpressed embryos.TG; trigeminal ganglion, IN; interneuron, SN; sensory neuron, MN; motor neuron. Scale Bar: 100 μm.

subcloning into the pCR2.1 vector (Invitrogen). This DNA fragment was introduced into the phsp70-EGFP plasmid [3] to create phsp70-mib:EGFP. Transgene preparation and injection were performed as described in [3]. Two lines, *Tg[hsp70-mib:EGFP]-1W* and *Tg[hsp70-mib:EGFP]-2S*, had the Mib/EGFP fusion protein induced to different levels, and *Tg[hsp70-mib:EGFP]-2S* was used for further analysis. For heat-shock treatment, the *Tg[hsp70-mib:EGFP]* homozygotes were maintained at 38.5 °C for 30 min.

2.3. Retinoic acid and citral preparation

All *trans* RA (Sigma) was prepared as a stock solution at 10^{-2} M in 95% ethanol. The stock solution was diluted directly into 30% Danio solution to obtain the desired concentration, 5×10^{-7} M, for zebrafish embryos. For citral, which is antagonist of RA [5], treatments of live embryos, stock solutions of 100 mM citral were made in 5% DMSO to obtain the desired concentration; 5 mM for zebrafish embryos.

2.4. *Whole-mount* in situ *hybridization*

Whole-mount *in situ* hybridization was performed as described in Yeo and Chitnis [3]. Anti-sense riboprobes were transcribed from cDNA of zebrafish GAD67, which is a marker for GABAergic KA′, VeLD and KA″ cells in developing spinal cord [8] and *elavr3*, which is a marker for all type of neurons [9]. Photos were taken using a differential interference contrast microscope (Axioplan2, Carl Zeiss).

3. Results

3.1. Specification of primary neurons by Notch signaling and the retinoid pathway

During primary neurogenesis, neurons were distributed in a salt-and-pepper like manner in the neural plate due to the lateral inhibition mechanism (Fig. 1A). To test the RA effects on primary neurogenesis, RA was administered to wild-type, *mib* mutants, and a transgenic embryo that expresses EGFP-tagged Mib under transcriptional control of the zebrafish *heat shock 70* promoter (*Tg[hsp70-mib:EGFP]*). In wild-type zebrafish embryos exposed to RA at mid-gastrulation (8.5 h post-fertilization, hpf), there were far denser neurons in the motor neuron domain (Fig. 1A′), whereas sensory and interneurons were still distributed in a salt-and-pepper like manner in the lateral and intermediate regions (Fig. 1A). The stripes of sensory neurons and interneurons were merged and shifted anteriorly (Fig. 1A′). This is consistent with the observation that endogenous RA can promote primary neurogenesis *in vivo*, and implies that the lateral inhibition mechanism normally functions in RA-treated embryos (Fig. 1A′, [10]). When RA was added to *mib* mutants, the neurogenic phenotype of *mibm*^{178} and *mib*^{ta52b} embryos (Fig. 1B and C) was strongly magnified in the stripes of motor neurons, as well as that of sensory neurons and interneurons (Fig. 1B′ and C′). The neurogenic phenotype could also be observed in the *Tg[hsp70-mib:EGFP]* embryos heat-shocked during the early gastrulation stage, suggesting that the overexpression of Mib:EGFP effectively inhibited Notch signaling (Fig. 1D). In *Tg[hsp70-mib:EGFP]* embryos exposed to RA at 8.5 hpf, far denser neurons were observed in the sensory neuron and interneuron domain, whereas the motor neuron domain of *Tg[hsp70-mib:EGFP]*