Slits and Robos help guide Olfactory Sensory Neuron Axons in the Zebrafish Olfactory System

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Abstract

The axons of olfactory sensory neurons (OSNs) project to individually identifiable neuropil regions called protoglomeruli in the Olfactory Bulb (OB), and later segregate into smaller odorant receptor (OR)-specific neuropils called glomeruli. We are investigating the contributions that the highly conserved axon guidance related Slits and Robos make to OSN axon guidance through the use of F0 CRISPR/Cas9 gene knockdowns in embryonic zebrafish. We observe striking abnormal midline crossing of transgene labelled OMP:RFP and TRPC2:Venus axons in *robo2* knockdowns, consistent with a role for *robo2* in preventing midline crossing during development. Transgene-labeled BacOR111-7:Gal4 OSN axons escape from the Central Zone protoglomerulus in *slit1a;slit1b* double knockdowns. The rate of these ectopic misprojections in *slit1a;slit1b* double knockdowns is significantly elevated as compared to wildtype, *slit1a*, or *slit1b* knockdowns. These findings suggest that slit1a and slit1b normally work together to keep OSN axons confined to the Central Zone protoglomerulus.

Introduction

- The olfactory system discriminates between distinct odors by organizing odorant experiences into a spatial topographic map on the olfactory bulb, the early development of which is the initial step to characterization of odorant perception (Lodovichi, 2021).
- Each olfactory sensory neuron (OSN) spanning the olfactory epithelium (OE) mono-allelically expresses one odorant receptor (OR) that selectively binds to specific odorant molecules (Serizawa, Miyamichi, & Sakano, 2004).
- During early development, OSN axons that express closely related ORs initially target specific protoglomeruli (large, individually identifiable neuropils) before they later segregate into OR-specific neuropil regions known as glomeruli (Shao, Lakhina, Dang, Cheng, Marcaccio, & Raper, 2017).
- The genes that encode Slit ligands and their Robo receptors are highly conserved, being present in invertebrates and vertebrates.
- Slit ligands bind Robo receptors to prevent axons from crossing the midline (Brose et al, 1999; Kidd, Bland, Goodman, 1999).
- While Slit-Robo signaling is known to be important in the development of the olfactory system, the precise mechanisms and resulting phenotypes of distinct interactions between the four Robo and four Slits in zebrafish OSN targeting have not yet been delineated. We are investigating the interactions of distinct Robo receptors and Slit ligands in OSN targeting in the developing olfactory system of larval zebrafish.

Materials and Methods

- We are utilizing an F0 approach as described by Kroll et al rapidly screen for phenotypic effects of *robo/slit* knockdowns.
- Three synthetic guide RNAs/Cas9 complexes with a high probability of causing frameshift mutations are injected into just fertilized zebrafish eggs and are screened for olfactory axon guidance errors three days later.
- This method is highly efficient and has been shown to cause 90% biallelic knockouts (Kroll et al., 2021).
- By using the F0 knockdown approach, the amount of time to knockdown a gene and screen for resulting phenotypes was largely reduced.
- The *robo* and *slit* genes are knocked down one or two at a time in the offspring of two transgenic lines: OMP:RFP and TRPC2:Venus for gross visualization of nearly all OSN axons, or UAS:Citrine and either BacOR111-7:Gal4 or BacOR130-1:Gal4 to label small sub-populations of OSNs expressing these ORs (Sato, Miyasaka, Yoshihara, 2005; Lakhina et al., 2012).
- Uninjected and RNP injected embryos are fixed in paraformaldehyde, processed, and compared via confocal microscopy at three days post fertilization (dpf) to assess the effects of the knocked down gene on protoglomerular targeting.
- OSN trajectories are reconstructed by confocal microscopy of wholemount preparations. These are randomized and scored blind by two scorers to determine the presence/absence of misprojections. The scores are then analyzed via Fisher's exact test to test for statistical significance.

Results

OR111 expressing OSNs terminate ectopically in *slit1a;slit1b* double knockdowns

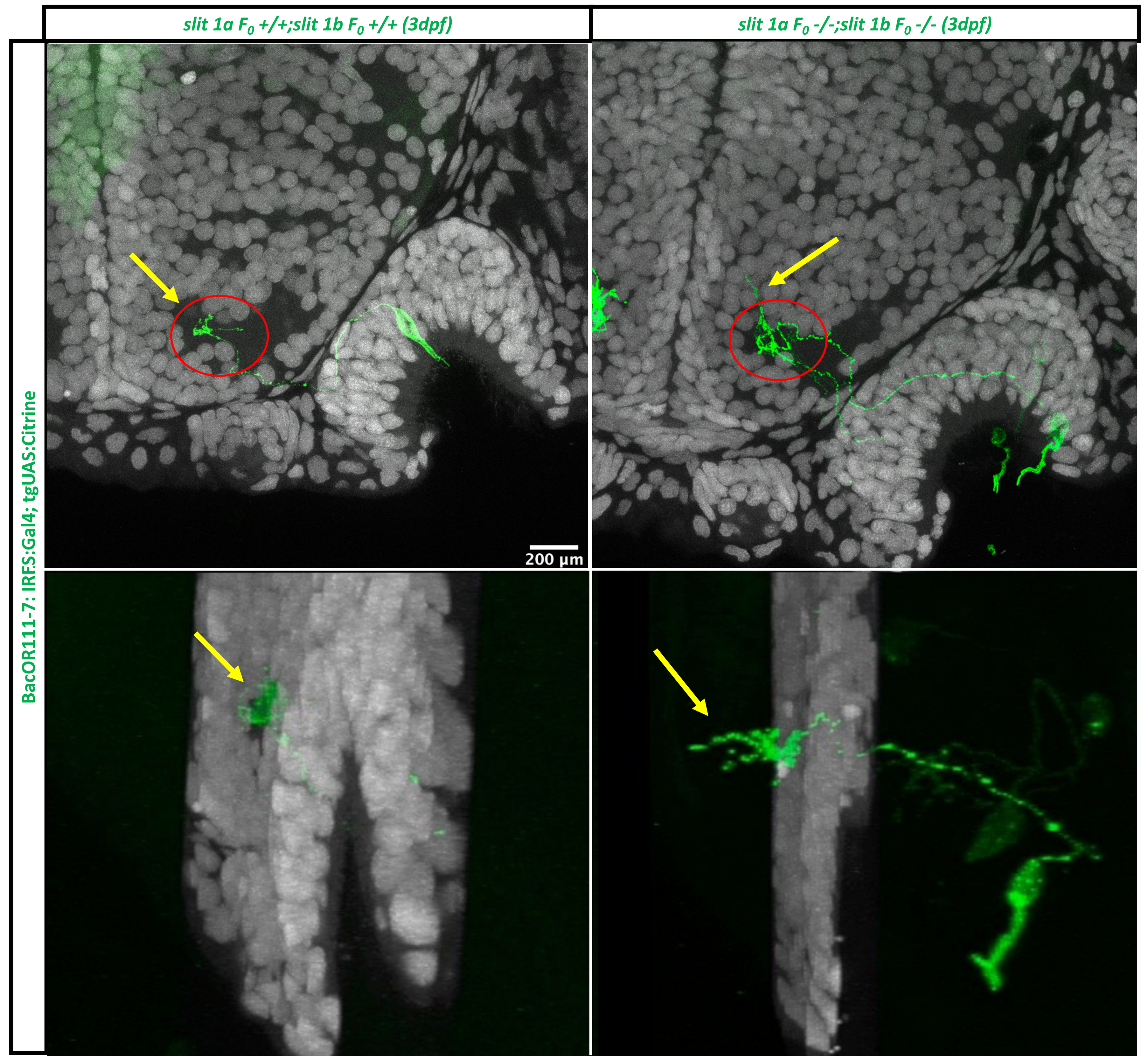
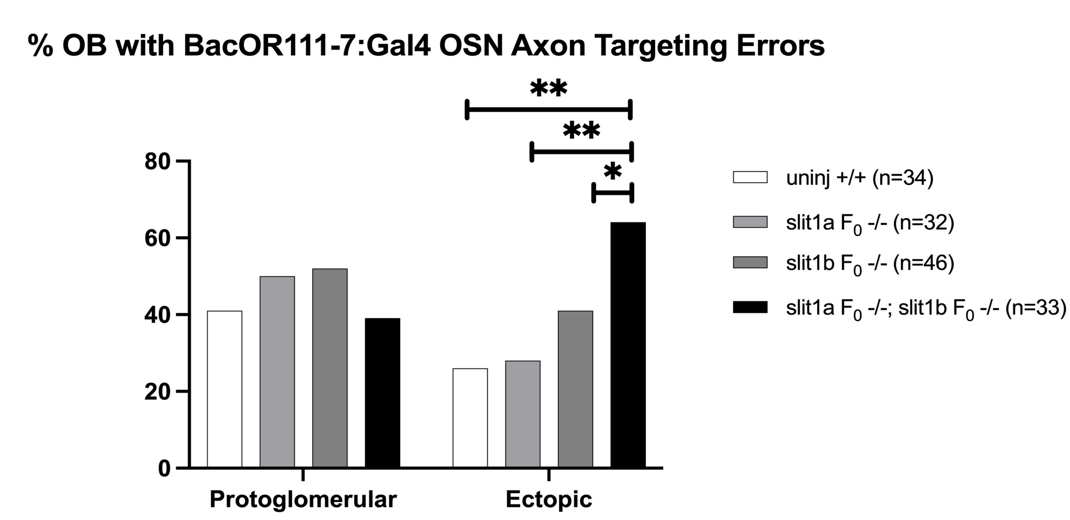
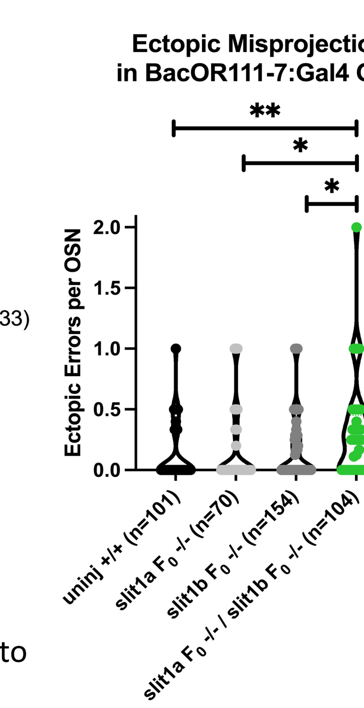


Figure 1. Example ectopic mispreojections in OR111-7 expressing OSNs from the Central Zone protoglomerulus in *slit* F_0*1a;slit1b* double knockdowns (right) as compared to control (left). The 200 µm scale bar is applicable to the top panels, while the bottom panels are magnified.





Figures 2a and b. The rate of ectopic misprojections of OR111-1 expressing OSNs is significantly elevated in *slit 1a;slit 1b* double knockdowns compared to single knockdowns and uninjected embryos.

robo 2 knockdowns cross the midline

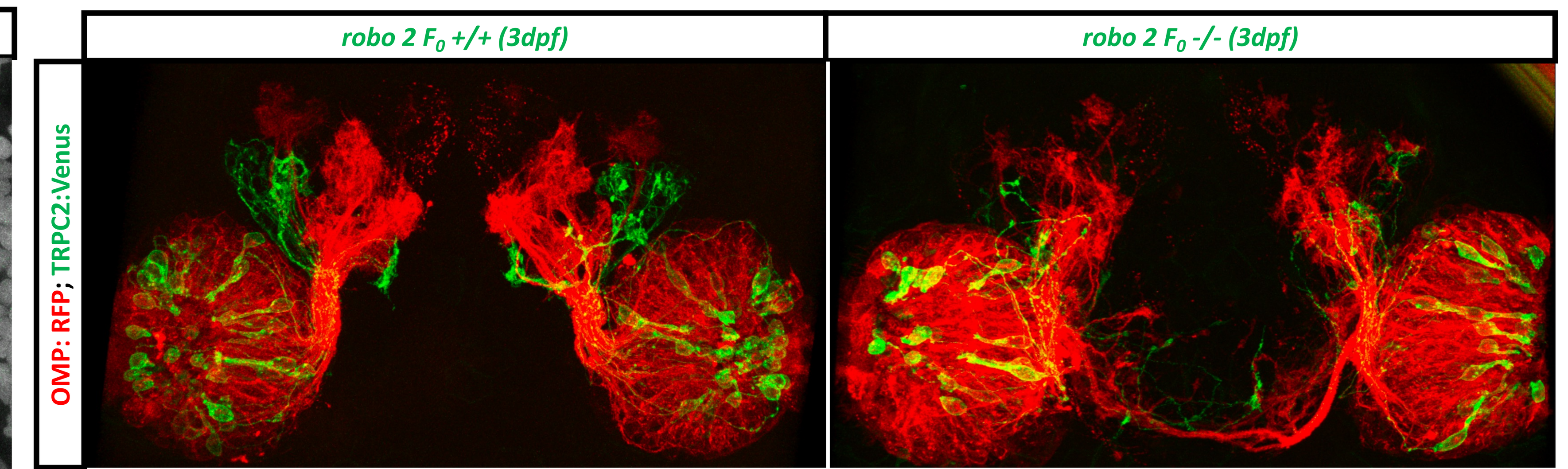


Figure 3. Striking abnormal ventral, posterior, and abnormal midline misprojections in *robo2* F_0 -/- knockdowns (right) as compared control (left).

Discussion

- *slit1a and slit1b*
  - The axons of BacOR111-7:Gal4 typically project to the CZ protoglomerulus
  - *slit1a* and *slit1b* single knockdowns project normally and terminate in the CZ
  - In *slit 1a;slit1b* double knockdowns, OR111-7 expressing axons successfully target the CZ, but they escape and terminate ectopically from the protoglomerulus
  - The rate of ectopic misprojections from the CZ is significantly elevated in *slit1a;slit1b* double knockdowns as compared to single knockdowns and uninjected controls
- *robo2*
  - We were able to phenocopy the *robo2* knockout mutant line described by the Yoshihara lab in 2005, validating that the F0 approach is applicable to our model system (Miyasaka et al., 2005)
  - OMP:RFP and TRPC2:Venus labeled OSNs misproject both ventrally and posteriorly as well as crossing the midline in *robo2* knockdowns

Conclusions

- *slit1a* and *slit1b* compensate for each other in single gene knockdowns
- *slit1a* and *slit1b* together suppress OSN axon escape from the CZ protoglomerulus
- The F0 approach is applicable to our zebrafish model system
- *robo2* is required for preventing midline crossing during development.

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