

Distorted coarse axon targeting and reduced dendrite connectivity underlie dysosmia after olfactory axon injury

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The glomerular map in the olfactory bulb (OB) is the basis for odor recognition. Once established during development, the glomerular map is stably maintained throughout the life of an animal despite the continuous turnover of olfactory sensory neurons (OSNs). However, traumatic damage to OSN axons in the adult often leads to dysosmia, a qualitative and quantitative change in olfaction in human. A mouse model of dysosmia has previously indicated that there is an altered glomerular map in the OB after the OSN axon injury; however, the underlying mechanisms that cause the map distortion remain unknown. In this study, we examined how the glomerular map is disturbed and how the odor information processing in the OB is affected in the dysosmia model mice. We found that the anterior-posterior coarse targeting of OSN axons is disrupted after OSN axon injury, while the local axon sorting mechanisms remained. We also found that the connectivity of mitral/tufted cell dendrites is reduced after injury, leading to attenuated odor responses in mitral/tufted cells. These results suggest that existing OSN axons are an essential scaffold for maintaining the integrity of the olfactory circuit, both OSN axons and mitral/tufted cell dendrites, in the adult.

Key words: dysosmia, olfactory sensory neurons, traumatic axon injury, regeneration, glomerular map, olfactory bulb

Significance Statement: Olfactory sensory neurons (OSNs) have a unique ability to regenerate throughout the life of animals. Therefore, OSNs are newly generated and project to the olfactory bulb (OB) even after injury. However, it has been known that severe head trauma, which accompanies the transection of OSN axons, often leads to dysosmia (qualitative and quantitative changes in olfaction). Currently there is no effective therapy for dysosmia patients. To gain mechanistic insights, we investigated the anatomical and functional changes of the olfactory system after OSN axon injury,

and found defects in coarse OSN axon targeting and dendrite connectivity of mitral cells. Based on these findings, possible future strategies for dysosmia will be discussed.

Introduction

The olfactory system is capable of detecting and discriminating a huge variety of airborne odorants. In addition, the olfactory system has an ability to judge the qualitative and social value of odors, being perceived as pleasant and unpleasant ones. While the olfactory perception is generally stable throughout life, perceived olfactory quality is altered in an olfactory disorder, known as dysosmia, in human. Dysosmia is often caused by a high energy trauma to the head region (Yousem et al., 1996; Doty et al., 1997; Doty, 2009). Olfactory sensory neuron (OSN) axons are easily damaged by the shearing stress of the trauma, and dysosmia is typically recognized after a transient loss of olfaction. Post-traumatic dysosmia patients typically have reduced sensitivity to odors (hyposmia) with unpleasant olfactory perception to many kinds of odors (parosmia)(Bonfils et al., 2005). The prognosis is bad and currently there is no effective therapy to prevent or cure post-traumatic dysosmia (London et al., 2008). Although olfaction is not essential for life, it impairs the quality of life and can become a serious issue for patients. To move toward more effective therapies, it is important to understand the molecular and cellular processes underlying dysosmia.

The logic of olfactory perception has been well studied using rodent models (Axel, 2005; Buck, 2005; Mori and Sakano, 2011). Odors are detected by odorant receptors (ORs) expressed by OSNs in the olfactory epithelium (OE) (Buck and Axel, 1991). There are ~350 ORs in human and ~1,000 ORs in mice. OSNs expressing the same type of OR converge their axons onto the same glomerulus in the olfactory bulb, forming a glomerular map. Thus, odor information is represented as a spatiotemporal activation pattern of ~1,000 sets of glomeruli in the olfactory bulb. Therefore the glomerular map is important for odor recognition and discrimination. The glomerular map is initially formed during embryonic development (Imai et al., 2010), and once established, this glomerular map is stable throughout the life of animals, even though

OSNs are continuously replaced by newly generated OSNs as a turnover process (Schwob, 2002).

OSNs are also regenerated after OSN injury (Schwob, 2002). Glomerular map formation after the OSN injury has been studied in mice with chemical or physical lesions of OSNs. The outcome was, however, variable between the different lesion protocols. For example, a single intraperitoneal injection of methimazole results in loss of most (though not all) mature OSNs, but they can reestablish the correct glomerular map without any defects (Blanco-Hernandez et al., 2012). An injection of dichlobenil or inhalation of Methyl Bromide causes more severe damage to OSNs, and regenerated OSN axons show mistargeting to the OB in the initial stage; however, in many cases, the glomerular map is largely recovered after sufficient recovery period (Schwob et al., 1999; St John and Key, 2003; Carr et al., 2004; Cheung et al., 2014). In contrast, glomerular map was poorly recovered when OSNs were injured by OSN axon transections (Costanzo, 2000; Christensen et al., 2001). It has been considered that the distorted glomerular map after diffuse OSN axon injury may underlie the altered olfactory perception in dysosmia patients. However, it has not been fully investigated how the glomerular map is disorganized after OSN injury. It has also remained unknown why newly generated OSN axons cannot reestablish the correct glomerular map when OSN axons were injured. Here we studied the cellular process that results in a distorted olfactory map after OSN injury, and found that anterior-posterior coarse targeting is disturbed. We also examined the OB circuitry and found that dendrite connectivity of mitral cells is reduced after the injury.

Materials and Methods

Mice. All the animal experiments were approved by the Institutional Animal Care and Use Committee of the RIKEN Kobe branch. Wild-type (C57BL/6N), *OMP-GFP* knock-in mice (RRID:IMSR_JAX:006667)(Potter et al., 2001) and *MOR29A/29B* transgenic mice (Tsuboi et al., 2011) were used to study OSN projection; *Thy1-YFP* (line G, RRID:IMSR_JAX:014130)(Feng et al., 2000) was used to label mitral cells in the OB; and *Thy1-GCaMP6f* (line GP5.11, RRID:IMSR_JAX:024339) was used for Ca^{2+} imaging of mitral cells *in vivo*. All of these mice were bred to C57BL/6N mice to maintain the mouse colony for this study. Male *OMP-GFP* mice were crossed with female ICR mice for *in utero* electroporation.

***In utero* electroporation.** *In utero* electroporation was performed as described previously (Saito, 2006). To label mitral cells, *in utero* electroporation was performed at E12 (Imamura and Greer, 2013). Two μl of plasmid solution (pCAG-tdTomato, 1 $\mu\text{g}/\mu\text{l}$) was injected into the lateral ventricles and electric pulses were delivered along the anterior-posterior axis with forceps-type electrodes (3-mm diameter) and a CUY21EX electroporator (BEX).

Axotomy. OSN axotomies were performed as described previously with some modifications (Costanzo, 2000). 8-12 week-old mice (both male and female) were anesthetized with ketamine (80 $\mu\text{g}/\text{g}$ body weight) and xylazine (7 $\mu\text{g}/\text{g}$), and then given dexamethasone (0.1 $\mu\text{g}/\text{g}$, Kyoritsu Seiyaku) and enrofloxacin (5 $\mu\text{g}/\text{g}$, Pfizer) by subcutaneous injection to prevent brain edema and infection. A dental drill was used to thin the nasal skull of the OE-OB border region (along the cribriform plate). OSN axons projecting to the dorsal OB were transected along the cribriform plate with a micro knife for ophthalmic surgery (Bvi Beaver® Xstar® Slit Knife 2.8mm 45° bevel up, Beaver-Visitec International, inc.). After axotomy, a coverslip was put onto the craniotomy with dental cement and the skin flap was sutured. In most experiments, only OSN axons projecting to the right OB were transected. In some experiments, the left or

both sides were axotomized. Mice were administered with daily subcutaneous injection of dexamethasone (0.1 µg/g, Kyoritsu Seiyaku) and enrofloxacin (5 µg/g, Pfizer) for 5 consecutive days. For recovery, mice were placed in single-housed cages and kept for 3 to 84 days. In Fig. 1B, a microknife was labeled with 5% TMR-Dextran (10kDa, Thermo Fisher, #D1868).

Immunohistochemistry of sections. Mice were deeply anesthetized with overdose Nembutal (Dainippon Sumitomo Pharma) injection, followed by intracardiac perfusion with 4% paraformaldehyde (PFA) in PBS. Dissected OE and OB samples were further fixed in 4% PFA/PBS. Fixed OE-OB samples were decalcified with 0.5M EDTA for 7 days, cryoprotected in 30% sucrose in PBS overnight, and then embedded in OCT Compound. Cryostat sections (16 µm thick) were collected on glass slides and fixed with 4% PFA/PBS for 15 min. In the anti-Nrp1 immunostaining, sections were treated with 10mM citrate buffer (pH 6.0) at 120°C, 20 min for antigen retrieval. Blocking of sections was performed with 5% donkey normal serum. Primary antibodies used in this study were goat anti-Neuropilin1 (Nrp1) (1:100, R&D systems, #AF566, RRID:AB_355445), goat anti-Kirrel2 (1:100, R&D systems, #AF2930, RRID:AB_2130975), chicken anti-GFP (1:500, aves, #GFP-1010, RRID:AB_2307313). Alexa488-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch Labs Cat# 703-545-155, RRID:AB_2340375) and Alexa555-conjugated donkey anti-goat IgG (Thermo Fisher, Cat# A-21432, RRID:AB_2535853) were used as secondary antibodies at 1/200-1/250. DAPI (Thermo Fisher) was used at 1/1000.

Optical clearing and dendrite tracing. *Thy1-YFP* line G mice were used for dendrite tracing experiments of mitral cells. Some experiments were performed with *OMP-GFP* mice with mitral cell labeling (tdTomato) by *in utero* electroporation. At 42 days after axotomy, OB was excised and fixed in 4% PFA/PBS and sections (500 µm thick) were obtained using a microslicer (Dosaka). OB slices were cleared with SeeDB2G as

described previously (Ke et al., 2016). A confocal microscope (TCS SP8, Leica Microsystems) with a 20x objective lens (HC PL APO 20x IMM CORR CS2, NA 0.75) was used to obtain three-dimensional images of EYFP and DAPI. Only the anterodorsal part of the OB was analyzed in sagittal slices, because this area is damaged by our axotomy protocol. Since the EYFP signals in this *Thy1-YFP* mouse line was heterogeneous among mitral cells, brightly labelled mitral cells (within the top 20%) were identified with ImageJ and their primary dendrites were analyzed in an unbiased manner in this study. Only primary dendrites were traced in Fig. 4 using Neurolucida software (MBF Bioscience). We only quantified neurons where whole primary dendrites were entirely contained within the acquired 3D volume. After the axotomy, mitral cells often possessed branches from the middle part of the primary dendrite. Tracing and quantification in Fig. 4 was performed by one observer who was not blinded to the sample types. Only for the *Thy1-YFP* day 42 samples, all images were re-analyzed by another observer who was blinded to the sample types, leading to the same conclusion (data not shown). In Fig. 5, primary and lateral dendrites, including dendritic tufts were reconstructed using Neurolucida.

***In vivo* two-photon Ca^{2+} imaging.** We analyzed odor evoked responses in the OB using *Thy1-GCaMP6f* transgenic mice (line GP5.11), in which GCaMP6f is specifically expressed in mitral cells in the OB. Mice (8-16 week old males, axotomized mice were 42 days post injury) were anesthetized with ketamine/xylazine and craniotomy was made onto the right OB. The skull overlying the dorsal OB was thinned with a dental drill and then removed with a fine forceps. Kwik-Sil (WPI) was applied on the OB, and a small coverslip (4 mm, MATSUNAMI) was placed onto the exposed OB. The coverslip was attached to the skull and surrounding thin wall was made with dental cement (GC UNIFAST II, Shofu). Butyric acid, Acetophene, and Guaiacol were diluted at 1/100 in mineral oil. Vanillin was dissolved in Dimethyl sulfoxide at the saturated concentration. Anesthetized mice were exposed to odorants diluted further

with air at 1:10 using an olfactometer (K.K. Matsumi Kogaku Keisoku). GCaMP6f was excited at 920 nm (InSight DS Dual, Spectra-Physics) and images were acquired with an upright two-photon laser-scanning microscope (FV1000MPE, Olympus) using a water-immersion 10x objective lens (UMPLFLN 10XW, NA = 0.30) and a GaAsP detector. In Fig. 6B, mean $\Delta F/F_0$ signals during the first 5sec of odor stimulation (single trial / animal) was averaged among samples after images were aligned based on the anterior and lateral edges of the OB. In Fig. 6C, cumulative plots were made based on pixel-based $\Delta F/F_0$ signals for individual samples using MATLAB.

Statistical analysis. Prism 5 and R was used for statistical analyses. Calcium imaging data were analyzed by custom-written MATLAB scripts.

RESULTS

Olfactory map disorganization by OSN axon transection

In humans, unmyelinated OSN axons connecting the OE and the OB are fragile, and dysosmia is often caused by damage to these axons following the head trauma. To recapitulate similar injuries in a mouse model, we performed a unilateral OSN axon transection (axotomy) as has been used in earlier studies (Costanzo, 2000; Christensen et al., 2001). To minimize unnecessary damage to the surrounding tissues, we made a small incision to the skull, and axon bundles projecting from dorsal OE to the dorsal OB were transected with microknives at the boundary between cribriform plate and OB (Fig. 1A, B). The ventral zone OSNs remained largely intact in our surgery. To minimize inflammatory responses, we administrated dexamethasone for 5 consecutive days (Kobayashi and Costanzo, 2009). Degeneration and regeneration was first examined with *OMP-GFP* knock-in mice in which all mature OSNs are labelled with GFP. The

unilateral axonal transection caused a gradual retrograde degeneration of OSNs in the ipsilateral OE, resulting in complete elimination of mature OSNs within 14 days (Fig. 1C, D). Distal axons were also degenerated (Wallerian degeneration) by this point (Fig. 1D, right). Out of 8 axotomies performed in our hands, all showed complete loss of OSNs in the dorsal zone OE by day 14. OSNs were then gradually regenerated and recovered by day 42 after the injury (Fig. 1E). Out of 12 *OMP-GFP* animals with unilateral axotomy, all demonstrated a recovery of OSNs at day 42.

We examined the overall axonal projection profiles of OSNs at 42 days post injury. Axons of newly generated OSNs extended to the glomerular layer of the OB. However, these OSN axons formed small glomerular-like structures within the glomerular layer in the anterior part of the OB (Fig. 1F, G), consistent with earlier studies done with chemical lesions (St John and Key, 2003; Cheung et al., 2014). Compared to the control, OSN axons tended to be scarcer in the posterior part of the dorsal OB (Fig. 1E, right).

Anterior-posterior coarse targeting is disturbed after axotomy.

Next we examined the axonal projection profiles for OSNs expressing transgenic *MOR29B* (Tsuboi et al., 2011). *MOR29B* (*Olf151l*) is a class II OR gene expressed in the dorsal zone of the OE. In the control side, *MOR29B*-expressing OSN axons projected to the dorsolateral part of the OB; however, they did not project to the original location in the OB, and instead formed smaller glomeruli in the anterior ($n = 4/6$, Fig. 2A) or more medial ($n = 2/6$) part of the OB after axotomy.

During development, the anterior-posterior topography of the OB is, at least partly, regulated by Neuropilin-1 (Nrp1) and Semaphorin3A (Sema3A) signaling (Schwartz et al., 2000; Taniguchi et al., 2003; Imai et al., 2009). Nrp1 expression levels in OSNs

determine the anterior-posterior positioning of glomeruli, with low Nrp1 to anterior and high Nrp1 to posterior OB. *Sema3A* is expressed not only in the anterior OB, but also by intermediate cues en route (ensheathing glia) and OSN axons projecting to anterior OB. As a result, *Sema3A*-Nrp1 signaling regulates both axon-axon and axon-target interactions to guide Nrp1-high axons to the posterior OB. Here we examined axonal projection of Nrp1-expressing OSNs. In the control OB, Nrp1-high axons are sorted to the superficial layer of the olfactory nerve layer and projected to the posterior OB. In contrast, on the axotomized side, Nrp1-high axons were often ectopically located in the deep layer of the nerve and projected to the glomeruli in the anterior part of the OB (n = 12/12) (Fig. 2B, C). Thus, the poor convergence of OSN axons after axotomy (Costanzo, 2000) is due to defects in coarse axon targeting (Imai et al., 2009), rather than those in local axon sorting (Yu et al., 2004; Serizawa et al., 2006).

Heterogeneous OSN axons form segregated glomerular-like structure within a glomerulus.

In the anterior part of the OB, Nrp1-high and Nrp1-low OSN axons were often intermingled within a glomerulus (Fig. 2C). To further examine the heterogeneity of OSN axons within a glomerulus, we also analyzed the localization of *Kirrel2*, another OR-regulated cell recognition molecule, involved in local axon sorting (Serizawa et al., 2006). In the control OB, each glomerulus showed a unique expression level of *Kirrel2*. Within each glomerulus, the *Kirrel2* level was almost homogeneous, reflecting the convergence of homogeneous OSN axons expressing a common OR. After the axotomy, the layout of periglomerular cells remained similar to the control, forming glomeruli of 50-80 μm diameter (Fig. 3). On the axotomized side, however, *Kirrel2*-high and *Kirrel2*-low OSN axons were often found in the same glomerulus, forming discrete glomerular-like structures within a glomerulus (Fig. 3). High magnification images showed that *Kirrel2*-high and *Kirrel2*-low axons were still segregated within a

glomerulus, indicating that local axon sorting mechanisms (Serizawa et al., 2006) remain intact even after axotomy. These results together demonstrate that heterogeneous OSN axons project to a common glomerulus after axotomy.

Connectivity of mitral cell dendrites to glomeruli was reduced after axotomy.

After recovering from axotomy, newly generated OSNs project to the OB (Costanzo, 2000; Christensen et al., 2001); however, it has not been fully examined whether these OSNs form functional connections in the OB. It is unknown whether mitral cell dendrites remain intact after the deafferentation of OSNs. To examine the connectivity of mitral cell dendrites after axotomy, we used *Thy1-YFP* (line G) mice (Feng et al., 2000), in which mitral cells are brightly labelled with EYFP. The OB slices were cleared with SeeDB2G (Ke et al., 2016), and 3D fluorescence images were acquired with confocal microscopy. To ensure tracing accuracy, primary dendrites of brightly labelled mitral cells (top 20th percentile) were traced in an unbiased manner from the somata to the tip of primary dendrites. We analyzed the anterodorsal part of the OB, where OSN axons de-innervated and re-innervated. In the control OB, most of mitral cells (80-90%) extended thick primary dendrites to the glomerular layer of the OB. However, 21 days after axotomy, thick primary dendrites were rarely observed in this OB region; in our light microscopy-based tracing, ~50% of mitral cells failed to show dendrite connection to the glomerular layer, suggesting a partial deinnervation or reduced dendrite thickness (Fig. 4A).

To more clearly visualize morphological changes in the dendritic tufts, we performed fluorescence labeling of mitral cells with *in utero* electroporation (Saito, 2006; Imamura and Greer, 2013). In this labeling, mitral/tufted cells were more sparsely and brightly labelled, facilitating visualization of dendritic tufts. In the control OB, highly ramified dendritic tufts were clearly visualized in the glomerular layer. In

contrast, mitral cells rarely innervated the glomerular layer and poorly ramified within glomeruli after axotomy (Fig. 5). It should be noted, however, with light microscopy, it is technically difficult to conclude whether some of mitral cells have completely lost connectivity to glomeruli, or if they still maintain very thin processes; at the minimum, our results indicate that primary dendrites become thinner and weakened in a substantial population of mitral cells after axotomy. The defective dendrite innervation did not fully recover even at day 42 or 84, when the projection of OSN axons has fully recovered in the anterior part of the OB (Fig. 4). We also obtained consistent results using *in utero* electroporation (data not shown).

Odor responses in the OB after OSN lesions and subsequent regeneration

To examine the functional consequences of aberrant regeneration after axotomy, we performed Ca^{2+} imaging of the OB using the *Thy1-GCaMP6f* transgenic mouse (line GP5.11), in which a genetically-encoded Ca^{2+} indicator GCaMP6f is specifically expressed in mitral cells in the OB (Dana et al., 2014). We analyzed odor-evoked responses in the glomerular layer. It should be noted that nearly half of the mitral cells still maintain connections to glomeruli even after axotomy (Fig. 4). In mice, dorsal OB can be divided into three distinct domains by genetic and/or molecular markers as well as by odor response signatures: D_I for OSNs expressing class I ORs; D_II for class II ORs; and D_III for Tarr ORs (Kobayakawa et al., 2007; Bozza et al., 2009; Pacifico et al., 2012). We used one odor that specifically activates the D_I domain (Butyric acid) and three odors that specifically activate the D_II domain (Acetophenone, Vanillin, and Guaiacol) (Bozza et al., 2009), and analyzed the anterodorsal part of the OB spanning these two domains. In the control OB, responses to these odors were robust, and activation domains for D_I and D_II odors were largely segregated. In contrast, on the axotomized side, the odor-evoked responses were much reduced as demonstrated by the cumulative plots of Ca^{2+} responses ($\Delta\text{F}/\text{F}_0$ of GCaMP signals) (Fig. 6). Thus, the odor-

evoked responses are dramatically changed after the axotomy, even though newly-generated OSNs project axons to the OB.

DISCUSSION

Qualitative changes of olfaction may be due to disrupted domain organization of the glomerular map

To recapitulate post-traumatic dysosmia, an earlier study performed axotomy for *P2-lacZ* knock-in mice in which an odorant receptor gene *P2* was genetically labelled (Costanzo, 2000). OSNs expressing *P2* gene project their axons to the anterior part of the OB. While this study demonstrated dispersed axonal projection after OSN regeneration, the overall changes of glomerular map have not been fully described. Therefore, it has been unknown whether it was due to defects in coarse axon targeting or local axon sorting (Imai et al., 2010). In the present study, we performed axotomy for dorsal zone OSNs and analyzed axonal projection of *MOR29B* OSNs. We found that *MOR29B* OSN axons were often mistargeted to more anterior or medial OB regions after the axotomy (Fig. 2A). The distorted anterior-posterior topography was also confirmed by anti-Nrp1 immunostaining of OB sections (Fig. 2B, C). In the anterior OB, multiple heterogeneous glomeruli were formed within a glomerulus (Fig. 3). Since different types of odors are represented by different domains of the OB (Mori et al., 2006; Bozza et al., 2009), the defective coarse axon targeting may underlie qualitative changes in odor perception after traumatic OSN axon injury. Because we only transected dorsal-zone OSN axons in our analysis, we do not exclude the possibility that dorsal-ventral topography of the OB may also be disrupted when both dorsal-zone and ventral-zone axons are injured (Costanzo, 2000).

Reduced olfactory sensitivity after OSN axon injury

It has been demonstrated that the regenerated OSNs after injury are functional and respond to odors (Cheung et al., 2014). However, the anatomy and odor responses in mitral cells have not been investigated so far. In the present study, we found that the connectivity of mitral cell primary dendrites is reduced after the OSN injury. It has been known that tufted structure of the mitral cell primary dendrites is stable in the normal situation in the adult (Mizrahi and Katz, 2003); however, our results indicate that OSN axons are required to stably maintain the dendrite morphology of mitral cells (Fig. 4, 5). Previously, atrophy of mitral cell dendrites has been reported for mice where OSN axons were eliminated from early developmental stages, before the formation of mature tufted structures (Leo and Brunjes, 2003; Kobayakawa et al., 2007). Our present study demonstrated that the dendritic retraction of mitral cells can occur in the adult, even after the maturation of dendrites. Furthermore, once retracted, it is difficult to re-establish the connectivity even after OSN projection recovered. It will be interesting to study in the future how OSN-derived signals control maturation and/or maintenance of mitral cell dendrites in the OB.

Consistent with the anatomical evidence, we also found that odor evoked responses in mitral cells are much reduced (Fig. 6). Thus, the reduced connectivity of mitral cell dendrites may be a cause for the reduced sensitivity after OSN axon injury (hyposmia) (Bonfils et al., 2005). It has been known that remodeling of mitral cell dendrites is active during an early postnatal period (Malun and Brunjes, 1996). In contrast, we could not find evidence for dendrite re-innervation/growth even at 84 days after axotomy, suggesting that the plasticity and growth of mitral cells are limited in the adult OB. It is possible that there is a critical period for mitral/tufted cell dendrite remodeling as has been known in other sensory systems (Hensch, 2005). In the OB, OR-specific convergence of OSN axons is the basis of odor discrimination and odor

information processing in the OB (Imai, 2014). Therefore, the convergence of multiple heterogeneous OSN axons to a glomerulus (Fig. 2, 3) may also have been a cause of reduced responses in mitral cells.

OSN axons are required to maintain the glomerular map and its connectivity with mitral cell dendrites in the adult

Why is the regeneration process after OSN axon damage different from the normal OSN turnover process? We found that Nrp1-positive OSN axons are often mistargeted to the anterior OB after axotomy (Fig. 2). This may indicate that the target cues are no longer functioning in the adult (Fig. 7A). In fact, one of the genes for positional cues, *Sema3a* is localized at the anterior OB only during embryonic stages (Schwartz et al., 2000). Our result is also consistent with recent studies using transgenic manipulations, showing critical period for correct olfactory map formation (Ma et al., 2014; Tsai and Barnea, 2014). In the adult, regenerating OSNs depend more on axon-axon interactions in the adult (Fig. 7B) (Imai and Sakano, 2011). Consistent with this idea, an earlier chemical lesion study with methimazole did not entirely eliminate OSN axons, and also did not lead to olfactory map distortion (Blanco-Hernandez et al., 2012). In contrast, OSN axons were almost completely degenerated by axotomy. Without scaffolds for follower OSN axons, the coarse mapping of OSN axons may be disturbed after traumatic OSN axon injury (Fig. 7C). In contrast, local axon sorting mechanisms remained functioning; as a result, Kirrel2-high and Kirrel2-low axons were nicely segregated to form discrete glomerular-like structures within glomeruli (Fig. 3). In the present study, we also found that primary dendrites of mitral cells partially de-innervated from glomeruli after OSN injury. This result also indicates the requirement of OSN axons in maintaining the mitral cell connectivity and OB circuits in the adult. Our results in olfactory system are also insightful toward regenerative medicine in the nervous system: regeneration and regrowth of axons are not sufficient for functional recovery.

What kind of therapeutic approaches could minimize these problems after post-traumatic OSN injury? Since the physical existence of OSN axons seems to be important to maintain olfactory map topography and its connectivity, one possible approach is to minimize the degeneration of OSN axons after injury. If we can delay the degeneration of injured OSN axons, the regenerated OSN axons may catch them up and form correct circuitry in the OB. It is known that the degeneration of distal axons after axonal injury is a result of active degeneration mechanism, known as Wallerian degeneration (Conforti et al., 2014). In several types of neurons, ectopic expression of *WldS* (a fusion protein between *Nmnat1* and *Ube4b*) or *Nmnat1* are shown to delay Wallerian degeneration up to a few weeks. Unfortunately, however, our experiments with *WldS* mice did not show any delays in degeneration after OSN axotomy (data not shown), suggesting alternative and/or additional mechanisms in OSNs. Identifying the active axonal degeneration mechanisms in OSNs may open a new therapeutic possibility for post-traumatic dysosmia.

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Figure legends.

Figure 1. Mistargeting of OSN axons after OSN lesion and recovery.

A, Schematic representation of unilateral axotomy of dorsal-zone OSNs. Axon bundles of dorsal zone OSNs projecting to dorsolateral OB were transected near the cribriform plate. Only the right side was injured. **B**, The microknife was coated with TMR-dextran dye to visualize the incision site. *MOR29B-EYFP* mice were used. Axotomy was limited to dorsal zone OSNs (broken line). **C**, OE and OB 7 days after the axotomy. **D**, OE and OB 14 days after the axotomy. Almost complete OSN lesion in the anterodorsal OE and OB was observed in 8 out of 8 mice at day 14. **E**, Recovery of OSNs 42 days after axotomy. OSNs were regenerated and the number of OSNs recovered to comparable levels to the control on the non-injured side. However, OSN axons were scarce in the posterior part of the dorsal OB (right, white broken line). **F**, High magnification images of **E**, middle, showing axotomized (orange) and control (gray) sides. OSN axons formed small glomerular-like structures in the anterior OB after axotomy. **G**, Quantification of diameter for glomerular-like structure in the control and axotomized sides. *OMP-GFP* mice were used in **C-G**. $p^{***} < 0.001$ (Mann–Whitney U test). $n = 559$ (axotomy) and 136 (control) from 12 mice. A, anterior; D, dorsal. Scale bars are $200\ \mu\text{m}$ in **C-E** and $100\ \mu\text{m}$ in **F**.

Figure 2. Anterior-posterior topography is disrupted after axotomy.

A, Defective axonal projection of OSNs expressing *MOR29B-EYFP*. On the control side, a major glomerulus for MOR29B was observed in the posterolateral area of the dorsal OB. However, MOR29B-expressing OSNs projected to the anterior and/or medial region of the OB after OSN axon injury (42 days recovery; in 6 out of 6 animals analyzed). A, anterior; L, lateral. **B**, Mistargeting of Nrp1-positive OSN axons to the anterior OB. **C**, High magnification confocal images of boxed areas in **B**. In the control

side, Nrp1-positive axons were sorted to the superficial layer of the olfactory nerve layer and projected to the posterior OB. However, on the axotomized side, Nrp1-positive axons were found in the deeper portions of the olfactory nerve layer, and invaded glomeruli in the anterior OB. Nrp1-positive and -negative axons formed small glomerular-like structures within glomeruli in the anterior OB (arrow heads). Glomerular layer is demarked by dotted lines. An OMP-GFP mouse was analyzed. Note that Nrp1-positive and -negative OSN axons formed distinct glomerular-like structures within glomeruli in the high magnification images. Scale bars are 500 μm in **A**, 200 μm in **B**, and 100 μm in **C**.

Figure 3. Heterogeneous OSN axons converge onto a glomerulus.

OB sections stained with anti-GFP (*OMP-GFP*) and anti-Kirrel2 antibodies were imaged with confocal microscopy. DAPI staining shows the distribution of periglomerular cells surrounding glomeruli. The layout of periglomerular cells remains unchanged. Glomerular layer is demarked by dotted lines. Anterior part of the dorsal OB is shown. In the control OB, Kirrel2 immunoreactivity was glomerulus-specific and homogeneous within a glomerulus. However, on the axotomized side, distinct glomerular-like structures with differential Kirrel2 levels were found within each glomerulus (arrowheads). Similar results were obtained with 17 experiments. Scale bars are 100 μm .

Figure 4. Connectivity of mitral cell dendrites is reduced after axotomy.

A, Stacked confocal images (20 μm thick) of OB slices from *Thy1-YFP* (G line) mice. Mitral cells are labelled with YFP in this mouse line. Sagittal OB slices were cleared with SeeDB2G and anterodorsal part (red) was analyzed with confocal microscopy. Note that control OB show thick primary dendrites extending to the glomerular layer (white arrowheads), whereas only faint signals were seen in the outer external plexiform

layer in the axotomized conditions. For unbiased quantification brightly-labelled mitral cells (top 20%) were traced for quantification. **B**, Representative Neurolucida tracing of mitral cell primary dendrites in the control and axotomized OBs. Primary dendrites of mitral cells partially de-innervated from glomeruli in the axotomized side. Innervated glomeruli are shown in blue and other glomeruli are shown in gray. **C**, Quantification of primary dendrite innervation. Mitral cell dendrites were often thinned and became untraceable at 21-days after axotomy. The partial de-innervation of primary dendrites persisted at least for 84 days after axotomy. Numbers of mitral cells (n) and animals (N) are indicated. $p^{***} < 0.001$ (Chi-square test for mitral cells with innervating dendrites, compared to the control side). Scale bars are 100 μm .

Figure 5. Atrophy of dendritic tufts in mitral/tufted cells.

A, Mitral cells were labeled with tdTomato using *in utero* electroporation at E12. *OMP-GFP* mice were used to visualize OSNs. Axotomy was performed at P56 and mitral cell morphology was analyzed 42 days after axotomy. Z-stacked confocal images (52 μm thick) are shown. High magnification images show the tufted structures within a glomerulus in the control, but not after axotomy. **B**, Representative Neurolucida tracing of mitral cell dendrites including tufted structures within glomeruli. We show five representative neurons including the ones shown in A. Boxed areas are shown in high magnification images in A. Scale bars are 100 μm .

Figure 6. Odor responses in mitral cells are reduced after axotomy.

A, Two-photon Ca^{2+} imaging of the olfactory bulb using *Thy1-GCaMP6f* mice (GP5.11). In this mouse line, GCaMP6f is specifically expressed in mitral cells. The glomerular layer in the anterodorsal part of the OB (boxed area) was imaged using two-photon microscopy. Right OBs (demarcated by orange broken lines) were analyzed. A, anterior; L, lateral. **B**, The odor map after axotomy was altered (day 42). Odor-evoked

responses in the OB were analyzed by two-photon Ca^{2+} imaging. Average activation maps are shown. In the control OB, Butyric Acid activates anteromedial part of the dorsal OB (class I), whereas Acetophenone, Guaiacol, and Vanillin activated more posterolateral area (class II). However, the response amplitude was much reduced and localization was not clear after the recovery after axotomy. **C**, Cumulative curves for odor-evoked responses (pixel-based $\Delta F/F_0$ during the first 5sec of odor exposure). After the recovery from axotomy, odor-evoked responses in the OB were much reduced. $p^* < 0.05$, $p^{***} < 0.001$ (Kolmogorov–Smirnov test). Scale bars are 1 mm.

Figure 7. Schematic representation of defective circuit recovery after axotomy.

A, Embryonic development of the olfactory map. Target cues (e.g., Sema3A) guide axons to correct positions in the OB. A, anterior; P, posterior. **B**, Normal OSN turnover process. We assume that axon-axon interactions (pioneer-follower interactions) are important to maintain the topographic olfactory map in the normal turnover process. **C**, Our proposed model for defective OSN projection and partial dendrite deinnervation after axotomy. Without positional cues and axon-axon interactions, regenerated OSN axons cannot find correct targets in the OB after axotomy. Reduced connectivity of primary dendrites may lead to reduced odor responses in mitral cells.

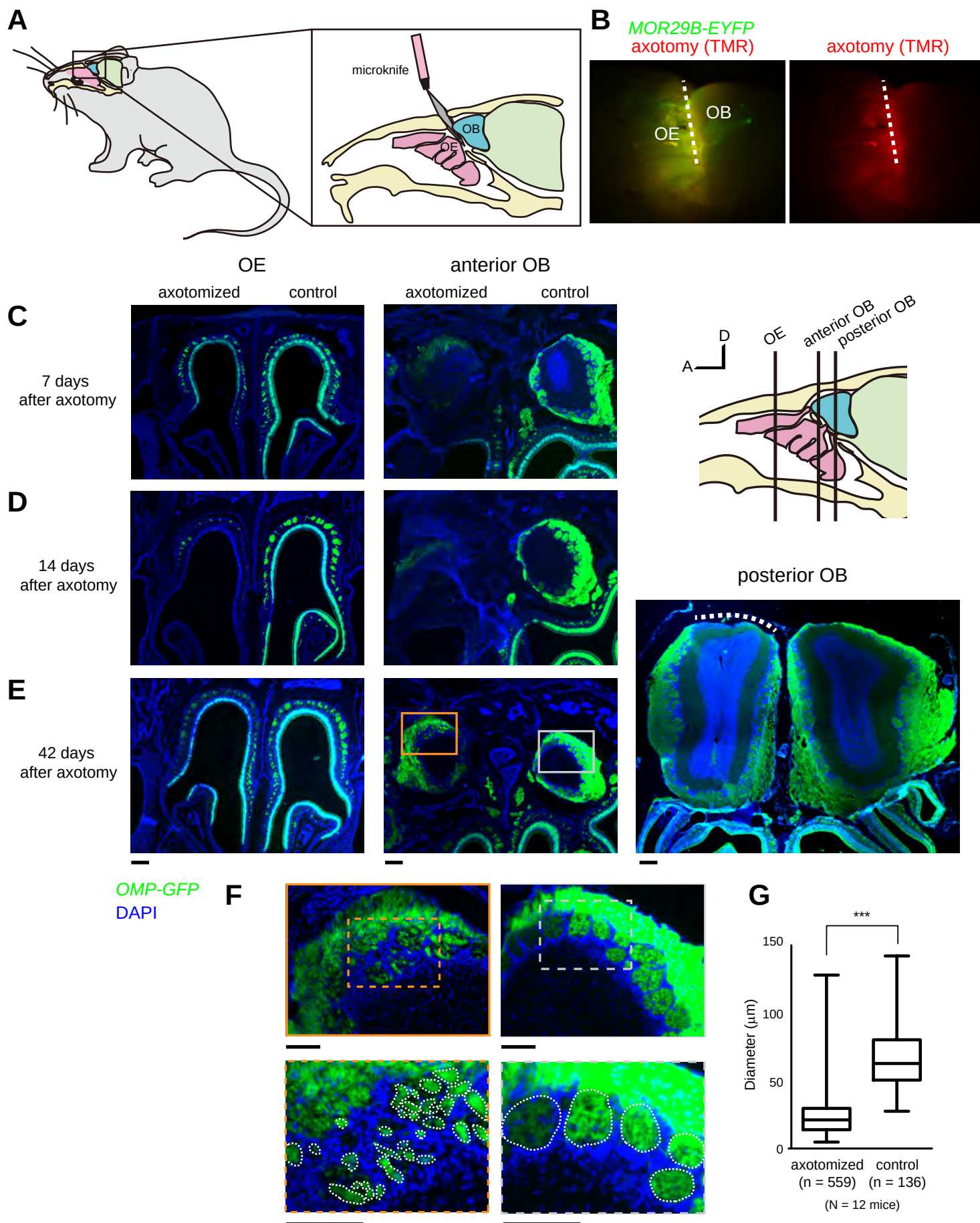


Fig. 1

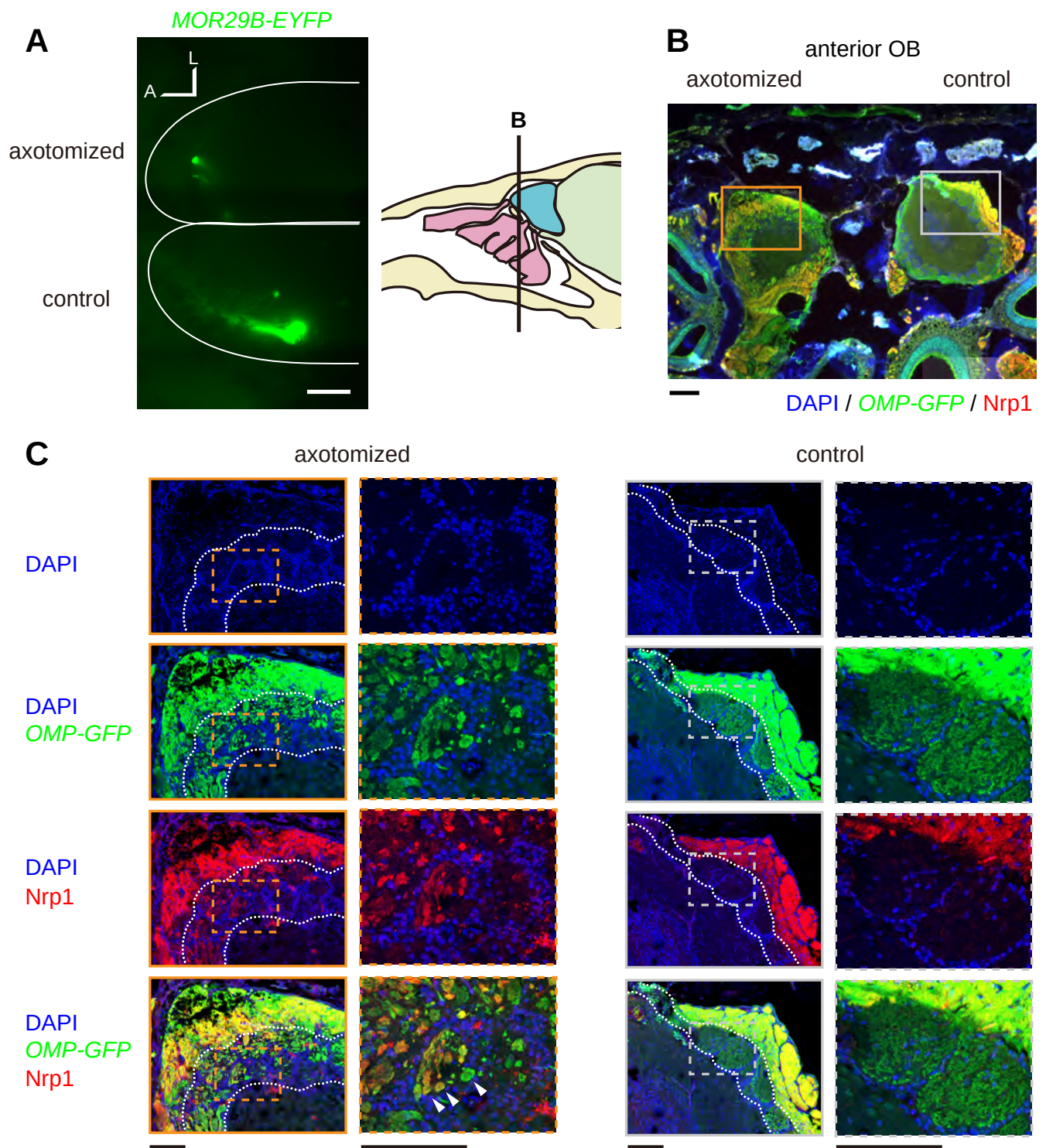


Fig. 2

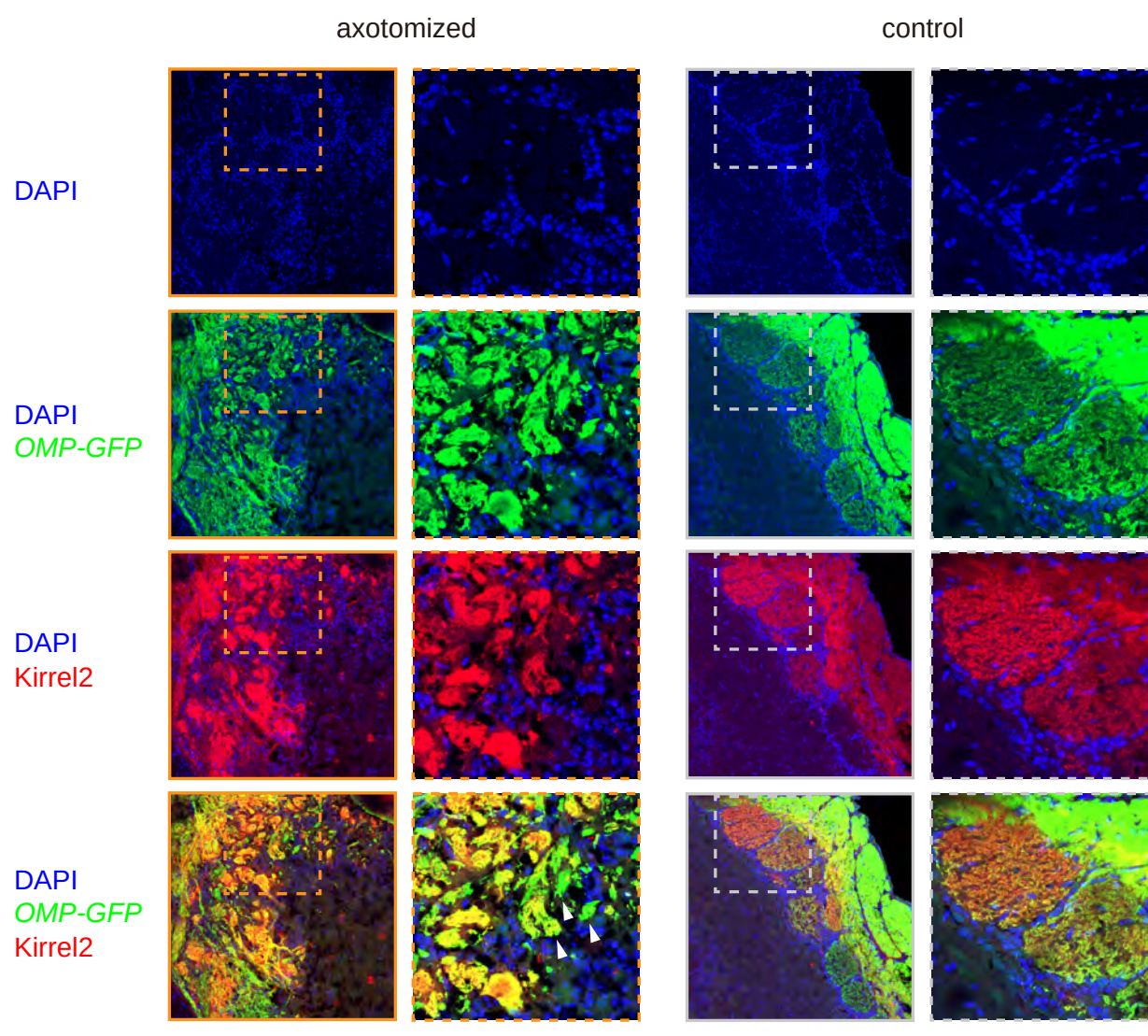


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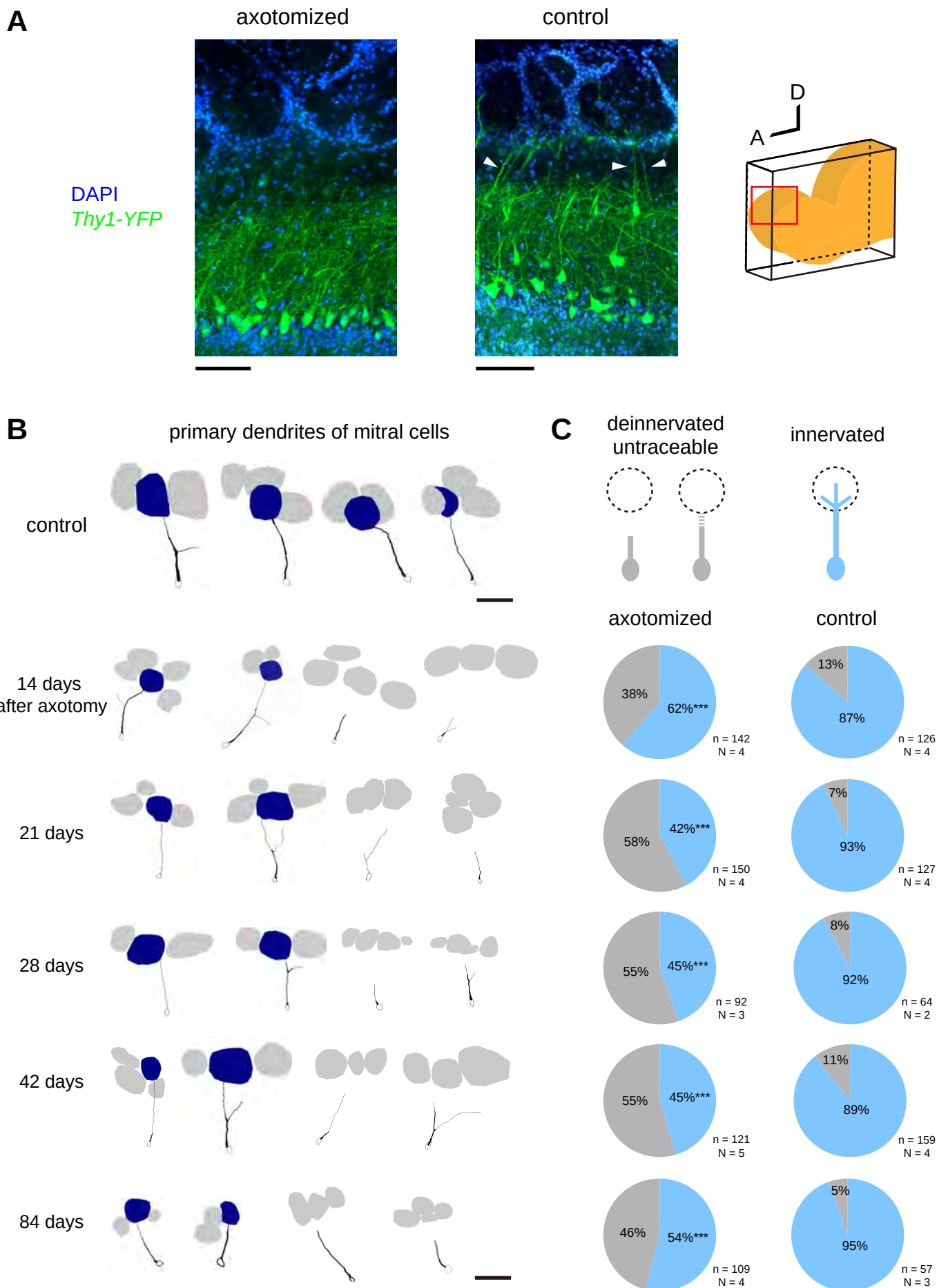


Fig. 4

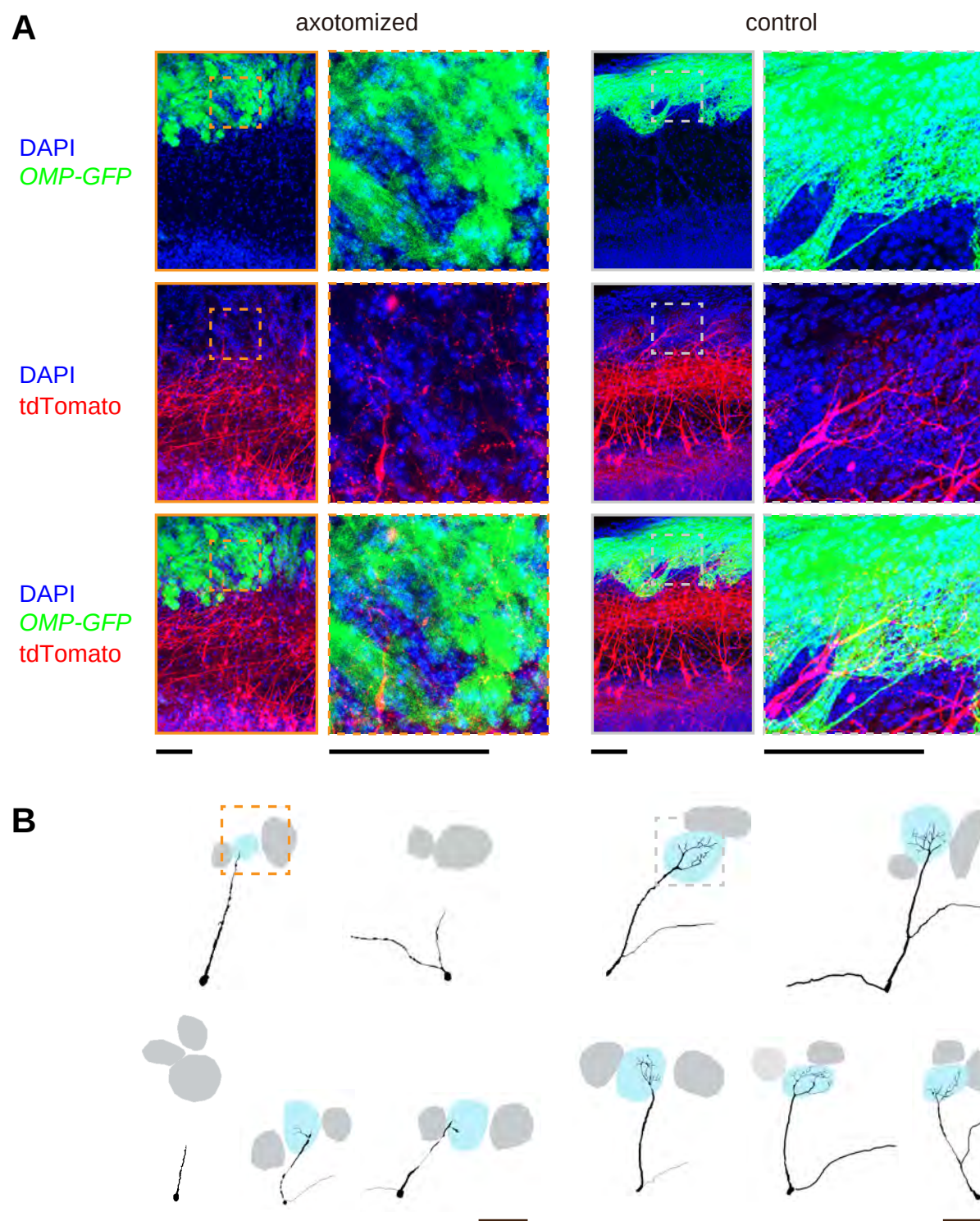


Fig. 5

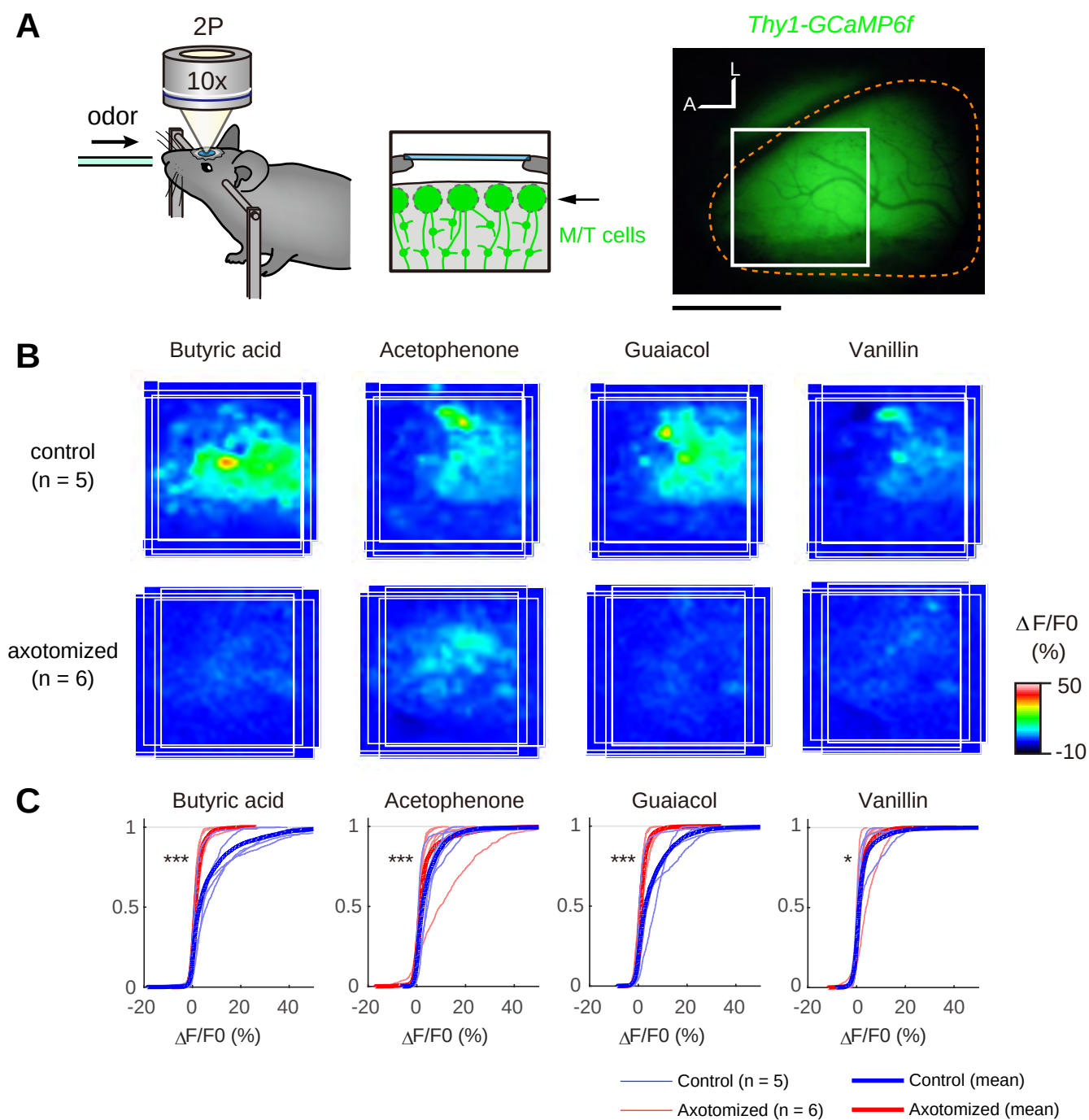


Fig. 6

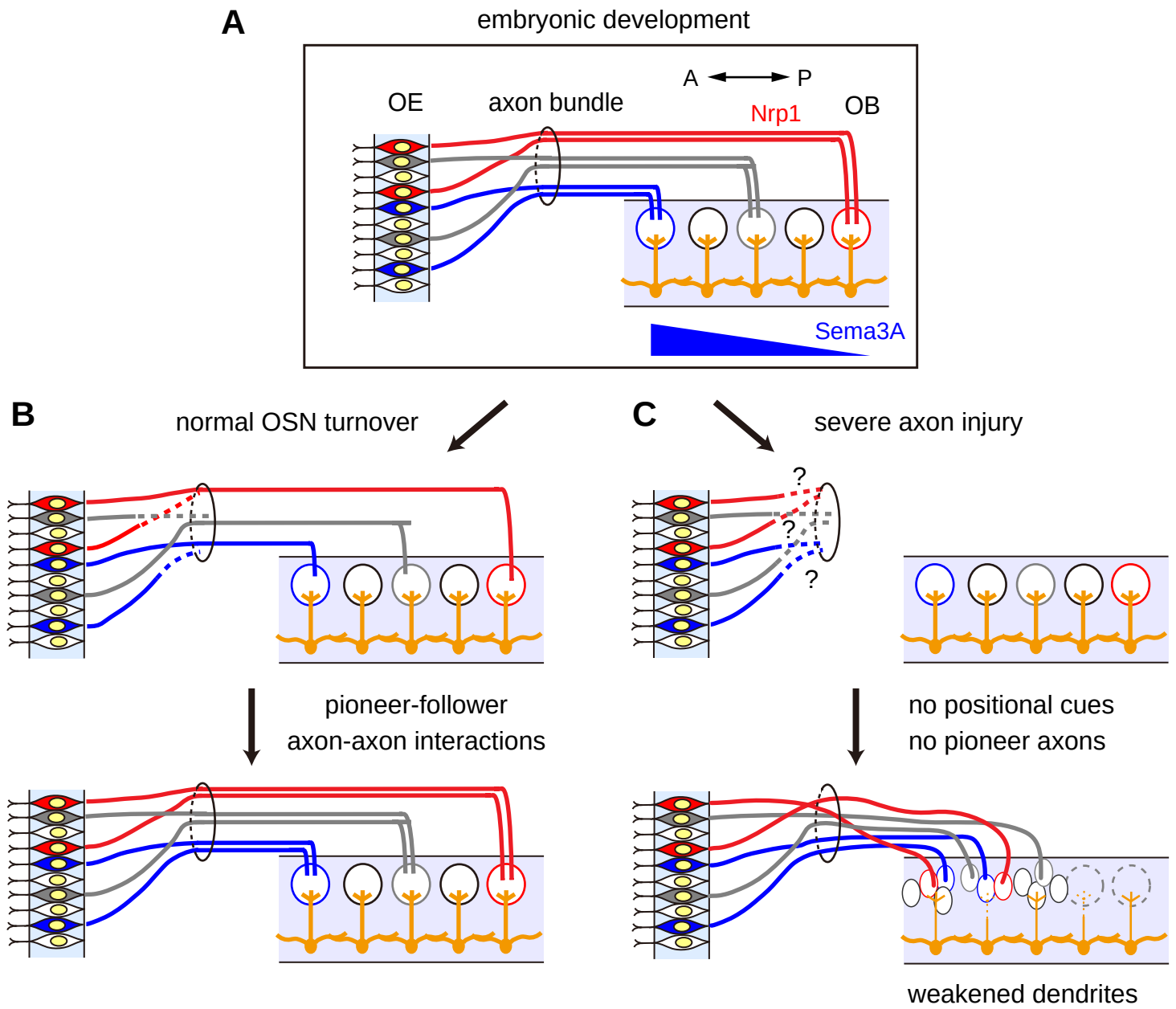


Fig. 7