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Projection neurons are necessary for the maintenance, but not for the assembly, of the mouse olfactory circuit

Luis Sánchez-Guardado, Peyman Razavi, Bo Wang, Antuca Callejas-Marín, Carlos Lois

Department of Biology and Biological Engineering, California Institute of Technology, E91125, Pasadena, United States • Department of Cell Biology, School of Science, University of Extremadura, E06071 Badajoz, Spain

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Abstract

The assembly and maintenance of neural circuits is crucial for proper brain function. Although the assembly of brain circuits has been extensively studied, much less is understood about the mechanisms controlling their maintenance as animals mature. In the olfactory system, the axons of olfactory sensory neurons (OSNs) expressing the same odor receptor converge into discrete synaptic structures of the olfactory bulb (OB) called glomeruli, forming a stereotypic odor map. The OB projection neurons, called mitral and tufted cells (M/Ts), have a single dendrite that branches into a single glomerulus, where they make synapses with OSNs. We genetically eliminated more than 95% of M/Ts in early postnatal mice, and observed that the assembly of the OB bulb circuits occurred normally. However, as the animals became adults the apical dendrite of remaining M/Ts grew multiple branches that innervated several glomeruli, and OSNs expressing single odor receptors projected their axons into multiple glomeruli, disrupting the olfactory sensory map. Moreover, ablating the M/Ts in adult animals also resulted in similar structural changes in the projections of remaining M/Ts and axons from OSNs Surprisingly, the ability of these mice to detect odors was relatively preserved despite only having 1-5% of projection neurons transmitting odorant information to the brain, and having highly disrupted circuits in the OB. These results indicate that a reduced number of projection neurons does not affect normal assembly of the olfactory circuit, but induces structural instability of the olfactory circuitry of adult animals.

eLife assessment

This **valuable** study shows that eliminating a large portion of the principal neurons in the mammalian olfactory bulb does not affect the initial establishment of the circuit but has an impact on its maintenance. The strength of the paper is that the anatomical changes induced by genetic ablation of neurons are clear-cut. There is a **solid** description of the structural and behavioral effects of ablating the majority of M/T neurons; however, the conclusions are **incompletely** supported by the findings.

Introduction

In order to produce behavior, the brain needs to respond to two contradictory demands. First, to acquire new information, the brain needs to be plastic. Second, to retain information, the brain needs to be stable. The brain of young animals is highly plastic, enabling early experiences to influence the development of many neuronal circuits (Wiesel and Hubel, 1963). In contrast, the adult brain is considered to be relatively hard-wired, with limited capacity for morphological changes in response to natural experiences or different types of experimental manipulations (Darian-Smith and Gilbert, 1994; Florence et al., 1998).

Two fundamental parameters for the function of brain circuits are the number of neurons that comprise the circuit and how they are connected to each other. Interestingly, within a given circuit and animal species, the number of neurons of a specific cell type in that circuit appears to be relatively conserved. Similarly, there is also significant regularity in the general connectivity trends between neurons in the circuit. These observations have raised the question of whether there is any relationship between the number of a specific type of neuron in the brain, and their patterns of connectivity. In adult *Drosophila*, reducing the number of neurons of specific cell types in the olfactory system does not alter the overall structure of the circuit (Berdnik et al., 2006). In contrast, ablation of a particular cell type during embryonic development induces structural changes in the neurites of the remaining neurons of the same type ("peer" cells) in *Drosophila* and mice (Johnson et al., 2017; Wang et al., 2021). However, less is known about how the reduction in the number of neurons affects the connectivity of their remaining "peer" cells and how these changes evolve as animals mature, impacting the organization and function of the entire circuit. To further investigate these questions, we focused on the mammalian olfactory bulb (OB).

The structural organization of the olfactory system makes it an ideal model to investigate the mechanisms underlying the connectivity, assembly, maintenance, and plasticity in brain circuits. The olfactory epithelium harbors olfactory sensory neurons (OSNs) that send their axons to the OB. Axons from OSNs expressing the same odor receptor converge into two stereotypic glomeruli within the OB, generating an odor map on its surface (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). The odor information received by the OSNs is transmitted from each glomerulus to cortical areas by the axons of the OB projection neurons, mitral and tufted cells (M/T cells) (Schwob and Price, 1984; Walz et al., 2006). During development, M/T cells initially extend several apical dendrites that innervate multiple neighboring glomeruli. At postnatal stages (P7-P10), M/T cells acquire their mature morphology with the vast majority (>95%) displaying a single apical dendrite innervating a single glomerulus (Blanchart et al., 2006; Hinds, 1968a, 1968b; Shepherd et al., 2004). Each glomerulus is innervated by the apical dendrites of ~40 peer M/T cells, and it has been demonstrated that peer M/T cells that their apical dendrites project into the same glomerulus differ in their intrinsic excitability and downstream connections (Burton and Urban, 2014; Inokuchi et al., 2017).

The OB is one of the areas of the adult mammalian brain with the highest levels of structural plasticity. OSNs, glomerular and periglomerular cells are some of the key synaptic partners for M/T cells and they turn over throughout the animal's life (Graziadei and Graziadei, 1979; Merkle et al., 2014). Surprisingly, despite this continuous turnover, the M/T dendrites are highly stable in adult animals (Mizrahi and Katz, 2003). Thus, the synaptic organization of the OB, where each M/T cell, and each set of OSNs expressing the same receptor innervate a single glomerulus, makes the OB circuit an ideal model for understanding how neurons achieve long-term stability.

To investigate the relationship between neuron number and connectivity, we genetically ablated >95% of M/T cells in perinatal transgenic mice. Despite this drastic reduction in M/T numbers, the initial assembly of the OB circuits occurred normally, and the remaining M/T cells refined their arbors to a single dendrite projecting to a single glomerulus, and OSNs expressing the same receptor targeted a single glomerulus. However, as animals matured, the OB wiring became progressively perturbed, and the apical dendrite of remaining M/T cells grew additional branches that innervated multiple glomeruli, indicating that long term stability of M/T cells morphology depends on the presence of peer M/T cells. In addition, after reducing the number of M/T cells the axons of OSNs expressing the same odorant receptor innervated multiple glomeruli, perturbing the olfactory sensory map in the OB surface, indicating that odor map maintenance also depends on a full set of M/T cells. Despite the significant disruptions in the connectivity of the OB circuits, several olfactory- dependent behaviors were preserved, demonstrating the high functional resilience of the olfactory system.

Results

Reducing the number of M/T cells in perinatal animals does not affect the refinement of the apical dendrite of remaining peer cells

M/T cells experience a drastic change in their morphology after birth. During the embryonic and early neonatal period (less than P7), M/T cells have multiple apical dendrites innervating multiple glomeruli. After the first postnatal week, the vast majority (>95%) of M/T cells prune all but one dendrite that innervates a single glomerulus. This refinement can also occur in the absence of sensory input (Lin et al., 2000; Ma et al., 2014). However, genetic deletion of NMDA receptors in the M/T cells prevents the refinement of their apical dendrites (Aihara et al., 2021; Fujimoto et al., 2023), indicating that synaptic inputs to M/T cells originating from spontaneous brain activity may regulate the process of dendrite refinement. In the mammalian retina, reducing the number of rod bipolar cells (RBCs) during development triggers dendritic sprouting on remaining RBCs, suggesting that interaction between peer cells sculpts the maturation of their dendritic arbors (Huckfeldt et al., 2009; Johnson et al., 2017). To investigate whether dendritic refinement of M/T cells depends on their density, we ablated M/T cells at perinatal stages by crossing the driver *Tbx21-Cre* (*Tbx* for short) mouse (Mitsui et al., 2011) with the reporter loxP-*DTA* mouse (Voehringer et al., 2008) (**Figure 1A**). The *Tbx* mouse expresses cre selectively in M/T cells, and the loxP-*DTA* mouse expresses diphtheria toxin (DTA) upon cre-mediated recombination, which triggers the death of M/T cells (**Figure 1B**). Based on the result obtained by crossing the *Tbx* mouse with the loxP-*tdt* reporter *Ai9* mouse (Madisen et al., 2010), we found the vast majority, if not all, M/T cells expressed tdTomato (tdT) fluorescent protein in *Tbx::Ai9* mice at P3 (**Figure 1—figure supplement 1**). Assuming that the time course of recombination of loxP-*tdt* and loxP-*DTA* trangenes is similar, one would expect that by P3 most M/T cells would be killed by DTA expression in *Tbx::DTA* mice.

Tbx::DTA mice survive and reach adulthood without any gross differences from their wild-type siblings (**Figure 1—figure supplement 2A** and **B**). Although the OB volume of *Tbx::DTA* mice was reduced to half, the typical OB layer organization was mostly preserved (**Figure 1—figure supplement 2C** and **D**). Interestly, using antibodies against the Tbr2 protein (a marker for M/T cells), we identified a small number of Tbr2+ cells remaining in the mitral cell layer (MCL) and external plexiform layer (EPL) after cell ablation (**Figure 1—figure supplement 2E**). In *Tbx::DTA::Ai9* mice we observed that there was a small percentage of M/T cells that were not killed and expressed *tdt.* Previous works have demonstrated that DTA is extremely potent, and that a single molecule of DTA is sufficient to kill a cell. These observations are consistent with the recombination of the loxP-*tdt* transgene being more sensitive to cre than the loxP-*DTA* transgene, a phenomenon that has been routinely reported in the literature (Becher et al., 2018; Kurachi et

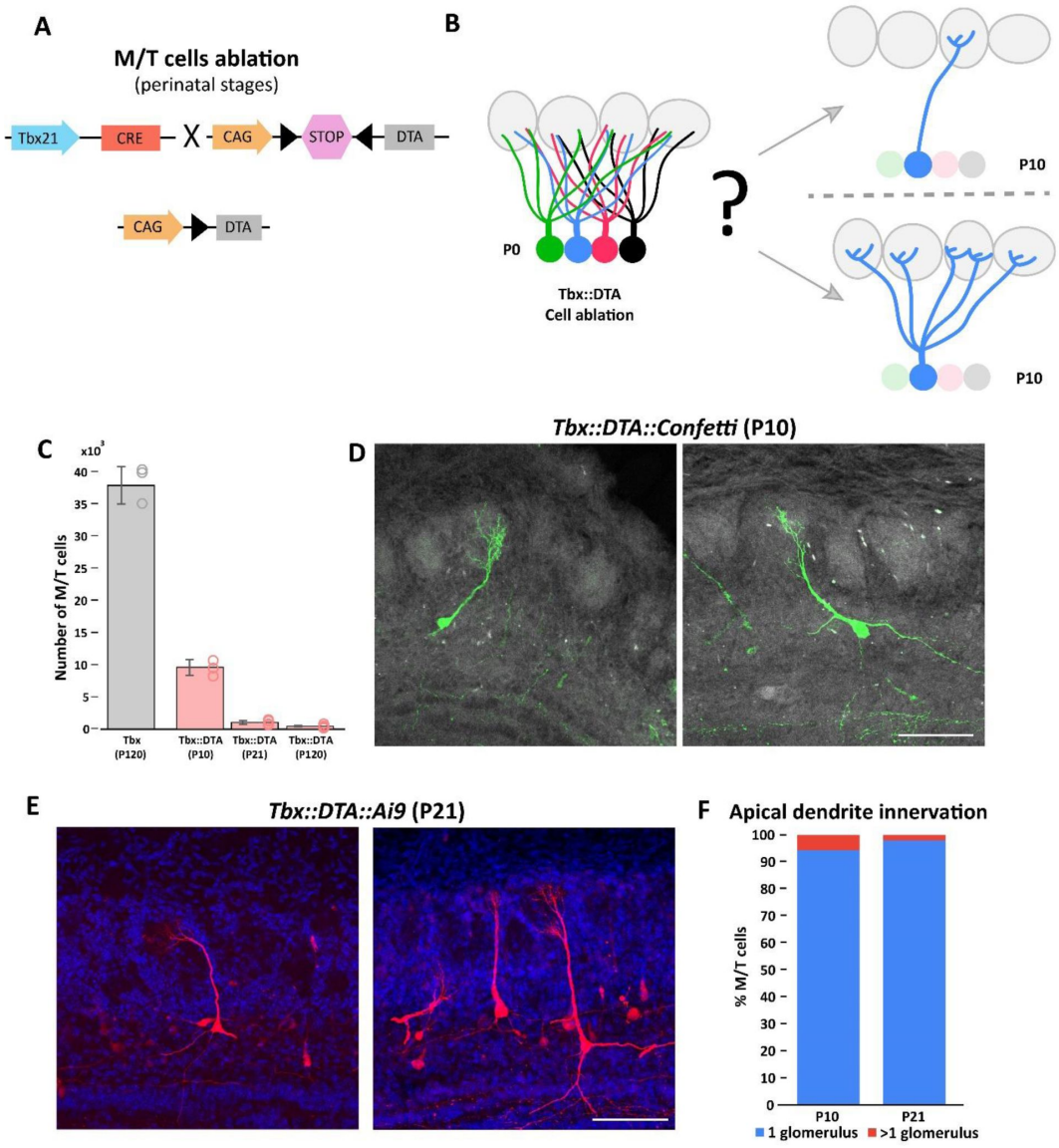

Figure 1

The refinement of mitral and tufted cell apical dendrite is not affected by massive cell ablation at perinatal stages.

A. Schematic representation of the transgenic strategy to ablate M/T cells at perinatal stages. The Tbx21 promoter is specific for M/T cells. The *Tbx::cre* transgene controls the expression of the Cre recombinase in M/T cells, thus regulating the selective expression in M/T cells of the subunit A of the diphtheria toxin (DTA), from a CAG::loxp stop loxP-*DTA* reporter mouse.
B. Schematic representation of the two possible scenarios expected after M/T cell ablation. (top) Reduction of M/T cells density during perinatal maturation does not affect the apical dendrite refinement. (bottom) The lack of peer M/T cells interferes with the normal refinement of the apical dendrite, thus retaining an immature morphology with multiple apical dendrites.
C. Quantification of the number of remaining M/T cells (tdT positive cells) in the OB at different times after perinatal cell ablation in *Tbx::DTA* mice crossed with the *Ai9* reporter mouse. In *Tbx::DTA::Ai9* mice, approximately 25% of the M/T cells remained at P10 (9,605.7 ± 1,229.6; n=4), ~3% at P21 (1,024.7 ± 282.2; n=9), and ~1% at P120 (437.6 ± 119.2; n=8) compared with wild type (*Tbx*) mice at P120 (38,546.8 ± 2,912.2; n=3). Data are shown as average ± standard deviation.
D. Confocal images of individually labeled M/T cells in the OB of a P10 *Tbx::DTA::Confetti* mouse after perinatal cell ablation. Note that M/T cells present a single apical dendrite innervating a glomerulus. Scale bar is 100 µm.

al., 2019; Madisen et al., 2010; Song and Palmiter, 2018). We observed that in *Tbx::DTA::Ai9* mice approximately 25% (P10), 3% (P21), and 1% (P120) of M/T cells remained after cell ablation (and expressed tdt), compared with wild type mice (*Tbx::Ai9* at P120) (**Figure 1C**).

Next, we crossed the *Tbx::DTA* mouse with the loxP-*Confetti* reporter mouse to label individual M/T cells with one out four possible fluorescent proteins(Sánchez-Guardado and Lois, 2019; Snippert et al., 2010). This sparse labeling of cells facilitates investigating whether the refinement of the dendrites of M/T cells depends on interactions between peer cells. At P10, ~95% of the remaining M/T cells had a single apical dendrite innervating a single glomerulus (**Figure 1D**; **Figure 1—figure supplement 3**). These results indicate that the refinement of the apical dendrite of M/T cells is not influenced by a drastic reduction in the density of M/T cells, suggesting that cell-autonomous mechanisms might control it.

M/T cells extend additional dendrites to multiple glomeruli in the absence of neighboring peer cells in adult animals

The decrease in neuronal plasticity with age is probably due to a combination of cell-autonomous, and non-cell autonomous processes. Cell-autonomous processes depend on genetic program of maturation intrinsic to the individual cells. An alternative model is that the reduction in plasticity with age may be due to non-cell autonomous processes, where each neuron loses plasticity because of the interactions with its neighbors. In normal conditions, despite the turnover of their synaptic partners (OSNs and periglomerular interneurons), the apical dendrite of M/T cells remains very stable (Mizrahi and Katz, 2003). To investigate whether the interactions between neighboring peer M/T cells regulate the stability of their dendrites in adult animals, we reduced the number of M/T cells in full adult animals (two month old) by crossing the *Tbx21-Cre* mouse with the *iDTR* mouse (Buch et al., 2005). Mice from this cross express the gene for diphtheria toxin receptor (dtR) on the M/T cells. Upon systemic injection of diphtheria toxin (DT), M/T cells are selectively eliminated (**Figure 2A** and **B**). DT injections into P60 *Tbx::iDTR::Ai9* mice were analyzed two months later (P120), and only 3% of the M/T cells remained compared with the wild type (*Tbx::Ai9*) mice (**Figure 2C**).

Next, we analyzed the dendrites of individual M/T cells using a two-component viral vector system to sparse and stochastically label cells that express Cre recombinase in the *Tbx* mice (Chan et al., 2017). Viral injections were performed in three types of animals: (i) *Tbx*, that had the normal set of M/T cells, (ii) *Tbx::iDTR*, in which >90% of M/T cells were killed by DT injection at P60, and (iii) *Tbx::DTA*, in which >90% of M/T (cells were killed around P3). In all three conditions, the morphology of M/T cells was examined in adult animals, at P120. As expected, most of M/T cells projected into a single glomerulus in *Tbx* mice (**Figure 2D** and **I**). Surprisingly, in *Tbx::iDTR* mice, 48% of the remaining M/T cells branched to several glomeruli (**Figure 2F** and **I**; **Figure 2—figure supplement 1B**). Interestingly, whereas in *Tbx::DTA* mice the apical dendrite of remaining M/T cells innervated a single glomerulus at P10, we also observed that by P120, 36% of remaining M/T cells projected their apical dendrite into multiple glomeruli, as in the *Tbx::iDTR* mice (**Figure 2D** and **I**; **Figure 2—figure supplement 1A**). We also investigated whether the plasticity of the apical dendrite is reduced with age. Cell ablation performed in six month old *Tbx::iDTR* mice (P180) showed that 45% of remaining M/T cells branched to several glomeruli, a percentage comparable to that of animals in which ablation was performed at P60, indicating that the ability of M/T cells to experience drastic morphological changes in their dendrites does not decrease with age (**Figure 2G** and **I**; **Figure 2—figure supplement 1C**). The tuft length of M/T cells in *Tbx::DTA* (perinatal cell ablation), and *Tbx::iDTR* (cell ablation at P60 and P120) was increased compared to apical dendrite tuft in control *Tbx* (**Figure 2—figure supplement 2**). These results indicate that a sufficient density of M/T cells are required for the maintenance of a normal mature morphology (one apical dendrite-one glomerulus), suggesting that structural plasticity is not locked even six months after M/T cells acquired their mature morphology.

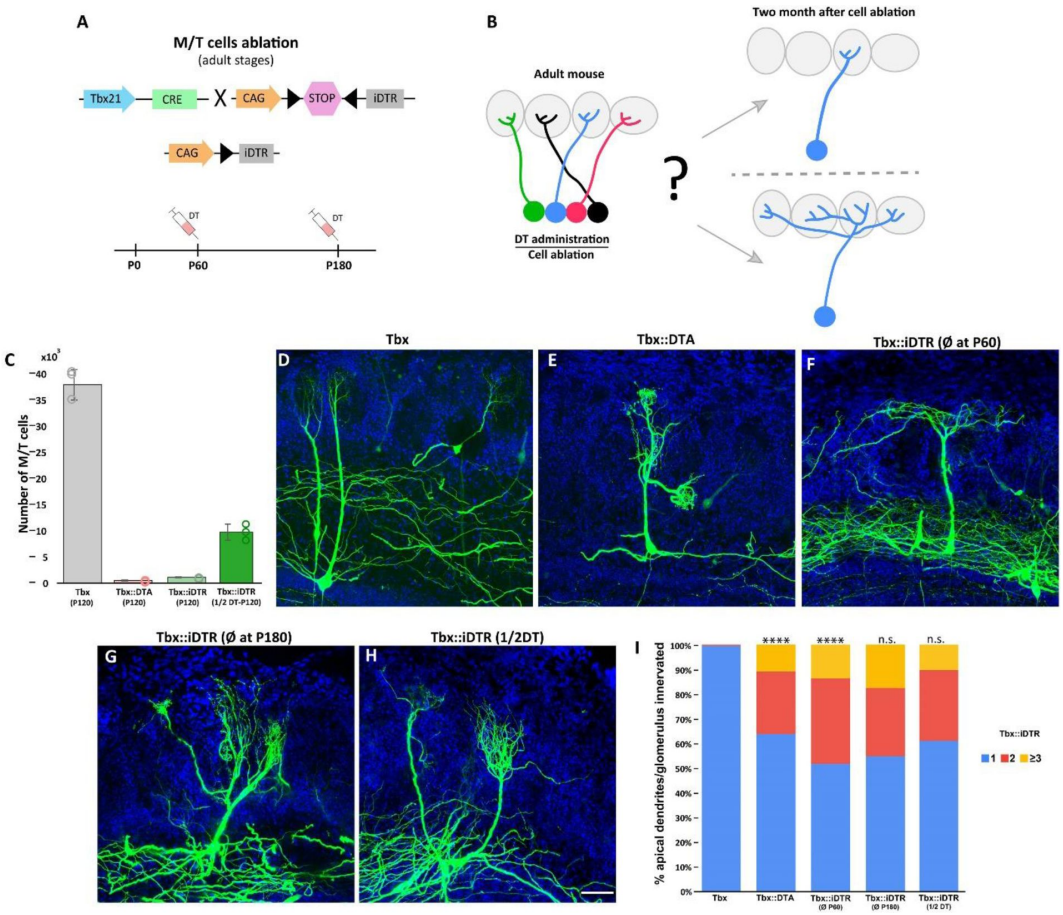

Figure 2

Massive ablation of M/T cells in adult mice induces plasticity of apical dendrite.

A. Schematic representation of the transgenic strategy to ablate M/T cells in adult animals. The Tbx21 promoter is specific for M/T cells. The *Tbx-cre* transgene controls the expression of the Cre recombinase in M/T cells, enzyme, thus regulating the selective expression in M/T cells of the receptor (iDTR) for diphtheria toxin (DT), from a CAG:loxp stop loxP-*iDTR* reporter mouse. Upon systemic injection of DT into the bloodstream, DT gets transported into M/T cells expressing iDTR, and selectively kills them.

B. Two possible scenarios are expected after ablating most of the M/T cells in adult animals. (top) M/T cells density reduction does not induce changes in the apical dendrite structure. (bottom) The absence of peer M/T cells induces structural plasticity in remaining M/T cells, whose apical dendrites extend into nearby glomeruli.

C. Quantification of the number of remaining M/T cells (tdTomato (tdT) positive cells) in the OB of adult mice (P120) in different cell ablation experiments. 437.6 ± 119.2 M/T cells remained in *Tbx::DTA::Ai9* mice (~1% of the full set of M/T counted in wt mice; n=8; light red). 1,158 ± 99 M/T cells remained in *Tbx::iDTR::Ai9* mice (~3% compared to wt mice; n=4; light green). 9,819 ± 1544 M/T cells remained in *Tbx::iDTR::Ai9* mice when half of the DT concentration (10 µg/Kg of body weight) was injected (~25% compared to wt; n=3; dark green). Wild type (*Tbx::Ai9*) mice had 38,546.8 ± 2,912.2; n=3; gray). Data are shown as average ± standard deviation.

D-H. Confocal images of M/T cells after sparse labeling using AAV-DiO-GFP. (C) *Tbx* (wild type) mice at P120. (D) *Tbx::DTA* mice at P120. (E) *Tbx::iDTR* mice at P120 (cell ablation induced at P60). (F) *Tbx::iDTR* mice at P240 (cell ablation induced at P180). (G) *Tbx::iDTR* mice at P120 (cell ablation induced at P60 using half of the DT dose). Blue = DAPI staining. Scale bar in H is 50 µm and applies to D-H.

I. Percentage of M/T cells with an apical dendrite innervating one, two, three or more glomeruli for experimental conditions in D-H. In *Tbx::DTA* ~35% of M/T cells had an apical dendrite innervating more than one glomerulus (n=149; p-value<0.0001; $\chi 2$ test). In *Tbx:iDTR* mice, when ablation was induced at P60, 48% of M/T cells had an apical dendrite innervating more than one glomerulus (n=188; p-value<0.0001). No significant differences were observed in *Tbx:iDTR* mice when comparing cell ablation performed at P60 or P180 (n=216; p=0.15) or using half of the DT dose (n=236; p=0.05).

Finally, we tested the hypothesis that if glomeruli are innervated by a drastically reduced number of M/T cells this may induce nearby M/T cells to expand their apical dendrites so that they innervate the neighboring empty or depleted glomeruli. In wild type animals, there are ~2,000 glomeruli, and each glomerulus is innervated by around 40-50 M/T cells. In animals injected with a high dose of DT (20 µg/kg mouse body weight), as shown above, around 3% (~500) of M/T cells remained, suggesting that in these animals most glomeruli were innervated by very few M/T cells (2,000 glomeruli innervated by ~ 500 M/T cells – roughly 1 M/T for every 4 glomeruli), or none, in many cases. In these animals, around 40% of the remaining M/T cells projected to several glomeruli. When we injected half (10 µg/kg) of the DT dose used in our previous experiments, the fraction of remaining M/T cells was 25% (~10,000 cells) compared to wild type mice (~40,000 cells) (**Figure 2C**). Thus, in animals injected with this lower dose of DT it is expected that each glomerulus would be innervated by around several M/T cells (~10,000 M/T cells for 2,000 glomeruli – roughly around 5 M/T per glomerulus). However, in these animals, ~40% of the M/T cells still projected to multiple glomeruli, a similar percentage as in the animals with higher doses of DT in which the vast majority of M/T cells were ablated (**Figure 2H** and **I**; **Figure 2—figure supplement 1D**). No significant differences in the length of the tuft M/T cells were observed when comparing with control mice (using high DT doses in P60 *Tbx::iDTR* mice) (**Figure 2—figure supplement 2**). These results suggest that the branching of M/T dendrites into multiple glomeruli does not simply occur because nearby glomeruli are empty. Instead, these observations suggest that the expansion of the M/T apical dendrites may be constrained by some putative interaction between M/T cells, and that below a certain density of M/T cells, apical dendrites can expand into multiple glomeruli, even if these glomeruli are occupied by dendrites from neighboring M/T neurons.

These results indicate that the ability of M/T cells to grow new dendrites, and to project them to multiple glomeruli is not restricted to neonatal stages and that it can be induced in adults (6 months old) mice, simply by reducing the density of their peer M/T cells. Interestingly, only around half of the surviving M/T cells their apical dendrites extended toward several glomeruli, while the other half maintained a single dendrite that targeted a single glomerulus. This observation suggests that the ability of M/T cells to change their structure and grow dendrites into adjacent glomeruli may be restricted to specific subtypes of M/T types.

Mechanisms controlling M/T cells structural plasticity in adult animals

Activity plays an important role in sculpting cortical sensory circuits (Dräger, 1978; Gordon and Stryker, 1996; Sawtell et al., 2003). However, M/T cells apical dendrite refinement occurs in the absence of sensory activity (Lin et al., 2000; Ma et al., 2014), indicating that it is independent of input from OSNs. To investigate whether the structural plasticity induced in remaining M/T cells after ablation is regulated by sensory input, we performed unilateral naris occlusion (**Figure 3A**). Sensory deprivation was carried out in P50 *Tbx::iDTR* mice, starting ten days before M/T cell ablation. We used a two-component viral vector system to sparse and stochastically label cells, which allowed us to visualize the dendrites of individual M/T cells (Chan et al., 2017). OBs were analyzed two months after naris occlusión (P120). Sensory deprivation causes a decrease of tyrosine hydroxylase (TH) expression in PG cells, and we confirmed that in these animals sensory deprivation was effective by TH immunostaining (**Figure 3—figure supplement 1**). M/T cells tuft length in sensory deprived OBs was reduced compared to apical dendrite tuft in control *Tbx::iDTR* mice (ablation at P60 and unmanipulated sensory input) (**Figure 3—figure supplement 4**). However, the percentage of M/T cells innervating multiple glomeruli was similar regardless of whether the animals had a closed (39%) or open naris (44%) compared to the *Tbx::iDTR* mice (48%; control mice) (**Figure 3B** and **E**; **Figure 3— figure supplement 2A** and **B**). This result suggests that sensory stimuli are not essential for the expansion of M/T apical dendrites into multiple glomeruli in remaining M/T cells.

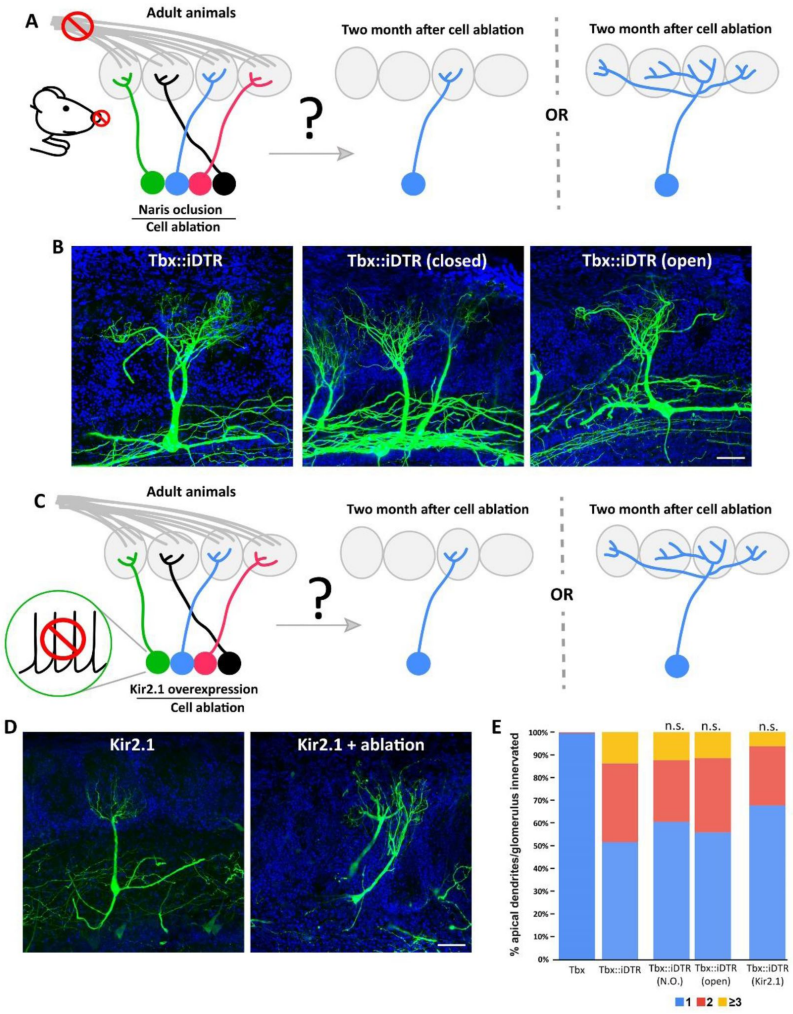

Figure 3

M/T cell apical dendrite plasticity caused by reduction in the number of neurons is independent of neuronal activity.

A. (left) Schematic representation of the strategy to perform sensory deprivation (naris occlusion) in *Tbx::iDTR* prior to cell ablation induced at P60. (right) Two possible scenarios are expected in the remaining M/T cells of sensory deprived OBs. (left cartoon) Sensory deprivation prevents the structural changes of M/T caused by ablation of M/T peers and retain a single apical dendrite, or (right cartoon) M/T cells undergo structural plasticity in sensory deprived OB, as shown in **Figure 2F**.
B. Confocal images of M/T cells labeled using AAV-DiO-GFP showing that remaining M/T cells in sensory deprived OBs still remodel their apical dendrites, such that they branch toward multiple nearby glomeruli, as the open OB. Blue = DAPI staining. Scale bar in B is 50 μm.
C. Schematic representation of the strategy to selectively reduce neuronal activity in M/T cells in *Tbx::iDTR* by expressing the Kir2.1 channel before inducing cell ablation at P60. Two possible scenarios are expected in the remaining M/T cells. Neuronal activity reduction prevents the structural changes of M/T caused by ablation of M/T peers and retain a single apical dendrite, or M/T cells with reduced neuronal activity undergo structural plasticity in sensory deprived OB, as shown in **Figure 2F**.
D. Confocal images of M/T cells labeled using AAV-DiO-GFP show that M/T cells expressing the Kir2.1 channel in the remaining cells when cell ablation was performed in *Tbx::iDTR* mice, showed structural changes in their apical dendrite. Blue = DAPI staining. Scale bar in D is 50 μm.
E. Percentage of M/T cells with apical dendrites innervating one, two, three or more glomeruli for experimental conditions shown B and D. Similar results were obtained in M/T cells from both OBs, closed (39%; n=231) or open (44%; n=228) nostril. No significant differences were observed between *Tbx::iDTR* mice with open or closed nostrils (p-value=0.17; $\chi 2$ test). Similarly, expression of Kir2.1 starting before cell ablation did not cause significant differences in *Tbx::iDTR* mice (p-value=0.05).
F.

To examine the role that the spontaneous activity of M/T cells may play in their apical dendrite plasticity in the absence of peer M/T cells, we overexpressed the inwardly rectifying potassium channels (Kir2.1) which reduces the firing of action potentials (**Figure 3C**)(Burrone et al., 2002). Kir2.1 channel was delivered into *Tbx::iDTR* mice 2-3 weeks before DT injection at P60 using a viral vector in which Kir expression depended on cre recombination. Thus, in this strategy, Kir was selectively expressed in M/T cells, and as expected, the firing of M/T cells was strongly reduced (**Figure 3—figure supplement 3**). As observed with sensory deprivation, kir expression caused a significant reduction of the tuft length in the apical dendrite of remaining M/T cells (**Figure 3—figure supplement 4**). However, the overexpression of the Kir2.1 channel in remaining M/T cells (32%) did not show significant differences in the structural changes of their apical dendrite compared to remaining M/T cells in *Tbx::iDTR* mice (48%) (**Figure 3D** and **E**; **Figure 3—figure supplement 2C** and **D**). These results indicate that apical dendrite plasticity in the remaining M/T cells in adult animals is not controlled by activity-dependent mechanisms.

Odor map disruption caused by a drastic reduction in the number of M/T cells

OSNs send their axons from the olfactory epithelium to the OB. During development, axons from OSNs expressing the same odor receptor initially project to several neighboring glomeruli. In the first postnatal week, OSNs axons undergo a refinement process such that all OSNs expressing one receptor project into a single glomerulus and form a stereotypic odor map on the OB surface (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994; Wang et al., 1998). Once the odor map is established, it is maintained throughout the animal's lifespan, despite the constant turnover of OSNs (Zou et al., 2004a). Above, we described that reducing the density of M/T cells in *Tbx::DTA* and *Tbx::iDTR* mice breaks the "one apical dendrite-one glomerulus" rule, such that remaining M/T cells branched into several glomeruli. Thus, we investigated whether M/T cells could also play a role in the refinement and maintenance of the odor map from the OSNs into the bulb.

To study whether M/T cells could regulate OSN refinement, we crossed the *Tbx::DTA* mouse (to produce perinatal cell ablation) with the *M72-IRES-ChR2-YFP* mouse (M72 for short; (Smear et al., 2013)) which expresses yellow fluorescent protein under the promoter of the M72 odor receptor (**Figure 4A** and **B**). At P10 and P21 there were no differences in the targeting of M72 axons between *Tbx::M72* and *Tbx::DTA::M72* mice (**Figure 4C** and **E**). These results suggest that a normal density of M/T cells is not necessary to refine OSN axons and odor map formation, and it is consistent with previous works (Bulfone et al., 1998).

Next, we investigated whether M/T cells are necessary for odor map maintenance by ablating M/T cells in adult *Tbx::iDTR::M72* mice (ablation induced by DT injection at P60). Surprisingly, M72 axons projected to several glomeruli when OBs were analyzed two months later (P120) (**Figure 4D** and **E**). Interestingly, whereas in *Tbx::DTA* mice M72 axons projected into a single glomerulus at P10 and P21, we also observed that by P120 the M72 axons projected into multiple glomeruli, as in the *Tbx::iDTR* mice (**Figure 4D** and **E**). These results suggest that the maintenance of the odor map on the OB surface depends on the presence of a sufficient density of M/T cells.

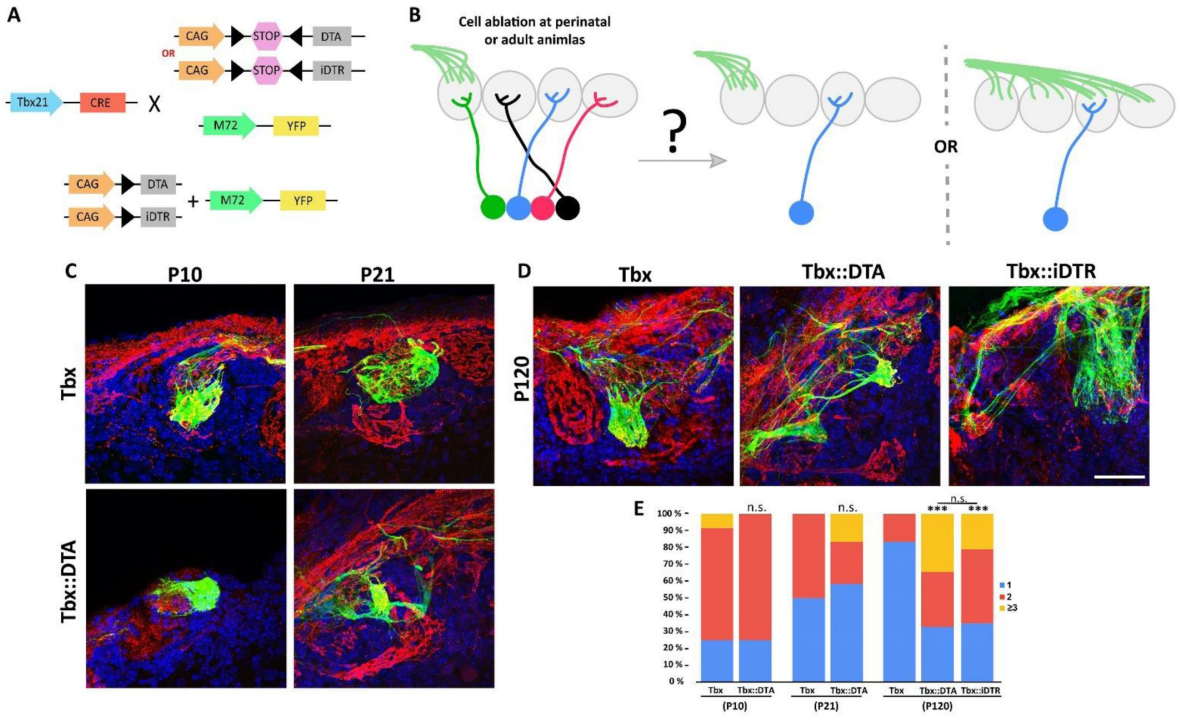

Figure 4

Odor map maintenance is disrupted by the drastic reduction in the number of M/T cells.

A. Schematic representation of the transgenic strategy to perform cell ablation in perinatal (*Tbx::DTA*) and adult (*Tbx::iDTR*) mice to analyze the axon projection of the M72 olfactory sensory neurons into the OB.
B. Two possible scenarios are expected for the OSN axon projection to the OB after M/T cells ablation. (left) M72 OSN axon project to a single glomerulus per hemibulb as in wt animals, or (right) M72 OSN axons project to more than one glomeruli per hemibulb, perturbing the odor map in the OB surface.
C. Confocal images of M72 OSN axons (green) in a wild type mouse (*Tbx*, top row) and *Tbx::DTA* mice (bottom row). Each column represents the analysis of the OSN axon at two different time points (P10 and P21). Immunostaining against olfactory marker protein (OMP, red) labels all OSN axons. Blue= DAPI staining.
D. Confocal images of M72 OSN axons (green) in *Tbx*, *Tbx::DTA* and *Tbx::iDTR* mice at P120. The glomerular projection was disrupted in adult mice, both in animals in which ablation occurred perinatally (*Tbx::DTA*) or as adults (*Tbx::iDTR* injected at P60 with DT). Immunostaining against OMP (red) labels all OSN axons. Blue = DAPI staining. Scale bar in D is 50 μm and applies to A-D.
E. Percentage of M72 OSN axons projecting to one, two, three or more glomeruli per hemibulb in *Tbx*, *Tbx::DTA* and *Tbx::iDTR* mice. No differences were observed at P10 and P21 between the M72 OSN axon in normal conditions or with reduced M/T cells (*Tbx*: P10 (n=12), P21 (n=20); *Tbx::iDTR*: P10 (n=12), P21 (n=12). At P10 (p-value=0.59; $\chi 2$ test), P21 (p-value=0.23). However, significant differences in M72 OSN axons were observed in *Tbx::DTA* (n=64; p-value<0.001) and *Tbx::iDTR* (n=52; p-value<0.001) mice at P120, projecting to multiple glomeruli instead of a single glomerulus as in *Tbx* mouse (n=24).

Olfactory resilience after a drastic reduction in the density of bulb projection neurons

Learned behavior: odor detection and discrimination

In rodents, many behaviors critically depend on olfaction. Previous experiments with mutant mice affecting the olfactory system have reported severe perturbations of behavior, including the inability to find food or choose mates (Belluscio et al., 1998). *Tbx::DTA* and *Tbx::iDTR* mice not only have very few M/T cells remaining (fewer than 5%), but also the architecture of their OBs is disrupted: OSNs expressing the same odor receptor and single M/T cells apical dendrite innervate multiple glomeruli. Thus, we investigated the ability of *Tbx::DTA* (perinatal cell ablation) and *Tbx::iDTR* (adult cell ablation) mice to identify odors using a go/no-go paradigm. Surprisingly, despite the massive loss of M/T cells and the disorganization of OB connectivity, all *Tbx::DTA* mice reached the performance criterion of 80% accuracy when exposed to 5% cineole (a chemical typically used for olfactory tests) concentrations on their first trial and even showed comparable accuracy to control *Tbx* mice at 0.5% cineole concentrations (**Figure 5A**). Whereas all the *Tbx::DTA* mice (with perinatal M/T ablation) succeeded in the go/no-go test, only 50% of *Tbx::iDTR* (in which M/T ablation occurred as adults) can perform this test on the first day of testing. Of the remaining 50% of *Tbx::iDTR* animals, 21% of the *Tbx::iDTR* mice were unable to perform this detection test, even after training on this test for 3 weeks. The remaining 29% of *Tbx::iDTR* mice were eventually able to complete the test after one week of training (**Figure 5A**; **Figure 5—figure supplement 1**). These observations indicate that assembly of an olfactory circuit with a reduced density of projection neurons (in *Tbx::DTA* mice) is compatible with preserved olfactory performance. In contrast, reducing the density of M/T cells in an adult animal after the circuitry of its olfactory bulb was assembled normally (in *Tbx:iDTR* mice) causes important deficits in olfactory performance; however, these deficits can be overcome after training.

After the animals were exposed to the detection test, we investigated the ability of these mice to perform a more challenging olfactory test: to discriminate between two odors with very different chemical properties (cineole (S+) and eugenol (S-)). Both *Tbx::DTA* and *Tbx::iDTR* mice reached more than 80% accuracy criterion when exposed to pure molecules and binary mixtures (80/20::20/80) (**Figure 5B**). However, they had difficulties discriminating between more similar binary mixtures (55/45::45/55). Next, *Tbx::DTA* and *Tbx::iDTR* mice were exposed to highly similar smells, such as the monomolecular enantiomers (carvone (+) (S+) and carvone (-) (S-)). *Tbx::DTA* mice failed to discriminate between carvone (+) and carvone (-), even when the odorants were presented as pure molecules (not as mixtures). In contrast, *Tbx::iDTR* mice reached 80% accuracy in this version of the discrimination test with pure molecules (**Figure 5B**). We should note that both in *Tbx::DTA* and *Tbx::iDTR* animals, the discrimination test was performed after they were exposed to the detection test. Thus, it is possible that the good performance of the *Tbx::iDTR* animals in the discrimination test could be due to the prior training in the detection test, in agreement with the improved performance of the *Tbx::iDTR* animals on the detection test after several days of practice, as indicated above.

These results suggest that despite the reduced number of M/T cells and disorganization of their OBs in *Tbx::DTA* and *Tbx::iDTR* mice, their olfactory system has a remarkable resilience that partially preserves their ability to smell.

Social behavior: sexual and aggressive behavior

In rodents, many key social behaviors depend on olfaction. In mice, litters are primarily taken care of by females. No apparent differences were observed in offspring nursing between both experimental mice, indicating that this behavior was mostly preserved in females (*Tbx::DTA* and *Tbx::iDTR*). However, the reduction of M/T cells affected the mating rate when wild type females were introduced to *Tbx::DTA* and *Tbx::iDTR* male mice. We scored that a male mated with a female

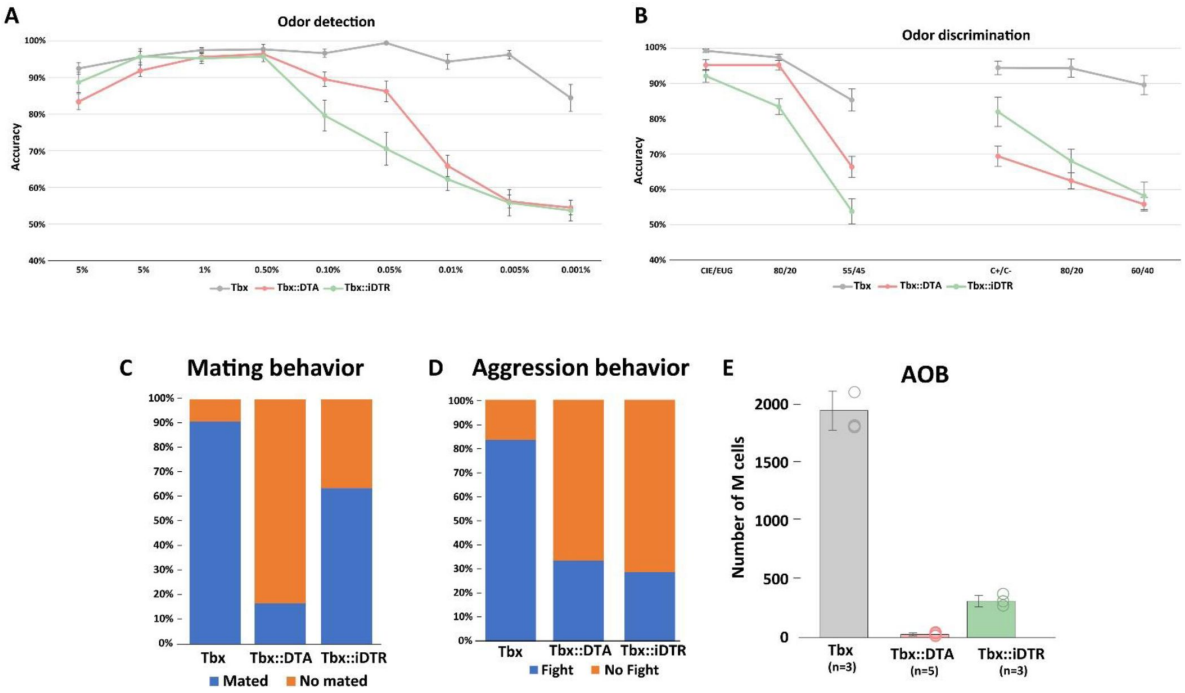

Figure 5

Mouse behavior after massive M/T cells ablation.

A. Olfactory performance of *Tbx* (n=12), *Tbx::DTA* (n=13), and *Tbx::iDTR* (n=11) mice in a go/no go paradigm to investigate their odor detection ability. Decreasing cineole concentrations were presented each experimental day. Dots represent the mean percentage of accuracy responses in a single day. Accuracy lower than 80% was considered as a deficit in olfactory detection. *Tbx::DTA* and *Tbx::iDTR* mice successfully performed odor detection with concentrations of cineole as low as 0.05%, but failed when the concentration of odorant was further reduced. Control mice (*Tbx*) successfully detected cineole at all concentrations tested (up to 0.001%).

B. Odor discrimination experiment exposing mice to increasing binary mixtures of cineole and eugenol (left) and (+)-carvone and (−)-carvone odors (right). Both experimental mice (*Tbx::DTA* and *Tbx::iDTR*) could successfully detect cineole and eugenol when they were presented as individual odors, or with 80/20 mixtures. However, they failed when mixtures of cineole::eugenol were 55/45. However, *Tbx::iDTR* mice discriminated between (+)-carvone and (−)-carvone odors presented individually, but not when odor mixtures were presented. *Tbx::DTA* could not discriminate between (+)-carvone and (−)-carvone even when they were presented as individual odors.

C. *Tbx::DTA* and *Tbx::iDTR* males mated with wt females with a much lower frequency (*Tbx::DTA*=16.67%; n=12; and *Tbx::iDTR*=63.63%; n=11) compared to control males (90.91%; n=11).

D. In a resident/intruder paradigm, then the resident and intruder were both wt male mice, the resident started a fight in >90% of the tests. When the resident was a wild type male they fought in 33.33% of the cases if the intruder was a *Tbx::DTA* male (n=9) or 28,57% if the intruder was a *Tbx::iDTR* male (n=7). When the residents were *Tbx::DTA* (perinatal cell ablation) or *Tbx::iDTR* (cell ablation at P60) males, they did not fight when the intruder was a male with the same genotype or a wild type male mouse.

E. Quantification of M/T cells in the accessory olfactory bulb (AOB) in *Tbx*, *Tbx::DTA*, and *Tbx::iDTR* showed that only 1,6 % of M/T cells remained (30 ± 7, average ± S.E.M.; n=5) in *Tbx::DTA* mice and 16,6% in *Tbx::iDTR* (315 ± 35; n=3) compared to wt *Tbx* mice (1,900 ± 119; n=3).

when a vaginal plug was observed. Only 16.67% of *Tbx::DTA* males mated, compared to 63.63% of *Tbx::iDTR* and 90.91% control *Tbx* animals (**Figure 5C**). These results are surprising because, as indicated above, *Tbx::DTA* mice can perform well on olfactory detection tasks (**Figure 5A**). Next, we used a resident/intruder paradigm to analyze the aggressive behavior of the male mice. *Tbx::DTA* and *Tbx::iDTR* mice did not initiate a fight when a wild type male or a male mouse from the same strain was introduced into its cage. Moreover, when *Tbx::DTA* or *Tbx::iDTR* male mice were the intruders, the resident wild type mice attacked them in 33% (*Tbx::DTA*) and 28% (*Tbx::iDTR*) of the cases, compared to 83% when the intruder was another wild type male mouse (**Figure 5D**).

The accessory olfactory bulb (AOB) plays a key role in innate olfactory behaviors. We quantified the number of M/T cells remaining in the male AOB. Wild type mice have around ~2,000 M/T cells in their AOBs, while only ~1.5% and 16% of M/T cells remained in the AOB of *Tbx::DTA::Ai9* mice and *Tbx::iDTR::Ai9* mice, respectively (**Figure 5E**). Thus, the drastically reduced number of AOB M/T cells in the *Tbx::DTA* mice could explain their inability to engage in social behaviors such as a mating or aggression, suggesting that behaviors depending on the AOB are hard-wired, less plastic, and less resilient than the learned behaviors that depend on the main OB.

Discussion

A full set of M/T cells is not necessary for dendrite maturation, but it is necessary for the maintenance of a stable dendrite

Dendrite morphology is one of the main morphological features of a neuron. The shape and size of the dendritic arbor are important determinants of the number and types of inputs that a neuron receives (London and Häusser, 2005). During development, each M/T cell extends several apical dendrites toward several glomeruli. In the first week after birth, M/T cells undergo a refinement process where they end up with a single apical dendrite innervating a single glomerulus (Blanchart et al., 2006; Hinds, 1968a, 1968b; Shepherd et al., 2004). Our cell ablation experiments at perinatal stages indicate that the refinement of M/T cell apical dendrites occurs normally in the absence of peer M/T cells. This suggests that the refinement process is independent of interactions with counterparts (**Figure 1**). In addition, M/T cells refine their apical dendrite in the absence of neurotransmitter release by OSNs (Lin et al., 2000). However, genetic ablation of the NMDA receptors in M/T cells prevents the refinement of their apical dendrites (Aihara et al., 2021; Fujimoto et al., 2023), indicating that glutamatergic input to M/T cells plays a role in the refinement of its dendrites. These glutamatergic input to M/T cells could originate from multiple sources, including external tufted cells, axonal inputs from the olfactory cortex, or from reciprocal connections between M/T cells.

Once the M/T cell apical dendrite refinement process is complete such that each M/T cell projects a single apical dendrite to a single glomerulus, this status stays stable throughout the life of the animal (Ma et al., 2014; Mizrahi and Katz, 2003). In the OB each glomerulus is innervated by approximately 40 M/T peer cells where they make synapses with OSN axons expressing the same odor receptor, juxtaglomerular cells (external tufted cells (ETCs) and periglomerular cells (PGCs), and short axon cells (SACs)). Despite the continuous turnover of OSN and PGCs, the apical dendrites of M/T cells remain stable throughout adulthood over long periods of time (Mizrahi and Katz, 2003). Similar dendrite stability has been observed in the projection neurons of the *Drosophila* olfactory system, even when OSNs were ablated in adult flies (Berdnik et al., 2006). Our results showed that the refinement of the M/T apical dendrite occurs even if M/T cells develop in the absence of most of their peers. In contrast, ablating a large fraction of the M/T cells in perinatal or adult animals, after the refinement process had occurred and a single dendrite remained projecting to a single glomerulus, induced the surviving M/T cells to expand their dendrites into multiple nearby glomeruli. These observations suggest that M/T cell density is not

necessary for dendrite refinement, but instead, it is essential for the maintenance of apical dendrite stability. Importantly, the main synaptic partners of M/T cells in the glomerulus, the OSNs and juxtaglomerular cells, had not been directly perturbed by our genetic manipulation. This observation suggests that M/T cells apical dendrite stability depends more on the cell density of the peer M/T cells than on their other glomerular synaptic partners (OSN axons, ETCs, PGCs, and SACs). Interestingly, M/T cell ablation after the initial refinement is complete, only induced half of the remaining M/T cells (~50%) to expand their dendritic tuft toward several glomeruli (**Figure 2**). This observation is consistent with previous works demonstrating that M/T cells are not a homogeneous cell type (Angelo et al., 2012; Kollo et al., 2014; Padmanabhan and Urban, 2010), and that different subtypes of M/T cells may respond differently to these perturbations.

Mechanisms controlling structural plasticity of the M/T cells in adult mice

A previous work has shown that reducing the number of bipolar cells in mouse embryonic retina induces dendrite expansion in remaining cells when the retinal circuit is being assembled (Johnson et al., 2017). The results from our perinatal cell ablation experiment in the OB suggest that the M/T cells apical dendrite refinement process is independent of cell density. However, our experiments indicate that cell density plays an important role in maintaining the stability of the apical dendrite. There is evidence that neurotransmission might play a role in the dendrite growth and establishment of new synapses (Andreae and Burrone, 2015; Bleckert and Wong, 2011; Rajan et al., 1999). In one scenario, after ablating a large fraction of M/T cells, OSN axons and ETCs cells that do not have a M/T synaptic partner in the glomerulus could increase the neurotransmitter levels in the glomerulus, thus triggering the dendritic growth from nearby M/T cells, as we observed in our experiments.

Neuronal activity plays an essential role in sculpting the neuronal circuit as it is being assembled during development (Andreae and Burrone, 2015; Bleckert and Wong, 2011; Martini et al., 2021). The refinement of M/T dendrites is independent of sensory stimuli (Lin et al., 2000), although it seems to depend on glutamatergic inputs (Aihara et al., 2021), suggesting that spontaneous circuit activity is necessary for the refinement of M/T cells. However, our experiments indicated that ablating a high percentage of M/T cells in adult animals (in *Tbx::iDTR* mice) caused the expansion of apical dendrites into multiple glomeruli, even when sensory input or intrinsic activity were reduced, by naris occlusion, or by Kir expression in M/T cells, respectively.

Projection neurons are dispensable to form an olfactory map of sensory axons on the olfactory bulb, but are necessary to maintain it during adulthood

The olfactory map formed by OSN axons on the OB is maintained throughout life despite OSNs turnover (Graziadei and Graziadei, 1979; Zou et al., 2004b). The flow of information between OSNs and M/T cells is an essential step for the transmission of odor information because M/T cells are the only connection from the OB to the rest of the brain. Several previous works have investigated how OSN manipulations affect the formation and maintenance of the odor map (Biju et al., 2008; Lin et al., 2000; Ma et al., 2014; Yu et al., 2004; Zheng et al., 2000). Here, we evaluated whether reducing the density of M/T cells affects odor map formation and/or maintenance. Our results showed that odor map formation is independent of M/T cells. These results are consistent with previous works that showed that initial convergence of OSN axons expressing the same odorant receptor into single glomeruli does not require M/T cells, as reported in Tbr1 mutant mice that cannot produce M/T cells (Bulfone et al., 1998). In contrast we observed that odor map maintenance requires a full complement of M/T cells (**Figure 4**). With a reduced density of M/T cells the axons of OSNs initially project to a single glomerulus, but as the animals mature, those axons eventually project into multiple neighboring glomeruli. Moreover, in

animals that developed a normal olfactory map with a full set of M/T cells, reducing the density of M/T cells as adults also caused OSN axons to project abnormally into multiple glomeruli. Therefore, the present work shows that while M/T cells are not necessary for the formation of a normal odor map on the OB, they are necessary for the maintenance of this map in adult animals.

The odor map disruption observed after ablation of M/T cells is interesting, because many of the other synaptic partners for OSN axons (ETCs and PGCs) were not directly targeted by our genetic manipulation. One may have anticipated that even without M/T cells dendrites, the neurites from ETCs and PGCs could have provided synaptic targets for the OSN axons, and this could have been sufficient to maintain the odor map on the OB surface. Indeed, there is some evidence that OSN axons make strong synapses contact with ETCs, and some works indicate that M/T cells are not the primary synaptic target of OSN axons (De Saint Jan et al., 2009). The fact that the drastic reduction in the number of M/T cells caused the OSN axons to miss their normal targets in the OB in adult animals suggests that the OSN-M/T cell (but not the OSN-ETC) connections are necessary for the maintenance of the olfactory map during adulthood. Alternatively, it is also possible that ablating M/T cells may cause changes in other cells that make synapses in the glomeruli (ETCs, PGCs, sAC, etc…), and that the misrouting of OSN axons that we observed in our experiments may be a secondary effect caused by the elimination of M/T cells.

Robustness of learned but frailty of innate olfactory behaviors

Previous experiments with mice carrying mutations affecting the olfactory system have reported severe behavioral perturbations, such as the inability of mothers to nurse their pups, inability to breed, and inability to find food (Belluscio et al., 1998). Ablation of M/T cells in adult mice (in *Tbx::iDTR* mice) showed that 25% of mice were unable to perform odorant detection tests, regardless of how many days they were exposed to the test. In contrast, the remaining ~75% mice retained significant olfactory function, although these animals needed odor training (**Figure 5**). Surprisingly, all mice in which M/T cells were ablated at perinatal stages (*Tbx::DTA*) performed well on olfactory tests, even when only 1% of their M/T neurons remained, the only connection between the OB and the olfactory cortex. Moreover, in these mice, the organization of the projections from the OSNs to the glomeruli (the “odor map”), was severely disrupted, and the dendrites of M/T cells projected abnormally into multiple glomeruli. These results suggest that the brain of young mice exhibit high resilience in the performance of learned behaviors, whereas this functional resilience decreases with age. However, although mice with reduced numbers of M/T cells were able to perform satisfactorily in tests that measured learned behaviors (such as odor detection), their performance of innate behaviors such as mating with females or aggression against intruders was much impaired. Although it is thought that M/T cells in the main olfactory bulb (MOB) are sufficient to mediate some innate behaviors (Kobayakawa et al., 2007), we observed highly impaired innate behaviors. In *Tbx::DTA* mice that performed well in tests for learned behaviors (such as odor detection), were unable to perform some innate behaviors such as mating with females or fighting intruders (**Figure 5**). Our results suggest that learned behaviors may be more resilient to experimental perturbations than innate behaviors, perhaps indicating that the performance of innate behaviors depends on hardwired circuits that cannot be easily reconfigured. Indeed, this hypothesis is consistent with our observation that ablating M/T cells in adult animals (in *Tbx::iDTR* mice) caused an initial failure to perform odor detection tests (a learned behavior), which could be overcome after several days of training.

In summary, our results indicate that the full complement of M/T cells, the projection neurons that transmit odor information from the OB into the rest of the brain, is not required for normal assembly of the olfactory circuit, but it is essential for its maintenance. The initial steps of several developmental processes in the assembly occurred normally despite a drastically (>95%) reduced number of M/T cells. M/T cell ablation did not affect the appropriate targeting of OSN axons into the OB, or the refinement to a single apical dendrite of remaining M/T cells that projected into a single glomerulus. However, after the OB circuitry was normally assembled despite a reduced density of M/T cells, we observed that the maintenance of the OB wiring was perturbed in multiple

ways, including expansion of several dendrites from M/T cells that projected into multiple glomeruli, and misrouting of OSN axons. Importantly, we observed that both of these changes in circuit wiring (apical dendrite expansion and OSN axon misrouting) could also be triggered in animals in which the OB assembly had occurred under normal circumstances, but in which the M/T cell ablation was induced in full adult animals. These experiments highlight that in some brain regions, a full density of cells may be dispensable for the assembly of its neuronal circuits, but it may be required for its maintenance during adulthood. Finally, these observations indicate that olfactory circuits have a remarkable ability to reorganize such that after a severe (>95%) loss of an essential neuronal type during development and adult ages, olfactory function is surprisingly preserved.

Methods

Animals

Tbx21-Cre, *DTA*, *iDTR*, *M72-YFP*, *Ai9* and *Confetti* were obtained from the Jackson laboratory. The *Tbx21-Cre* driver mouse (JAX#024507) was used to express the Cre recombinase in M/T cells, the olfactory bulb projection neurons (Mitsui et al., 2011). The reporter mouse *DTA* (JAX#009669) expresses the subunit A of the diphtheria toxin upon cre recombination (Voehringer et al., 2008), inducing death of M/T cells at perinatal stages when crossed with *Tbx21-Cre mouse*. The *iDTR* reporter mouse (JAX#007900) expresses the diphtheria toxin receptor upon cre recombination (Buch et al., 2005), allowing for the controlling the timing of M/T cell ablation after injecting the diphtheria toxin into the *Tbx::iDTR* mouse. The *Ai9* (JAX#024507) and *Confet*ti (JAX#013731) reporter mouse expresses the tdTomato fluorescent protein or produces four different fluorescent proteins (RFP, YFP, GFP or CFP) upon cre recombination (Madisen et al., 2010; Snippert et al., 2010), labeling M/T cells when crossed with the *Tbx21-Cre* mouse. The *M72-ChR2-YFP* reporter mouse (JAX#021206) expresses the Yellow fluorescent protein in the M72 olfactory sensory neurons (Smear et al., 2013), allowing the visualization of the glomeruli innervated by their axons in the OB.

All mice were handled according to the protocols approved by the California Institute of Technology (Caltech) Institutional Animal Care and Use Committee (IACUC). Mice colonies were maintained at the animal facility at Caltech.

Ablation of M/T cells

Ablation of perinatal M/T cells was directly achieved in the transgenic mice *Tbx::DTA*, as a result of crossing the *Tbx* driver mouse with the reporter *DTA* mouse. Ablation of adult M/T cells was induced by intraperitoneal injection of the diphtheria toxin A (DT) subunit (Sigma D0564) at a concentration of 20 µg/Kg body weight into *Tbx::iDTR* transgenic mice (Dong et al., 2021; Tien et al., 2016), with two injections separated by 24 hours starting at either P60 or P180. To ablate 75% of M/T cells, we used half of the DT dose (10 µg/Kg body weight).

Tissue processing, immunohistochemistry, and imaging

Mice were deeply anesthetized with a single intraperitoneal injection of Ketamine/Xylazine (100mg/10mg per animal kg) and fixed by intracardiac perfusion with 4% PFA in 0.1 M phosphate buffer (PBS), pH 7.4. Brains were postfixed overnight in 4% PFA at 4°C. The next day, all samples were washed three times, 10 min each, with 0.1 M PBS. Then, brains were embedded into 3% agarose, and the OBs were cut in a vibratome into 50 µm thick sections for immunostaining and cell quantification, and analysis of OSN axon projection into the OB, or into 300 µm thick sections for M/T cells apical dendrite analyses. Immunohistochemistry was performed in 50 µm thick sections using the following blocking solution: 10% fetal bovine serum (FBS) and 0.1% Triton X-100 in 0.1 M PBS. Sections were incubated overnight at 4°C with primary antibodies diluted in blocking

solution: rat anti-Tbr2 (1:500, Invitrogen 14-4875-80), rabbit anti-tyrosine hydroxylase (TH; 1:250, Abcam AB76442), mouse anti-calbindin (1:1.000, Synaptic Systems 214 011) and rabbit anti-GFP (1:1.000, AB3080P Millipore). The next day, sections were washed and incubated with secondary antibody diluted in blocking solution: Goat anti-Rabbit IgG Alexa-488 (Invitrogen A-11008), Goat anti-Rat IgG Alexa 594 (Invitrogen A-11007), Goat anti-Mouse IgG Alexa 647 (Invitrogen A-21235). 300 µm thick OB sections were clarified using the ScaleSQ(5) method (Hama et al., 2015). All sections were incubated with DAPI (D9542, Sigma) to label cell nuclei and treated with TrueBlack (23007, Biotum) to eliminate lipofuscin autofluorescence.

Z-stack images were acquired using 20x, 40x, or 60x objectives on a confocal microscope (Zeiss LSM 800). Z-stacks were merged and analyzed using ImageJ or Imaris and edited with Photoshop (Adobe) software.

Measurement of OB volume

OBs were sectioned in 50 µm thick transversal sections, collected in sequence, and stained with DAPI. The OB volume and each of its layers (glomerular, external plexiform, granular and rostral migratory stream) were calculated using the Neurolucida software (MBF Bioscience Inc, Williston, VT).

Mounting and analyzing morphology of M/T cells

Quantification of M/T cells was performed on *Tbx::Ai9*, *Tbx::DTA::Ai9,* and *Tbx::iDTR::Ai9* mice using Neurolucida software. All coronal sections from each OB were collected and stained with antibodies against RFP to amplify the tdTomato signal.

For analyses of apical dendrites, M/T cells were labeled by systemic intravenous injection of AAV vectors carrying EGFP gene under the human synapsin promoter. Intravenous administration of AAV vectors was performed by injection into the retro-orbital sinus of adult mice 2-3 weeks before perfusing animals for histological analysis. In wild-type animals, we performed systemic injections with a two-component AAV viral vector system to achieve sparse labeling of M/T cells (Chan et al., 2017).

Analysis of M/T cell apical dendrites and OSN axon projections

Confocal images of the M/T cells apical dendrite tuft, taken with a 60x objective, were analyzed with the filament tracer tool from the Imaris software (*https://imaris.oxinst.com/*). Mitral and tufted cells were included in the same group without distinguishing between the two cell types. The number of glomeruli innervated by M/T cells apical dendrite or OSN axons was estimated using a confocal microscope and complemented by inspection with a stereo fluorescence microscope.

Olfactory sensory deprivation and reduction of neuronal activity

Naris occlusion

Mice were anesthetized with a single intraperitoneal injection with ketamine/xylazine (100mg/10mg per animal kg). Unilateral naris occlusion was performed by cautery with a high-temperature cautery tip (Bovie Aaron Change-A-TipTM) ten days before inducing ablation of M/T cells in *Tbx::iDTR* mice by injecting DT intraperitoneally (Lin et al., 2000). To confirm the efficiency of naris occlusion, we measured the levels of tyrosine hydroxylase in PGs by immunostaining (Baker et al., 1993).

Reduction of neuronal activity

AAVs carrying the Kir2.1 gene connected by a 2A linker to EGFP in a DiO conformation (inverted and flanked by loxP sites) was injected systemically into *Tbx::iDTR* mice 15 days before inducing M/T ablation by DT injection. The efficiency of silencing by the Kir2.1 channel in the M/T cells was evaluated by electrophysiological recordings.

Slice Electrophysiology

Mice were deeply anesthetized by intraperitoneal injection of ketamine/xylazine and perfused transcardially with ice-cold sucrose slicing solution (in mM, sucrose 213, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10, $MgSO_4$ 7, $CaCl_2$ 1, pH 7.35). After decapitation, the brain was removed and immersed in the same ice-cold slicing solution. Horizontal slices (300 µm) of OB were cut using a vibratome (VT-1200s, Leica). We first let the slices recover in artificial cerebrospinal fluid (ACSF. NaCl 124, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 24, glucose 25, $MgSO_4$ 1, CaCl2 2) at 33 °C for 30 minutes and then held them at room temperature (~22 °C) until use.

During recording, slices were perfused continuously (~2 mL/min) with ACSF at 25°C. Neurons were visualized and targeted using an upright IR-DIC microscope (BX51WI, Olympus). Mitral cells were identified by their characteristic large soma within the mitral cell layer. Whole-cell recordings were achieved using glass pipettes with an impedance of 3 to 6 MOhm when filled with intracellular solution (K-gluconate 145, NaCl 2, KCl 4, HEPES 10, EGTA 0.2, Mg-ATP 4, Na-GTP 0.3, pH 7.25). Electrical signals were sampled at 20 kHz and filtered at 2.9 KHz using an EPC-10 amplifier (HEKA Elektronik). Liquid junction potential was not corrected. Passive and active membrane properties were tested by injecting 500 ms-long current steps with amplitudes ranging from 50 to 1000 pA.

Behavior experiments

Learned behavior

The ability of mice to detect odorants was evaluated in a four channel olfactometer (Slotnick and Bodyak, 2002), two months after cell ablation. Several days before starting with the go/no-go paradigm training, mice were partially water-deprived (85 % of their initial body weight). First, mice were trained to introduce their snout into the odor sampling port and lick the water valve to get the water reward. During this initial phase of training, only mineral oil was presented. Next, we began with the go/no-go paradigm using Cineole (843788, Sigma) as a reinforced stimulus (S+) and mineral oil as an unreinforced stimulus (S−). Every day, mice underwent 10 training blocks (20 trials per block, with 10 trials with a positive stimulus, and 10 trials with a negative stimulus) per day, for a total of 200 trials per day. A single random stimulus was presented as S+ or S- in each trial. The accuracy was calculated for each block of 20 trials. Mouse accuracy >80% was considered as successful learning of the positive (S+) and negative (S-) stimuli. During 2 days (40 trials), mice were trained with 5% cineole concentration to ensure that mice can learn the task. After this initial phase, we decreased the concentration of the reward stimulus at 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, to determine the odor detection threshold.

Odor discrimination was first tested using two different odors: cineole (S+) and eugenol (S-) (818455, Sigma) both diluted at a concentration of 1%. First, pure odors were presented to the mice: only cineole as S+ and only eugenol as S-. On subsequent days, a mixture of cineole and eugenol was presented at either 80/20 (80% cineole/20% eugenol for S+, and 80% eugenol/20% cineole for S−), or 55/45 ratios. Finally, we tested a more challenging odor discrimination task using enantiomer molecules: (+)carvone as S+ (22070, Sigma) and (−)carvone as S− (22060, Sigma) both diluted to 1%.

Social behavior

For *mating experiments*, male mice without sexual experience were used to test the mice's mating ability when a wild type female was introduced into the male cage and left there for three days. Females were exposed to male bedding for four days before being introduced to the male cage. This procedure was repeated with two more females (a total of 3 females). Successful mating was annotated when a vaginal plug was detected in the morning with any of the females presented.

For *aggression experiment*s, male mice were housed with a female for at least two weeks before the testing day. The resident/intruder paradigm was used to evaluate the male aggressive behavior. A male was introduced to the home cage of the resident male for 10 min. Aggression behavior was considered to occur when the resident male incited the aggression.

Data analyses

Excel and Matlab were used for statistical analyses. Wilcoxon rank-sum test was used to compare the tuft length of M/T cells apical dendrites (for two groups). $\chi 2$ test with Bonferroni correction was used for comparison of M/T cell apical dendrites and OSN axons glomeruli innervation (for more than two groups).

Figures

Author contributions

Conceptualization: L.S-G. and C.L.; methodology, L.S-G., P.R., B.W., C.L.; formal analysis, L.S-G., P.R., B.W., and A.C.-M.; investigation, L.S-G., P.R., B.W., and A.C.-M.; writing-original draft preparation, L.S-G.; writing-review and editing, L.S-G. and C.L.; supervision, L.S-G. and C.L.; funding acquisition, C.L. All authors have read and agreed to the published version of the manuscript.

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Declaration of Interests

The authors declare no competing interests.

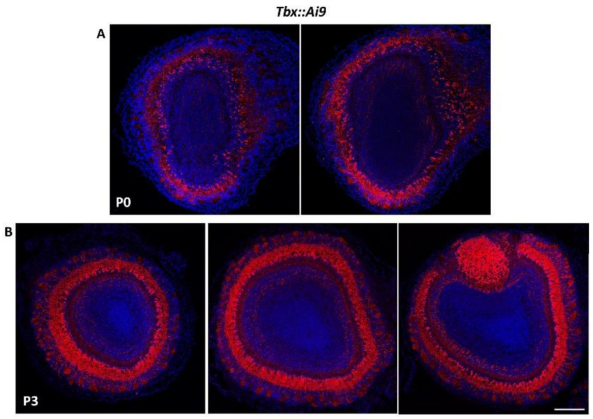

Figure 1 - figure supplement 1

M/T cells labeled at early postnatal stages in *Tbx::Ai9* mice.

A. Coronal section to the OB in a P0 *Tbx::Ai9* mouse. There is a high number of M/T cells that express tdT, indicating that Cre enzyme is active at embryonic stages in a significant fraction of M/T cells.
B. Coronal section to the OB in a P3 *Tbx::Ai9* where most, if not all, the M/T cells are expressing tdT protein. DAPI staining (blue) labels cell nuclei. Scale bar in B is 200 μm and applies to A-B.

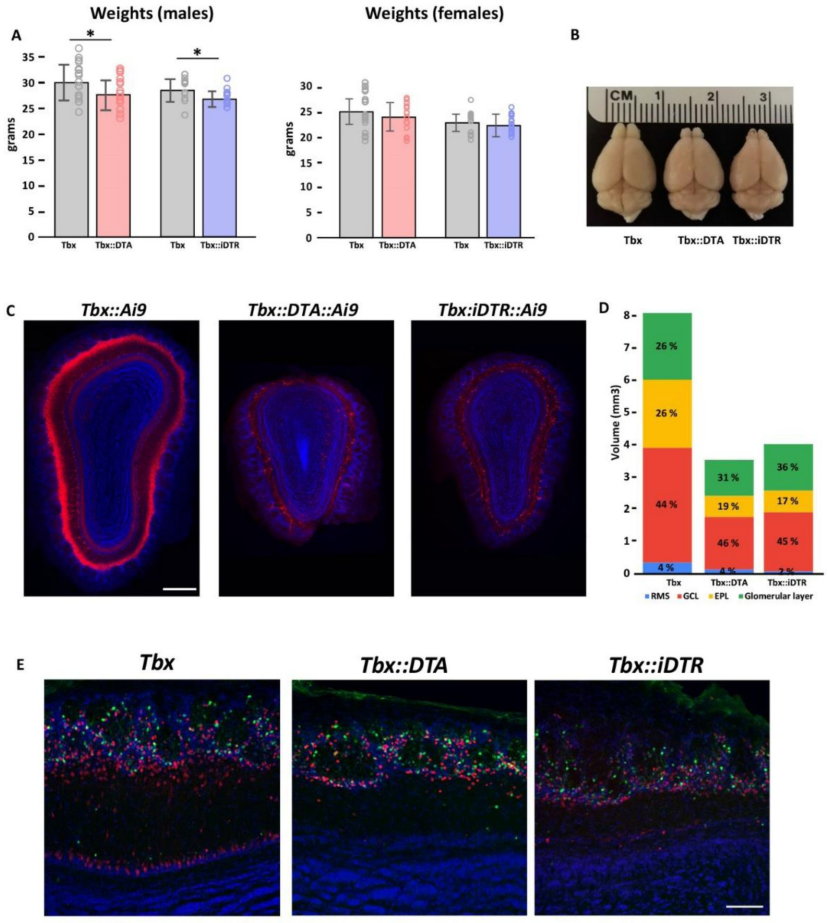

Figure 1 - figure supplement 2

Histological analyses of olfactory bulbs after M/T cells ablation.

A. (left) Weight of *Tbx::DTA* (red bar) and *Tbx::iDTR* (blue bar) males mice compared with their wild type siblings (grey bar). Significant differences were observed in their weights. *Tbx::DTA* (27.7 ± 2.8 gr; n=19) vs *Tbx* (30.1 ± 3.4 gr; n=19), p-value=0.02. *Tbx::iDTR* (26.5 ± 1.5 gr; n=12) vs *Tbx* (28.2 ± 2.2 gr; n=11), p-value=0.02. (right) Weight of *Tbx::DTA* (red bar) and *Tbx::iDTR* (blue bar) females mice compared with their wild type siblings (grey bar). No significant differences were observed in their weights. *Tbx::DTA* (24 ± 2.8 gr; n=19) vs *Tbx* (25.4 ± 3.9 gr; n=19), p-value=0.22. *Tbx::iDTR* (22.3 ± 1.7 gr; n=22) vs *Tbx* (22.7 ± 2.2 gr; n=11), p-value=0.19.
B. Dorsal view of *Tbx*, *Tbx::DTA*, and *Tbx::iDTR* brains. Note that the OBs of the *Tbx::DTA* and *Tbx::iDTR* brains are significantly smaller than in *Tbx* brains (wild type).
C. Coronal sections through the *Tbx::Ai9*, *Tbx::DTA::Ai9*, and *Tbx::iDTR::Ai9* OBs. M/T cells expressing the tdT fluorescent protein are labeled in red. Note that in the OB of *Tbx::DTA::Ai9* and *Tbx::iDTR::Ai9* mice there are much fewer M/T cells labeled compared with the OB in the *Tbx::Ai9* mouse (left). blue=DAPI staining. Scale bar is 300 µm.
D. Quantification of the OB volume in the wild type (*Tbx*; n=3) and the two experimental mice (*Tbx::DTA* (n=3), and *Tbx::iDTR* (n=3)), including the volume for each layer in the OB. The OB volume in *Tbx::DTA* and *Tbx::iDTR* mice is reduced to half of wild type OB.
E. Confocal images of coronal sections to the OB after immunostaining with Tbr2 (red; a marker for OB excitatory neurons), TH (green; a marker for a subpopulation of PGCs), and DAPI (blue; a nuclear stain). Note that the number of Tbr2+ cells is strongly reduced in *Tbx::DTA* and *Tbx::iDTR* mice compared to *Tbx* mice. Scale bar is 100 µm.

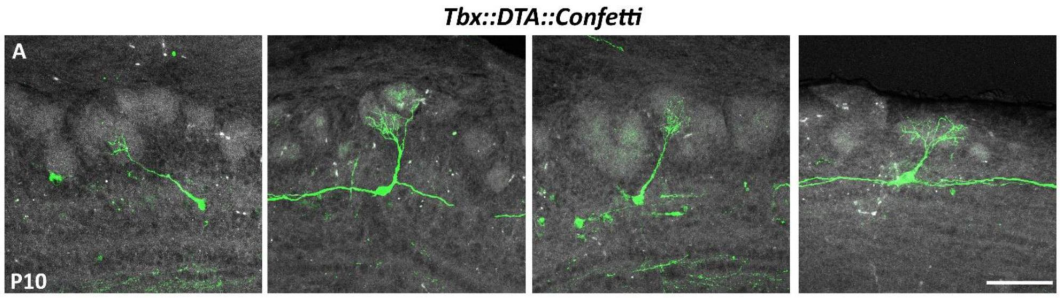

Figure 1 – figure supplement 3

M/T cells refine their apical dendrite in the absence of peer M/T cells.

A. Confocal images of M/T cells in P10 *Tbx::DTA::Confetti* mice after perinatal cell ablation. Most M/T cells showed a normal morphology, with a single apical dendrite innervating a single glomerulus. Scale bar is 100 µm.

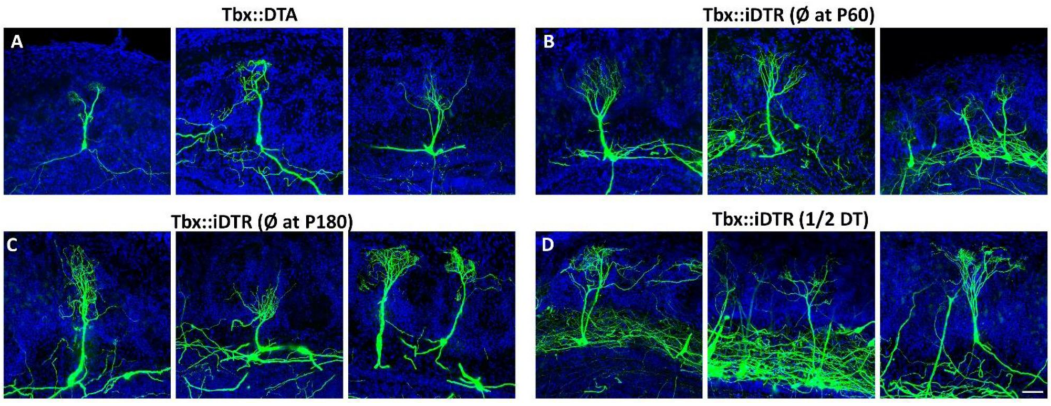

Figure 2 – figure supplement 1

Plasticity of M/T cells apical dendrite is induced by the reduction of peer cells.

A. Confocal images of M/T cells at P120 labeled with AAV-DiO-GFP in *Tbx::DTA* and (B) *Tbx::iDTR mice* (cell ablation performed at P60). (C) M/T cells in *Tbx::iDTR* mice at P240 (cell ablation at P180). (D) M/T cells in *Tbx::iDTR* at P120 (cell ablation at P60 using half the DT dose). Blue = DAPI staining. Scale bar in D is 50 µm and applies to A-D.

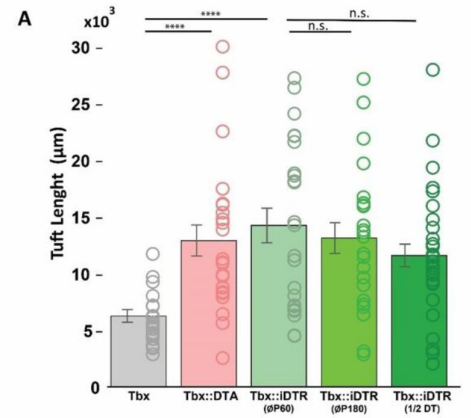

Figure 2 – figure supplement 2

Tuft length of remaining M/T cell apical dendrites.

A. Quantification of the apical dendrite tuft length of remaining M/T cells after cell ablation in experimental conditions as in **Figure 2 D-H**. Significant differences were observed when the tuft length of M/T cells in *Tbx* mice (6,234.2 ± 554.8 µm, n=22) was compared to M/T cells in *Tbx::DTA* (12,889.5 ± 1,378.1 µm; n=24; p<0.0001), and *Tbx::iDTR* mice (cell ablation at P60; 14,211.5 ± 1,527.3 µm; n=24; p<0.0001)(Mann Whitney U test). No significant differences were observed when comparing ablation performed at P60 versus P180 in *Tbx::iDTR* mice (cell ablation at P180; 11,583.4 ± 1,005 µm; n=23; p=0.72) or using half of the DT doses in P60 *Tbx::iDTR* mice (13,122.8 ± 1,005 µm; n=30; p=0.29). Data are shown as average ± S.E.M.

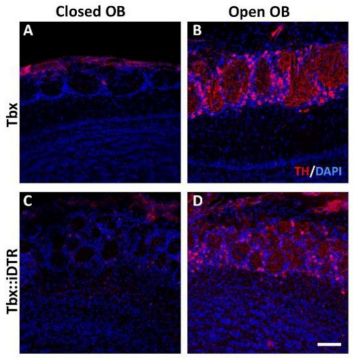

Figure 3 – figure supplement 1

Confirmation of sensory deprivation by naris occlusion.

A-D. Confocal images of coronal sections through the OB of a *Tbx* (A and B) and *Tbx::iDTR* (C and D) mice labeled with tyrosine hydroxylase antibody (TH, red) which labels a subset of periglomerular cells input. Upon sensory deprivation, the levels of TH are strongly reduced in PGs (A, C) Sensory deprived OBs in wild type (A) and *Tbx::iDTR* (C) mice. (B, D) Open OBs in wild type (B) and *Tbx::iDTR*
(D) mice. Blue = DAPI staining. Scale bar in D is 50 µm and applies to A-D.

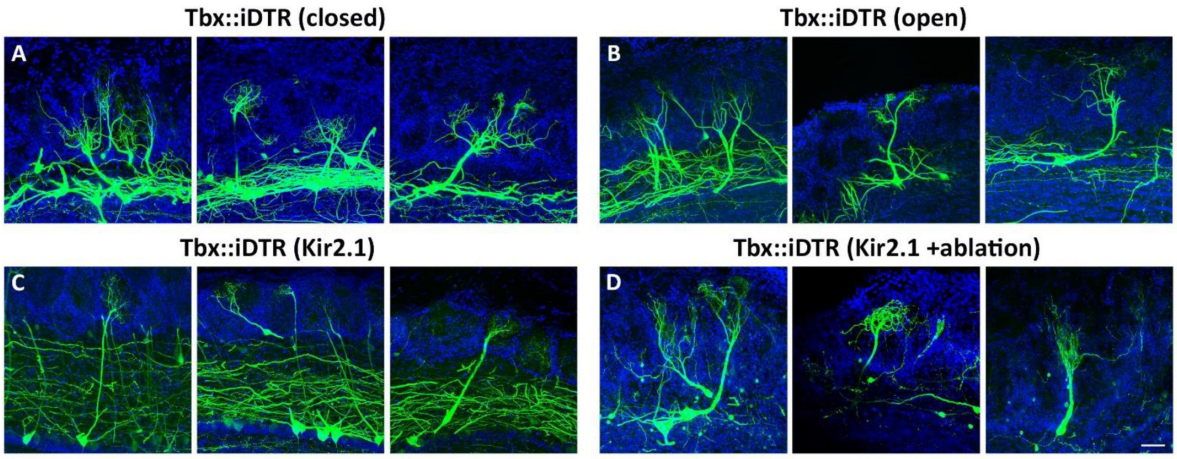

Figure 3 – figure supplement 2

M/T cell apical dendrite plasticity caused by reduction in the number of neurons is independent of neuronal activity.

A-B. Confocal images of remaining M/T cells labeled with AAV-DiO-GFP in the closed (A) and open (B) nostril in P120 *Tbx::iDTR* mice where sensory deprivation was performed before cell ablation at P60.
C-D. Remaining M/T cells in *Tbx* (C) and *Tbx::iDTR* (D) mice at P120 expressing the Kir2.1 channel before cell ablation at P60. Blue= DAPI staining. Scale bar in D is 50 µm and applies to A-D.

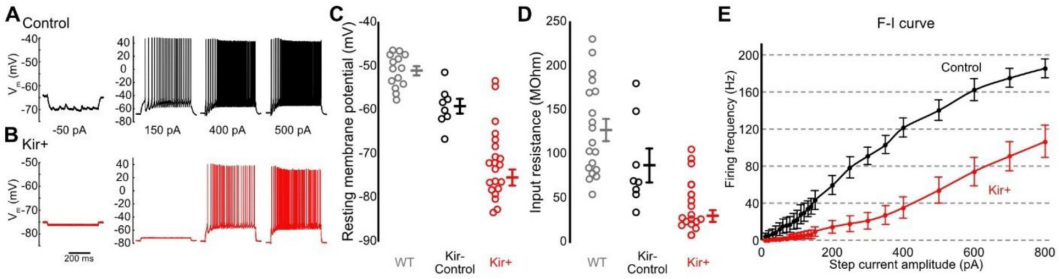

Figure 3 – figure supplement 3

M/T cells activity reduction by overexpressing the Kir2.1 channel.

A. Example of membrane potential traces in response to current steps of various amplitudes. Recorded from one M/T cell in a control (*Tbx::Ai9*) mice.
B. Example of membrane potential traces recorded from one M/T cell expressing Kir, with the same stimuli as in A.
C-D. Resting membrane potential (C) and input resistance (D) of M/Tss in control (*Tbx::Ai9*) mice (n=14 in C and n=19 in D), Kir- negative cells MCs in *Tbx* mice injected with Kir virus (Kir- control; n=8 in C and n=8 in D), or Kirexpressing MCs (Kir+; n=21 in C and n=17 in D).
E. F-I (firing frequency/injected current) curve of control and Kir+ MCs.

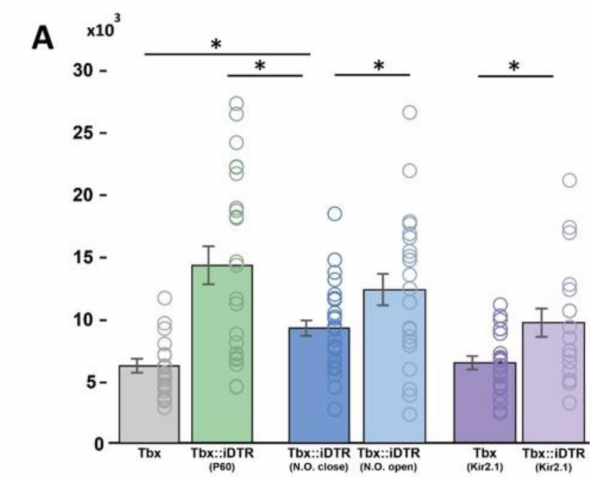

Figure 3– figure supplement 4

Apical dendrite tuft length of remaining M/T cell with reduced activity.

A. Quantification of the apical dendrite tuft length of remaining M/T cells after cell ablation in experimental conditions as in **Figure 3B** and **D**. Significant differences were observed when comparing the tuft length of M/T cells in the sensory deprived OB (closed nostril; 9,176.9 ± 618 µm; n=30) to those in the contralateral open OB (open nostril; 12,276.9 ± 1,272.2 µm; n=23; p=0.045), and control mice (*Tbx::iDTR*; 14,211.5 ± 1,527.3 µm; n=24; p=0.025) (Mann Whitney U test). Significant differences were observed when comparing the tuft length of remaining M/T cells expressing the Kir2.1 channel in *Tbx::iDTR injected with DT*(9,642.7 ± 618 µm; n=19) with M/T cells expressing the Kir2.1 channel in control *Tbx::iDTR* mice that did not receive any DT injection(*Tbx::iDTR*; 6,417.9 ± 546.7 µm; n=20; p=0.019). Data are shown as average ± S.E.M.

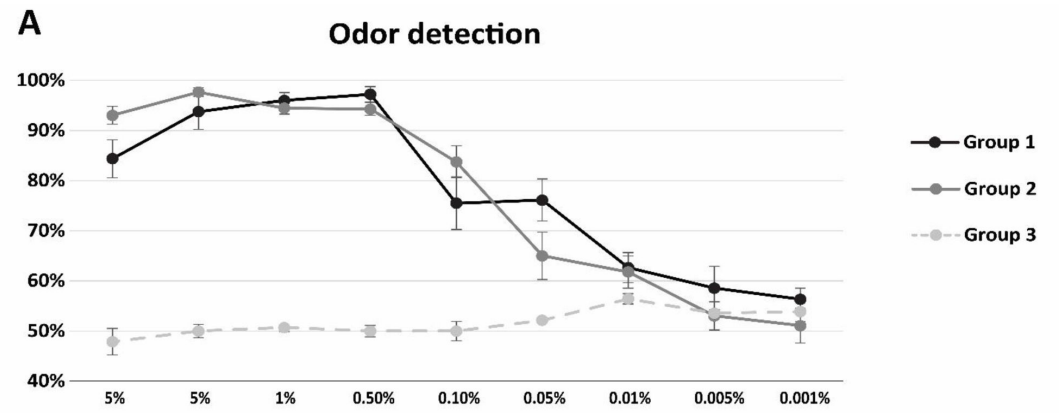

Figure 5 – figure supplement 1

Odor detection accuracy after reduction of M/T cells in adult mice.

A. The *Tbx::iDTR mice* (n=14) could be grouped in three different groups depending on the type of behavior that they exhibited in the go/no go paradigm for olfactory detection. Group 1: 50% of the mice (n=7) performed the test accurately from the first day [black line]; Group 2: 29% (n=4) were able to perform the task after one week of training [dark grey line], and Group3: 21% of the mice (n=3) were unable to do the test even after several weeks of training [stippled gray line]. Dots represent the mean percentage of accuracy responses in a single day. Accuracy lower than 80% was considered as a deficit in odor detection.

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Article and author information

Luis Sánchez-Guardado
Department of Biology and Biological Engineering, California Institute of Technology, E91125, Pasadena, United States, Department of Cell Biology, School of Science, University of Extremadura, E06071 Badajoz, Spain
For correspondence: guardado@unex.es

Peyman Razavi
Department of Biology and Biological Engineering, California Institute of Technology, E91125, Pasadena, United States

Bo Wang
Department of Biology and Biological Engineering, California Institute of Technology, E91125, Pasadena, United States

Antuca Callejas-Marín
Department of Biology and Biological Engineering, California Institute of Technology, E91125, Pasadena, United States, Department of Cell Biology, School of Science, University of Extremadura, E06071 Badajoz, Spain

Carlos Lois
Department of Biology and Biological Engineering, California Institute of Technology, E91125, Pasadena, United States
For correspondence: clois@caltech.edu

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Editors

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K VijayRaghavan

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

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K VijayRaghavan

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

Reviewer #1 (Public Review):

This paper aims to address the establishment and maintenance of neural circuitry in the case of a massive loss of neurons. The authors used genetic manipulations to ablate the principal projection neurons, the mitral/tufted cells, in the mouse olfactory bulb. Using diphtheria toxin (Tbx21-Cre:: loxP-DTA line) the authors ablated progressively large numbers of M/T cells postnatally. By injecting diphtheria toxin (DT) into the Tbx21-Cre:: loxP-iDTR line, the authors were able to control the timing of the ablation in the adult stage. Both methods led to the successful elimination of a majority of M/TCs by 4 months of age. The authors made a few interesting observations. First, they found that the initial pruning of the remaining M/T cell primary dendrite was unaffected. However, in adulthood, a significant portion of these cells extended primary dendrites to innervate multiple glomeruli. Moreover, the incoming olfactory sensory neuron (OSN) axons, as examined for those expressing the M72 receptor, showed a divergent innervation pattern as well. The authors conclude that M/T cell density is required to maintain the dendritic structures and the olfactory map. To address the functional consequences of eliminating a large portion of principal neurons, the authors conducted a series of behavioral assays. They found that learned odor discrimination was largely intact. On the other hand, mating and aggression were reduced. The authors concluded that learned behaviors are more resilient than innate ones.

The study is technically sound, and the results are clear-cut. The most striking result is the contrast between the normal dendritic pruning during early development and the expanded dendritic innervation in adulthood. It is a novel discovery that can lead to further investigation of how the single-glomerulus dendritic innervation is maintained. The authors conducted a few experiments to address potential mechanisms, but it is inconclusive, as detailed below. It is also interesting to see that the massive neuronal loss did not severely impact learned odor discrimination. This result, together with previous studies showing nearly normal odor discrimination in the absence of large portions of the olfactory bulb or scrambled innervation patterns, attests to the redundancy and robustness of the sensory system. The discussion should take into account these other studies in a historical context.

Main comments:

1. In previous studies, it has been concluded that dendritic pruning unfolds independently, regardless of the innervation pattern or activity of the OSNs. The new observation bolsters

this conclusion by showing that a loss of neighboring M/T cells does not affect the developmental process. A more nuanced discussion comparing the results of these studies would strengthen the paper.

2. The authors propose that a certain density of M/T is required to prevent the divergent innervation of primary dendrites, but the evidence is not sufficient to support this proposal. The experiment with low-dose DT injection to ablate a smaller portion of M/T cells did not change the percentage of cells innervating two or more glomeruli. The authors suggest that a threshold must be met, but this threshold is not determined. It would be possible to adjust the DT injection dose to find this threshold.

3. The authors suggest that neural activity is not required for this plasticity. The evidence was derived primarily from naris occlusion and neuronal silencing using Kir2.1. While the results are consistent with the notion, it is a rather narrow interpretation of how neural activity affects circuit configuration. Perturbation of neural activity also entails an increase in firing. Inducing the activity of the neurons may alter this plasticity. Silencing per se may induce a homeostatic response that expands the neurite innervation pattern to increase synaptic input to compensate for the loss of activity. Thus, further silencing the cells may not reduce multi-glomerular innervation, but an increased activity may.

4. There is a discrepancy between this study and the one by Fujimoto et al. (Developmental Cell; 2023), which shows that not only glutamatergic inputs to the primary dendrite can facilitate pruning of remaining dendrites but also Kir2.1 overexpression can significantly perturb dendritic pruning. This discrepancy is not discussed by the authors.

5. An alternative interpretation of the discrepancy between the apparent normal pruning by p10 and expanded dendritic innervation in adulthood is that there are more cells before P10, when ~25% of M/T cells are present, but at a later date only 1-3% are present. The relationship between the number of M/T cells and single glomerulus innervation has not been explored during postnatal development. It would be important to test this hypothesis.

6. The authors attribute the change in the olfactory map to the loss of M/T cells. Another obvious possibility is that the diffused projection is a response to the change in the olfactory bulb size. With less space to occupy, the axons may be forced to innervate neighboring glomeruli. It is not known how the total number of glomeruli is affected. This question could be addressed by tracking developmental changes in bulb volume and glomerular numbers.

7. The retained ability to discriminate odors upon reinforced training is not surprising in light of a number of earlier studies. For example, Slotnick and colleagues have shown that rats losing ~90% of the OB can retain odor discrimination. Weiss et al have shown that humans without an olfactory bulb can perform normal olfactory tasks. Gronowitz et al have used theoretical prediction and experimental results to demonstrate that perturbing the olfactory map does not have a major impact on olfactory discrimination.
Fleischmann et al have shown that mice with a monoclonal nose can discriminate odors. The authors should discuss their results in these contexts.

8. It should be noted that odor discrimination resulting from reinforcement training does not mean normal olfactory function. It is a highly artificial situation as the animals are overtrained. It should not be used as a measure of the robustness of the olfactory sense. Natural odor discrimination (without training), detection threshold, and innate appetitive/aversive response to certain odors may be affected. These experiments were not conducted.

9. The social behaviors were conducted using relatively coarse measures (vaginal plug and display of aggression). Moreover, these behaviors are most likely affected by the disruption of the AOB mitral cells and have little to do with the dendritic pruning process described in the paper. It is misleading to lump social behaviors with innate responses to odors.

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Reviewer #2 (Public Review):

The authors make the interesting observation that the developmental refinement of apical M/T cell dendrites into individual glomeruli proceeds normally even when the majority of neighboring M/T cells are ablated. At later stages, the remaining neurons develop additional dendrites that invade multiple glomeruli ectopically, and similarly, OSN inputs to glomeruli lose projection specificity as well. The authors conclude that the normal density of M/T neurons is not required for developmental refinement, but rather for maintaining specific connectivity in adults.

The observations are indeed quite striking; however, the authors' conclusions are not entirely supported by the data.

1. It is unclear whether the expression of diphtheria toxin that eventually leads to the ablation of the large majority of M/T neurons compromises the cell biology of the remaining ones.

2. The authors interpret the growth of ectopic dendrites later in life as a lack of maintenance of dendrite structure; however, maybe the observed changes reflect actually adaptations that optimize wiring for extremely low numbers of M/T neurons. The finding that olfactory behavior was less affected than predicted supports this interpretation.

3. The number of remaining M/T neurons is much higher at P10 than later. Can the relatively large number of remaining neurons (or their better health status) be the reason that dendrites refine normally at the early developmental stages rather than a (currently unknown) developmental capacity that preserves refinement?

4. While the effect of reduced M/T neuron density on both M/T dendrites and OSN axons is described well, the relationship between both needs to be characterized better: Is one effect preceding the other or do they occur simultaneously? Can one be the consequence of the other?

5. Page 7: the observation that not all neurons develop additional dendrites is not a sign of differences between cell types, it may be purely stochastic.

6. Page 8: the fact that activity blockade did not affect the formation of ectopic dendrites does not suggest that the process is not activity-dependent: both manipulations have the same effect and may just mask each other.

7. It remains unclear how the observed structural changes can explain the behavioral effects.

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