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Odor Enrichment Sculpts the Abundance of Olfactory Bulb Mitral Cells

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Abstract

Mitral cells are the primary output cell from the olfactory bulb conveying olfactory sensory information to higher cortical areas. Gene-targeted deletion of the *Shaker* potassium channel Kv1.3 alters voltage-dependence and inactivation kinetics of mitral cell current properties, which contribute to the “Super-smeller” phenotype observed in Kv1.3-null mice. The goal of the current study was to determine if morphology and density are influenced by mitral cell excitability, olfactory environment, and stage of development. Wildtype (WT) and Kv1.3-null (KO) mice were exposed to a single odorant (peppermint or citralva) for 30 days. Under unstimulated conditions, postnatal day 20 KO mice had more mitral cells than their WT counterparts, but no difference in cell size. Odor-enrichment with peppermint, an olfactory and trigeminal stimulus, decreased the number of mitral cells in three month and one year old mice of both genotypes. Mitral cell density was most sensitive to odor-stimulation in three month WT mice. Enrichment at the same age with citralva, a purely olfactory stimulus, decreased cell density regardless of genotype. There were no significant changes in cell body shape in response to citralva exposure, but the cell area was greater in WT mice and selectively greater in the ventral region of the OB in KO mice. This suggests that trigeminal or olfactory stimulation may modify mitral cell area and density while not impacting cell body shape. Mitral cell density can therefore be modulated by the voltage and sensory environment to alter information processing or olfactory perception.

Keywords

Kv1.3; plasticity; odor enrichment; trigeminal

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the writing and content of the paper.

Author Contributions

Designed experiments: DF; Conducted experiments and image analysis: KCB and JH; Statistical analysis: MCJ; Created figures and wrote manuscript: MCJ and DF.

1. Introduction

Olfactory sensory information is transported by olfactory sensory neurons (OSNs) that synapse with mitral cells in the olfactory bulb (OB), which in turn relay the information to the piriform cortex where olfactory perception occurs [2]. The majority (60–80%) of the outward current in mitral cells is carried by the *Shaker* voltage-gated potassium channel, Kv1.3 to regulate the resting membrane potential and cell excitability [1;7]. Mitral cells recorded from Kv1.3-null (KO) mice exhibit altered biophysical properties [7;14] and notable anatomical changes [1] that underlie an observed “Super-smeller” phenotype, in which the KO mice have an increased ability to detect and discriminate odors [8]. Mitral cell responses to odors are plastic and can be modified based on environmental cues such as reward learning [6]. Passive stimulation with odorants enhances olfactory discrimination and memory [11;17]. Odor enrichment also accelerates the refinement of olfactory maps [8;12], while increasing the number of inhibitory interneurons in the OB by decreasing cell death in the glomerular and granule cell layers [10;23]. Exposure to cyclohexanone or deodorized air for two months decreases mitral cell size in rats when compared to control animals exposed to normal laboratory air, and this effect is age dependent [9;15;16]. It is not clear how voltage-gated activity of the mitral cell influences these enrichment-induced responses. Previous studies indicate that voltage-gated activity influences anatomical changes at the level of the OSN in an odor-receptor specific manner [4]. It is plausible that combined odor-enrichment and voltage-gated activity may concomitantly induce changes in mitral cell numbers.

We proposed that chronic odor enrichment under conditions of a highly sensitive olfactory system, as in our KO “Super-smeller mice”, would result in anatomical changes at the level of the mitral cell. Mitral cell density (number), area, and shape were compared between WT and KO mice in response to an olfactory stimulus, citralva, and in response to peppermint, which stimulates the olfactory and trigeminal systems [5]. We chose to use peppermint in addition to a purely olfactory stimulus to parallel our previous studies comparing WT and KO mice [7]. There are very few studies that have accessed OB structure and function in response to trigeminal stimuli [19;23]. We also chose to analyze different developmental ages (P20, 3 mo, and 1yr) because these time points showed the most morphological plasticity in OSNs [4]. Previous work has demonstrated that OSNs are highly plastic, and anatomical changes are influenced by changes in mitral cell activity in KO mice [1] and in response to odor enrichment [4].

2. Materials and Methods

2.1 Ethics Statement

This work has been carried out in accordance with *EC Directive 86/609/EEC for animal experiments*, American Veterinary Medical Association (AVMA) approved methods, and guidelines set by the National Institutes of Health. This manuscript also adheres to the *Uniform Requirements for manuscripts submitted to Biomedical journals*.

2.2 Solutions

All salts and other reagents were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA).

2.3 Animals

All mice were housed at the Florida State University vivarium in accordance with the institutional requirements for animal care. Kv1.3-null mice (KO) were a generous gift from Drs. Leonard Kaczmarek and Richard Flavell (Yale University, New Haven, CT, USA) and

were generated by excision of the Kv1.3 promoter region and one-third of the 5' coding region in a C57BL6/J background [24]. Male and female wildtype (C57BL6/J; WT) and KO mice were maintained under a standard 12/12 h light/dark cycle with *ad libitum* access to 5001 Purina Rodent chow. Following weaning, all mice were housed individually in conventional style rodent cages; room air circulation was standardized at 19 changes/hour.

2.4 Odor Enrichment

Odor enrichment protocols have been described previously [4;22]. Briefly, three month old and one year old WT and KO mice were exposed to cotton swabs soaked with 200 μ l peppermint extract (1:1000; McCormick and Co., Inc., Hunt Valley, MD.). A separate group of three month old WT and KO mice were exposed to cotton swabs soaked with 200 μ l citralva (1:1000; Intercontinental Fragrances catalog # RM0429, Houston, TX). Each odorant was presented five sessions per day for 30 days. Cotton swabs were introduced to the testing cage for five 10-minute trials separated by a 10-minute recovery interval.

2.5 Histochemistry and Image Analysis

At the completion of the odor enrichment protocol, mice were sacrificed with a lethal dose of pentobarbital and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The OBs were processed as previously [1] and then stained with 0.05% cresyl violet (Fisher Scientific). A Zeiss Axiovert S100 microscope (Jena, Germany) equipped with Hoffman optics was used at 40X magnification to acquire images of the mitral cell layer (Zeiss Axiocam;Oberkochen, Germany) from a randomly chosen side of each OB section. All sections along the rostral-caudal axis from each animal were measured (NIH Image J Software). Mitral/tufted cells chosen for quantification resided along a 250 μ m line within a 25,000 μ m² box for each of four regions within the OB (Fig. 1A). Mitral cell area was normalized to the sample area of the region of the OB containing the mitral cell (Fig. 1A). Cell shape was determined by using a circularity scale of 0 – 1.00, with 1.00 being a perfect circle, which has been used to describe triangular shaped cells such as pyramidal cells [21] and mitral cells in the accessory olfactory bulb [20]. Data for mitral cell populations were collected by randomly drawing a box with an area of 25,000 μ m² in each of the dorsal, ventral, lateral, and medial regions of the OB being analyzed (Fig. 1A). Only mitral cells with a prominent nucleus within this defined region were counted and measured, which eliminated duplicating measurements for the same cell across serial sections. All data are presented as the mean \pm standard error of the mean (SEM). Due to the size of the measured population and capability of the statistical software (GraphPad Prism, La Jolla, CA), 30 measurements per treatment were randomly analyzed through selection of a random number generator. Data contained in Figure 1 are compared by anatomical region using a Student's *t*-test and a Bonferroni correction for multiple comparisons for increased stringency (α 0.0125). Data contained in Figures 2 and 3 are analyzed within region using a randomized two-way analysis of variance (ANOVA) with genotype x odor stimulation as factors. Significant main or interactive effects were determined by Bonferroni's post-hoc test with adjusted error rate for number of comparisons (α 0.0125).

3. Results

3.1 Gene-targeted deletion of Kv1.3 Increases the Number of Mitral Cells in the Lateral, Medial, and Ventral Regions of the Early Postnatal OB

Because Kv1.3 is expressed by mitral cells and targeted deletion is known to affect mitral cell function and olfactory behavior, we wondered if there were also concomitant changes in the morphology of the mitral cell in the "Super-smeller" mice. Mitral cell area and density were analyzed from four regions (dorsal, lateral, medial, ventral; 25,000 μ m² each) on coronal sections of OBs from P20 aged WT and KO mice (Fig. 1A). The mitral cell area was

not significantly different across genotype for any sampled region (Fig. 1B–D). However, there was a 1.5 fold increase in the number of mitral cells in the lateral, medial, and ventral regions of KO mice vs. WT mice (Fig. 1B, C, E). This increase in cell number was only observed in early postnatal mice and was not present by age 3 months to 1 year (non-odor stimulated controls, Fig. 2–3). This suggests that the increased frequency of action potentials and shorter interspike interval observed in mitral cells in KO mice [7] modifies early mitral cell development by either proliferation or survival, which changes with age.

3.2 Odor Enrichment with Peppermint Decreases Mitral Cell Size in WT Mice at 3 Months But Not at One Year

Several studies have examined the effects of odor exposure on mitral cell morphology and density in rats [9;15;16;18]. We wanted to examine how altering olfactory sensitivity influences odor-induced changes in mitral cell anatomy and determine if this modulation was age-dependent, as observed for OSNs [4]. One year old (Fig. 2A–D, I–K) and 3 month old (Fig. 2E–H, L–N) WT and KO mice were exposed to peppermint five sessions per day for 30 days. In contrast to the lack of mitral cell size differences across genotype at P20, WT mice at both 1 year and 3 month had larger cells than those of KO mice in the dorsal ($F_{1,118}=9.125$, $p=0.0031$) and lateral ($F_{1,118}=7.737$, $p=0.0063$) regions (Fig. 2I) and the lateral ($F_{1,118}=12.11$, $p=0.0007$) region (Fig. 2L), respectively. Post-hoc analysis revealed that odor-enrichment with peppermint in 3 month old WT mice decreased the size of the mitral cell in the dorsal and lateral regions (Fig. 2L) whereas in 1 year old mice, cells in the lateral region exhibited an increase in size (Fig. 2I). Post-hoc analysis also revealed that KO mice exhibited larger cells in the dorsal region of 3 month old mice stimulated with peppermint (Fig. 2L), while in 1 year old KO mice, there was a decrease in cell size with odor-enrichment in the dorsal region and an increase in cell size in the lateral region (Fig. 2I). The 3 month old WT mice stimulated with peppermint (Fig. 2L) exhibited smaller mitral cell areas compared to P20-aged control mice (Fig. 1D), while 1 year old WT mice were similar in size to P20-aged mice (Fig. 2I). However, the mitral cells of KO mice regardless of age (Fig. 2I, L) were smaller than those of P20-aged mice (Fig. 1D). In addition, the shape of the mitral cell largely did not vary across genotypes at either the 3 month (Fig. 2M, no regions significantly different) or the 1 year time point (Fig. 2J, only medial region significantly different, $F_{1,118}=5.699$, $p=0.0186$). Post-hoc analysis revealed no significant differences within either genotype for peppermint odor stimulation in the medial region.

Both 3 month and 1 year old mice had significant genotype, odor, or interaction effects in multiple regions with regards to variance in mitral cell density (Fig. 2N and 2K, respectively; $F_{1,118}=7.039$ to 56.69; $p=0.0114$ to 0.0001). The most prominent significant effect was in the lateral region, where KO mice had lesser cell densities than WT mice at age 3 month ($F_{1,118}=14.75$, $p=0.0001$) but were greater in density in this region at 1 year ($F_{1,118}=7.039$, $p=0.0114$). Post-hoc analysis revealed that unlike mitral cell size, peppermint enrichment caused a decrease in cell density that was largely genotype independent in both the medial and ventral regions in 1 year old mice (Fig. 2K). However, post-hoc analysis also revealed that 3 month old WT mice were more sensitive than KO mice to odor-enrichment induced loss of mitral cell number in three regions across the bulb (Fig. 2N). Therefore, odor enrichment with peppermint extract affects mitral cell density and morphology in an age- and region-dependent manner. The plasticity of the mitral cells can be influenced not only by the voltage-gated activity of the mitral cell but also by the odor environment of the animal, and this plasticity is also sensitive to the age of the animal.

3.3 Odor Enrichment with Citralva Alters Mitral Cell Morphology and Density at 3 Months

Because peppermint extract is a stimulus for both the olfactory and trigeminal systems [5], we wanted to examine the changes in mitral cell morphology and density in response to a purely olfactory stimulus. Three month old WT (Fig. 3A and B) and KO (Fig. 3C and D) mice were therefore exposed to citralva for 30 days in the same manner as for peppermint stimulation. Since we observed more diverse anatomical changes in response to peppermint in the three month old age group, we elected to design this set of experiments only in the younger mice. WT mice had a greater mitral cell area than that of KO mice in three regions of the OB, however, these differences, while significant, were not marked ($F_{1,118}=4.420$, $p=0.0377$ dorsal; $F_{1,118}=4.371$, $p=0.0387$ medial; $F_{1,118}=7.27$; $p=0.0081$; ventral) and post-hoc analysis only revealed a decrease in area for KO mice in one area (ventral) in response to odor-enrichment (Fig. 3E). Again, there were few significant difference in mitral cell shape across genotype or odor factors ($F_{1,118}=5.121$, $p=0.0255$ to $F_{1,118}=5.816$, $p=0.0175$) and none revealed any changes in response to odor-enrichment with post-hoc analysis (Fig. 3F). Again, all four regions demonstrated a main effect of genotype with regards to mitral cell density ($F_{1,118}=21.37$, $p=0.0001$ dorsal; $F_{1,118}=32.54$, $p=0.0001$ lateral $F_{1,118}=19.26$, $p=0.0001$ medial; $F_{1,118}=15.22$; $p=0.002$; ventral) whereby WT mice had approximately a 30% greater density than WT mice (Fig.3G). Interestingly, post-hoc analysis revealed that citralva stimulation significantly decreased the density of mitral cells in most OB regions regardless of genotype (Fig. 3G). These results suggest that cell size and density appear to be influenced by the olfactory and trigeminal components of the stimulus.

4. Discussion

Mitral cell responses to odors are plastic, changing based on contextual cues accompanying the odor presentation [6]. We have shown that mitral cell density and morphology are modulated in an odorant-dependent manner and that previously observed changes in mitral cell activity [7] adds an additional level of modulation of mitral cell plasticity. Increasing the excitability of mitral cells, by gene-targeted deletion of Kv1.3 [7], increases the number of mitral cells in early developmental stages of the bulb without affecting the size of the cells. Chronic odor stimulation with peppermint decreases the density of mitral cells in WT mice regardless of age, however, KO mice exhibited both age- and region-dependent modulation. Odor enrichment with citralva also decreased the number of mitral cells regardless of genotype, while not altering cell shape. In comparison to the P20-aged mice, which did not receive odor enrichment, odor-stimulation with peppermint and citralva generally caused a decrease in cell size regardless of age (3 mo. or 1 yr.) or genotype. These results suggest that odor environment and mitral cell activity both influence mitral cell density.

Odor enrichment is known to enhance olfactory discrimination [11], to decrease mitral cell odor responses [3], and to alter density of OSNs [5]. Peppermint and citralva stimulation decrease the number of mitral cells in WT and KO mice. This response to peppermint is interesting because very few studies have investigated effects of trigeminal stimulation on mitral cells [23]. In contrast, once daily peppermint exposure, along with tactile stimulation, in rats did not affect mitral cell or granule cell density [13], but this may be due to differences in length of enrichment protocol and the number of daily stimulation trials. Laing and colleagues [9;15;16] have shown that continuous exposure to a single odorant, or even to deodorized air, significantly decreases the size of mitral cell bodies. However, we observed more variation in cell size across regions of the OB dependent on genotype, which may indicate a difference in the amount of odorant information being processed by the mitral cells [16]. We kept our analysis to within single regions of the OB because we were not targeting odor stimulation of molecularly or positionally-identified glomeruli within the OB. Mitral cell plasticity is potentially influenced by several factors. The current study demonstrates mitral cell excitability and odor environment alter mitral cell density and

morphology, similar to previous results with OSNs [4]. This regulation is complicated by the odorant used and the age of the animal. In addition, OSNs and mitral cells are regulated by metabolic state [14]. Olfactory perception can be dramatically different based on the compellation of these various factors, and this allows for modification of olfactory sensitivity based on context much like what is observed in reward-based olfactory learning [6].

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Abbreviations

KO	Kv1.3-null (−/−)
MOE	main olfactory epithelium
OB	olfactory bulb
OSN	olfactory sensory neuron
PBS	phosphate buffered saline
SEM	standard error of the mean
WT	wildtype

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HIGHLIGHTS

- Early postnatal Kv1.3-null mice have high density of mitral cells in the olfactory bulb.
- Enrichment with olfactory or trigeminal stimuli reduced mitral cell number.
- Mitral cell area is decreased with olfactory and increased with trigeminal stimulation.

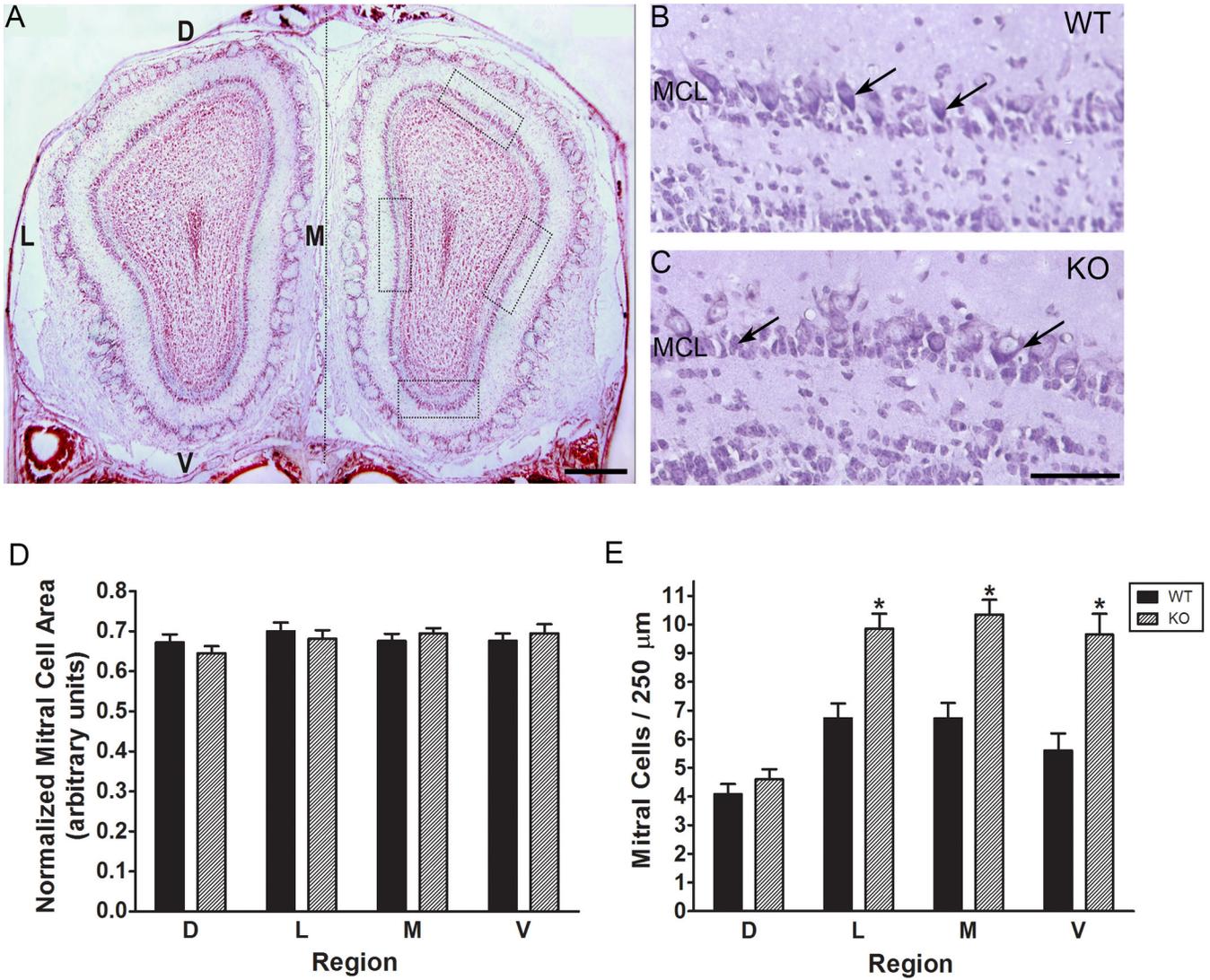
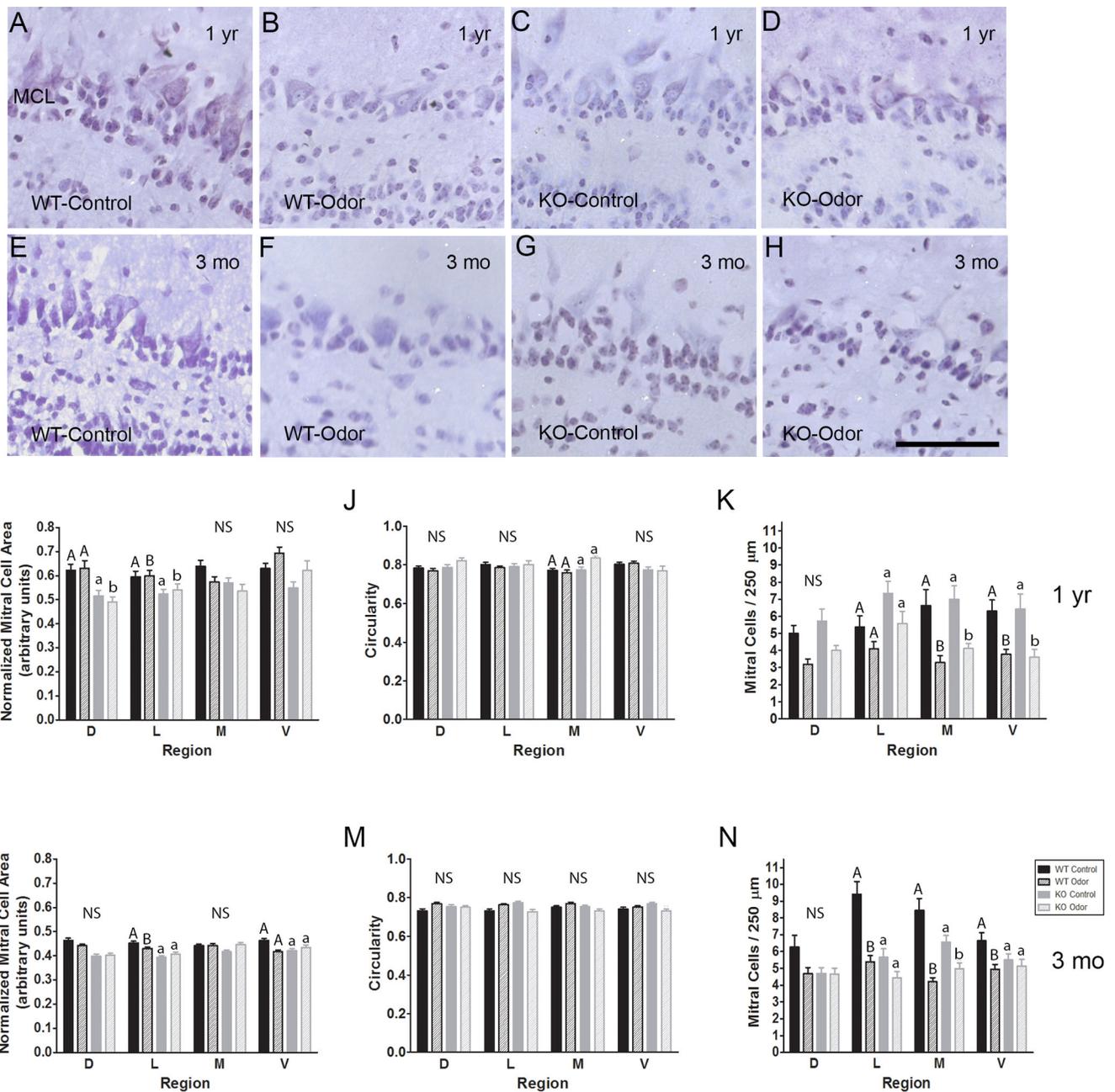


Figure 1. *Kv1.3*^{-/-} (KO) mice have a greater number of mitral cells in the lateral, medial, and ventral regions of the olfactory bulb (OB) at postnatal (P) day 20 compared to wildtype (WT) mice. A: Representative photomicrograph of a coronal section of the OB. Boxes represent the four regions used for analysis (D=dorsal, M=medial, V=ventral, L=lateral; scale bar=500 μm). B–C: Representative photomicrographs of the mitral cell layer (MCL) of the OB (arrows=representative mitral cells) acquired from the lateral (L) region from WT and KO mice (scale bar = 50 μm). D–E: Bar graph of the mean normalized mitral cell area and the mean number of mitral cells along a 250 μm line by OB region. Data represent mean ± standard error of the mean (SEM) for mitral cells measured in 10 mice (5 per genotype) across the four regions (black bars = WT; hatched bars = KO). Although all neurons were measured rostral to caudal for each animal, a subpopulation of 30 neurons was randomly selected per mouse and region to be analyzed. * = Significantly-different by Student’s *t*-test with Bonferroni correction for multiple comparisons, *p* 0.0125.

**Figure 2.**

Mitral cell size is decreased in WT three month old mice over that of one year old mice following peppermint odor enrichment whereas cell density decreases with odor enrichment regardless of genotype or age of development. A–D: Representative photomicrographs of the mitral cell layer (MCL) of the OB acquired from the lateral (L) region from one year old (1 yr) WT (A–B) and KO (C–D) mice under control (A, C) and peppermint-stimulated (B, D) conditions. E–H: Same as A–D, but for 3 month old (3 mo) mice. Scale bar=50 μm. I–N: Bar graphs of normalized mitral cell area (I, L), circularity index (J, M), and density (K, N) from one year old (I–K) vs. three month old (L–N) mice. Legend in N applies to all bar graphs. Data represent mean ± standard error of the mean (SEM) for mitral cells measured in 5 mice per treatment across the dorsal (D), lateral (L), medial (M), and ventral (V) regions

(black bars=WT; gray bars=KO). NS=not significantly different, two-way ANOVA (genotype x odor stimulation as factors). Upper case letters (WT) and lower case letters (KO) indicate significant difference by follow-up Bonferroni's post-hoc test (α 0.0125) comparing control versus odor stimulation when ANOVA was significant. If the letters are different, the values are significantly different (e.g. 'A' is different than 'B' and 'a' is different than 'b').

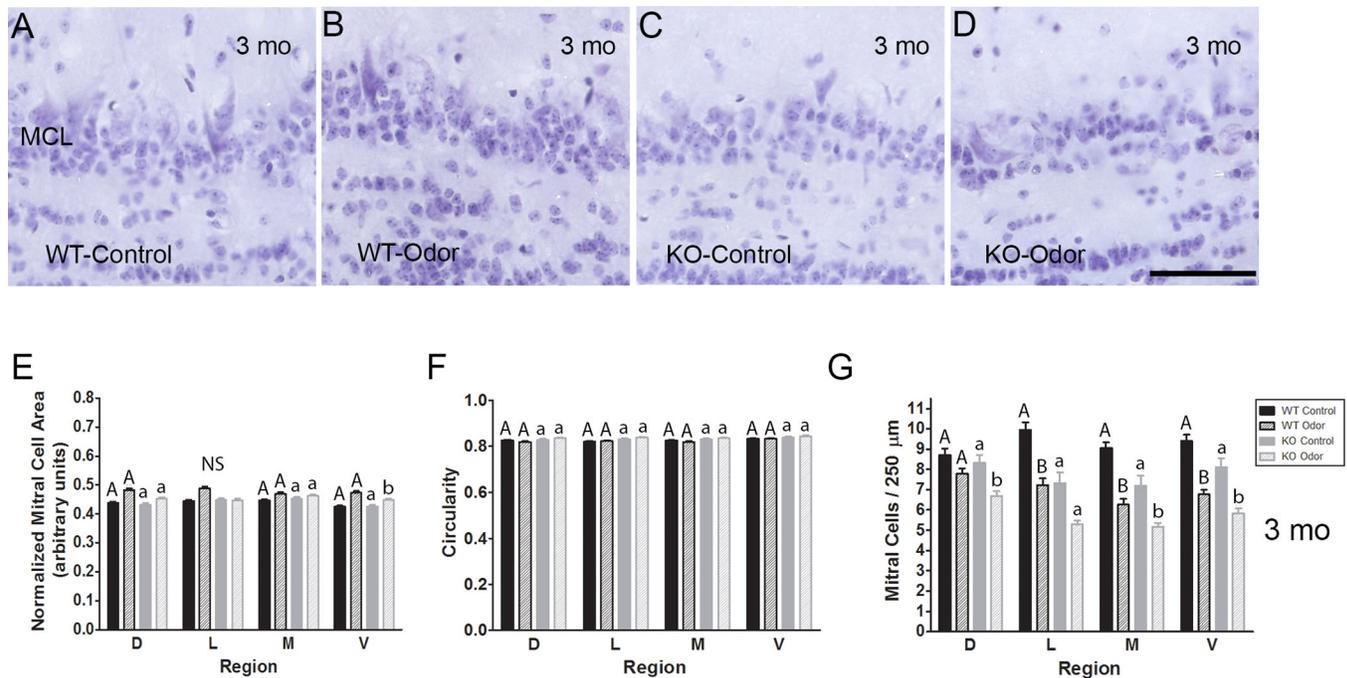


Figure 3. Mitral cell size is increased in WT and KO three month old mice following citralva odor enrichment whereas cell density is decreased. A–D: Representative photomicrographs of the mitral cell layer (MCL) of the OB from three month old (3 mo) WT (A–B) and KO (C–D) mice under control (A, C) and citralva-stimulated (B, D) conditions (scale bar = 50 μ m). E–G: Bar graphs of mitral cell area (E), circularity index (F), and density (G) by OB region. Legend in G applies to all histograms. Same sample size, notations, and statistical analysis as in Fig. 2.