Subpopulations of vomeronasal sensory neurons with coordinated coexpression of type 2 vomeronasal receptor genes are differentially dependent on Vmn2r1

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Abstract

The mouse vomeronasal organ is specialized in the detection of pheromones. Vomeronasal sensory neurons (VSNs) express chemosensory receptors of two large gene repertoires, V1R and V2R, which encode G-protein-coupled receptors. Phylogenetically, four families of V2R genes can be discerned as follows: A, B, C, and D. VSNs located in the basal layer of the vomeronasal epithelium (Bouchard & D'Orleans-Juste, 2002) are distributed in two subdomains: vomeronasal receptor genes V1R (Dulac & Axel, 1995) and V2R (Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). The cell bodies of V1R+ and V2R+ VSNs reside in the apical and basal layers of the VNO, respectively. The basal layer of VSNs can be subdivided into two sublayers based on the expression of nine non-classical class I major histocompatibility complex genes termed H2-M1 (Ishii et al., 2003; Loconto et al., 2003; Ishii & Mombaerts, 2008, 2012). Axons of VSNs from the apical and basal layers coalesce into multiple glomeruli in the anterior and posterior halves of the accessory olfactory bulb (AOB), respectively (Belluscio et al., 1999; Rodriguez et al., 1999; Del Punta et al., 2002; Ishii & Mombaerts, 2008, 2012). V2R+ VSNs respond physiologically to peptides and proteins (Leinders-Zufall et al., 2004, 2009, 2014; Kimoto et al., 2005; Chamerot et al., 2007, 2011, 2012; Haga et al., 2007, 2010; Sturm et al., 2013). The deduced amino acid sequences of V2Rs contain a long N-terminal extracellular region, which may form the ligand-binding site. The mouse genome contains 120 intact V2R genes, which can be grouped in four families (A, B, D, and C) based on amino acid sequence homology (Yang et al., 2005; Shi & Zhang, 2007; Young & Trask, 2007; Francia et al., 2014). The largest V2R gene family, family A, can be further grouped in nine subfamilies A1 to A10; family A7 exists in rat but not in mouse. Family C, which was originally referred to as mV2R2 (Ryba & Tirindelli, 1997) and then mouse V2R2 (Martini et al., 2001), comprises seven genes in mouse, Vmn2r1 through Vmn2r7 (Silvotti et al., 2007, 2011; Francia et al., 2014).

The consensus of numerous in situ hybridization (ISH) studies is that mouse family-ABD V2R genes are expressed in a punctate and mutually exclusive manner, most likely as a single gene per basal
VSN. When we knocked out the family-A9 gene V2rf2/Vmn2r81 and replaced it by the β-galactosidase marker using gene targeting in embryonic stem (ES) cells, we found that ~25% of VSNs that express the marker express another family-ABD V2R gene (Ishii & Mombaerts, 2011). This second choice of another family-ABD V2R gene can be interpreted in terms of a hypothetical negative feedback mechanism that helps restrict expression of family-ABD V2R genes to a single gene per VSN.

In sharp contrast to observations with family-ABD V2R genes, ISH probes or antibodies for family-C V2Rs label large populations of basal VSNs (Martini et al., 2001; Silvotti et al., 2007, 2011; Ishii & Mombaerts, 2011). Immunoreactive signals with antibodies against family-C members Vmn2r1 and Vmn2r2/Vmn2r5 are detected in a mutually exclusive manner, each in approximately half of basal VSNs (Silvotti et al., 2007, 2011). VSNs expressing V2Rs of families A8–10, B, and D are preferentially colabeled with anti-Vmn2r1 antibody, and VSNs expressing V2Rs of family A1-6 are preferentially colabeled with Vmn2r2/Vmn2r5 antibody (Silvotti et al., 2007, 2011). Vmn2r2 through Vmn2r7 are coexpressed in various combinations, and many VSNs appear to coexpress all six receptors (Silvotti et al., 2011). Surprisingly, more than twenty years after the discovery of mouse V2R genes, there are no reports about mouse strains with a knockout mutation in a family-C gene.

Here, we have generated two novel mouse strains carrying a knockout mutation in Vmn2r1. We found that various subpopulations of V2R+ VSNs are differentially affected by the absence of Vmn2r1; that there is no compensatory expression of Vmn2r2 through Vmn2r7; and that glomeruli form normally if there are sufficient VSNs left. We discovered that Vmn2r65 (family A5) and Vmn2r120 (family A6) are C1 type of V2Rs, in discordance with their phylogenetic classification as C2 type of V2Rs.

Materials and methods

Animal ethics

Mouse studies were carried out in accordance with the German Animal Welfare Act, the European Communities Council Directive 2010/63/EU, and the institutional ethical and animal welfare guidelines of The Rockefeller University, the Max Planck Institute of Biophysics, and the Max Planck Research Unit for Neurogenetics. Approval came from the IACUC of The Rockefeller University, the Regierungspräsidium Darmstadt and the Veterinäramt of the City of Frankfurt.

Generation of gene-targeted mouse strains

We have previously described the gene-targeted strains V2r1b-IRES-tauGFP (Del Punta et al., 2002) and V2r2-IRES-tauGFP (Ishii & Mombaerts, 2011). The internal ribosome entry site sequence (IRES) affords bicistronic translation of transcripts. We have here generated four novel mouse strains by gene targeting in the parental ES cell line E14. For the ΔC1 targeting vector, a 2.6 kilobase (kb) upstream fragment and a 4.8 kb downstream fragment flanking exon 1 of the Vmn2r1 gene were used as the 5′ and 3′ homologous arms, respectively. The Hprt (5′)-loxP-neo cassette (Ramirez-Solis et al., 1995) was ligated to replace a 1065 basepair (bp) sequence from the SpeI site at the 28th nucleotide (nt) before the initiation codon of the HindIII site at the 801st nt after the end of exon 1. For the ΔC1-GFP targeting vector, a 2.6 kb upstream fragment and a 5.6 kb downstream fragment of exon 1 of the Vmn2r1 gene were used as the 5′ and 3′ homologous arms, respectively. The tauGFP-pA cassette and the self-excisng neo selectable marker cassette (ACNF) were ligated to replace a 230 bp sequence from the initiation codon in-frame to the 4th nt before the end of exon 1. For the V2rf4-IRES-tauVenus targeting vector, a 8.2 kb Hpal-Nhel fragment containing exon 6 of the Vmn2r83 gene was used to construct the homologous arms. The IRES-tauVenus-ACNF cassette was inserted into an Ascl site that was created one nt after the stop codon of Vmn2r83. For the V2rf1-IRES-tauCherry targeting vector, a 7.8 kb SpeI fragment containing exon 6 of the Vmn2r82 gene was used to construct the homologous arms. The IRES-tauCherry-ACNF cassette was inserted into an artificial Ascl site one nt after the stop codon of Vmn2r82. All DNA sequences for homologous arms were from mouse BAC clones of 129/SvEv genomic origin. The targeting vectors were linearized and electroporated into ES cells as described (Mombaerts et al., 1996). Homologous recombination events were screened and confirmed by Southern blot hybridization with external probes. Cells from gene-targeted ES clones were injected into C57BL/6J blastocysts, and germline transmission was obtained by mating male chimera with C57BL/6J females. The ACNF cassette got removed during transmission through the male germline, leaving a loxp sequence behind in the targeted mutation. The strains are publicly available from The Jackson Laboratory in a mixed (129P2/OlaHsd x C57BL/6J) background, as follows: ΔC1 as stock number JR#24643 and strain name B6;129P2-Vmn2r1<tm1Mom>/MomJ, ACNF-GFP as JR#26765 and B6;129P2-Vmn2r1<tm2Mom>/MomJ, V2rf1-mCherry as JR#7885 and B6;129P2-Vmn2r82<tm1Mom>/MomJ, and V2rf4-Venus as JR#7886 and B6;129P2-Vmn2r83<tm1Mom>/MomJ.

Tissue preparation

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine (210 mg/kg and 10 mg/kg body weight, respectively), and perfused transcardially with phosphate-buffered saline (PBS) at room temperature, followed by ice-cold 4% paraformaldehyde/PBS. The VNO and the brain including the AOB were dissected separately, and post-fixed for 3 h at 4 °C. The samples were then decalcified in 0.45 m EDTA/PBS at 4 °C overnight (for 4-week-old mice) or two nights (for 10-week-old mice), cryoprotected in 15 and 30% sucrose/PBS successively, and frozen in OCT compound (Sakura Finetek #4583). The decalcification step was omitted for brain samples and 0-day-old samples.

Immunohistochemistry and cell counts in the VNO

Coronal 14 μm cryosections were cut through the entire VNO and collected on glass slides. Sections were treated with 0.5% SDS/PBS for 30 min at room temperature. This step was omitted for V2Rp5 or GFP IHC alone. After washing with PBS, sections were blocked in 5% normal goat serum (NGS) and 0.3% Triton X-100/PBS, and incubated with primary antibodies in the same blocking solution for 1–2 h at room temperature and then overnight at 4 °C. After washing in 0.1% Triton X-100/PBS, sections were incubated with secondary antibodies and counterstained with DAPI (1:10 000, Invitrogen #D1306) for 2–4 h at room temperature. Primary antibodies and working dilution of each antibody were as follows: rabbit anti-C1 (anti-Vmn2r21) antibody (1:500; Silvotti et al., 2007), rabbit anti-C2 (anti-Vmn2r25) antibody (1:400; Silvotti et al., 2007), rabbit anti-panC antibody (also referred to as V2R2; 1:4000; Martini et al., 2001), rabbit anti-V2R[p5 antibody (1:1500; Haga et al., 2010), and chicken anti-GFP antibody (1:1500; Aves Labs #GFP-1020). To improve the signal-to-noise ratio of anti-C1, anti-C2, and anti-GFP,
anti-GFP IHC, the antibodies were pre-absorbed with acetone powder prepared from brain homogenates of mice with a deletion of the family-C V2R gene cluster generated by genome engineering (AV2RCΔ, unpublished). The antibody solution was incubated with 1% acetone powder overnight at 4 °C, and cleared by centrifugation and filtration before use. The following secondary antibodies were used at a 1:1000 dilution: Alexa 488 goat anti-rabbit IgG, Alexa 546 goat anti-rabbit IgG, Alexa 647 goat anti-rabbit IgG, and Alexa 488 goat anti-chicken IgG (Invitrogen #A1034, A11035, A12245, A11039). Sections were examined under a Zeiss LSM 710 confocal microscope without knowing the genotype. Labeled cells were counted on every tenth section (for 4-week and 10-week-old mice) or on every fifth section (for 0-day-old mice). The total cell number per mouse was estimated by multiplying the count by ten or five, respectively. The numbers given are numbers of VSN cell profiles, but for the sake of simplicity, they are referred to as numbers of VSNs.

**IHC of the AOB**

Serial sagittal 25 μm sections of the AOB were cut and collected in PBS. To visualize the glomerular layer, free-floating sections were blocked in 5% NGS and 0.2% Tween 20/PBS, and incubated with rabbit anti-VGLUT2 antibody (1:3000; Synaptic Systems #135403) in the same blocking solution for 1–2 h at room temperature, and then overnight at 4 °C. After washing in 0.1% Tween 20/PBS, sections were incubated with Alexa 488 goat anti-rabbit IgG (1:1000) and counterstained with DAPI (1:10 000) for 2 h at room temperature. Sections were mounted on glass slides and examined under a Zeiss LSM 710 confocal microscope. For visualization of GFP+ axons, images of the intrinsic fluorescence were taken from serial AOB sections. Multiple sections were aligned using Photoshop, then combined in a z-stack file using IMAGE J. The 2D images were created by z-projection with maximum intensity method.

**NanoString multiplex gene expression analysis**

Whole VNO mucosa was dissected in RNA Stabilization Reagent RINLafer (Qiagen #76106). Tissue from a single mouse was placed in a tube and homogenized by TissueLyser LT (Qiagen #85600). Total RNA was extracted using the RNeasy Micro kit with DNase I treatment on the column (Qiagen #74004), and stored at −80 °C until use. A 0.75 μg aliquot of RNA from each mouse was tested with the custom CodeSet Pao (NanoString Technologies, Seattle, WA, USA). Samples from six wild-type mice and six homozygous mice were analyzed in one cartridge using the nCounter SPRINT Assay system (NanoString Technologies) according to the manufacturer’s instructions. Data processing and statistical analyses were performed using NSOLVER DATA ANALYSIS Software 3.0 (NanoString Technologies). We confirmed that samples passed quality control (QC) criteria based on imaging QC, binding density QC, and positive and negative control probes. We then processed the raw count data into normalized count values in three steps: normalization using a scaling factor calculated from the geometric mean of the six positive control counts, second normalization using a scaling factor calculated from the geometric mean of the counts of four reference genes (Ano2, Cnga4, Omp, Trpc2), and background correction by subtracting the mean ± 2 standard deviations of eight negative control counts for each sample. Values < 1 after background subtraction were set as 1. Genes with a median count < 100 in wild-type mice were eliminated from further analysis. Changes in gene expression were estimated by calculating the fold change (FC) of the geometric mean of the counts in mutant mice over the geometric mean of the counts in wild-type mice, and the log2 FC values were plotted.

**In situ hybridization**

RNA probes were prepared for the coding sequences of Vmn2r65 (nt 948-1603 of RefSeq NM_001105180.1), Vmn2r76 (mix of nt 508-1028 and nt 1219-1590 of RefSeq NM_001102580.1), Vmn2r118 (nt 822-1306 of RefSeq NM_001104582.1), and Vmn2r120 (nt 1044-1739 of RefSeq NM_001104591.1; Silvotti et al., 2007). Digoxigenin (DIG) labeling of RNA probes, tissue preparation, and ISH were performed as described previously (Ishii et al., 2004) with some modifications. Coronal 12 μm sections were cut through the entire VNO, and every 12th section was collected onto a slide for each probe. The incubation time of Proteinase K (Roche #03115828001) was 12 min. Hybridized probes were detected with an alkaline phosphatase-conjugated anti-DIG antibody (1:1000; Roche #11093274910) and BM purple substrate solution (Roche #11442074001). Labeled cells were counted on digital images of sections taken by a Parannomic MIDI slide scanner (3D HISTECH) without knowing the genotype. The total cell number per mouse was determined by multiplying the count by twelve.

**ISH combined with IHC**

Tissue preparation and ISH were carried out as described above, except that the incubation time of Proteinase K was 5 min. After hybridization and washing, sections were blocked in 5% NGS and 0.3% Triton X-100/PBS, and incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:1000) and anti-C1 antibody (1:500) or anti-C2 antibody (1:4000) in the same blocking solution for two nights at 4 °C. After washing in 0.1% Triton X-100/PBS, sections were incubated with Alexa 488 goat anti-rabbit IgG (1:1000) and DAPI (1:10 000) for 8 h at room temperature. Sections were washed with 0.1% Triton X-100/PBS, and DIG-labeled probes were detected by incubation with HNPP/Fast Red substrate solution (Roche #11758888001) for 30 min. After washing with TN buffer (100 mM Tris pH 7.5, 150 mM NaCl), sections were examined under a Zeiss LSM 710 confocal microscope, directly in TN buffer without mounting.

**Experimental design and statistical analysis**

For statistical evaluations of cell numbers, the Mann–Whitney test was performed using GRAPHPAD PRISM 5. For NanoString analysis, statistical analysis was performed by NSOLVER DATA ANALYSIS Software 3.0. The false discovery rates (FDR; Benjamini & Yekutieli, 2001) from conducting multiple testing were computed based on the P-values of the t-test. Genes were considered differentially expressed if FDR < 0.05.

**Results**

**Generation of gene-targeted strains with a knockout mutation in Vmn2r1**

The mouse V2R gene family C comprises seven intact genes, which are located in a single cluster extending over a 640 kb region on chromosome 3 (Fig. 1A). We generated two mouse strains with a gene-targeted mutation in the Vmn2r1 gene (referred to as C1), which is composed of six coding exons (Fig. 1B).
In the first strain (ΔC1), we replaced a 1065 bp sequence including coding exon 1 with a selectable marker cassette. Immunohistochemistry (IHC) with anti-C1 antibody revealed immunoreactivity in the basal layer of the VNO in wild-type mice (WT) but not in homozygous (ΔC1) mice (Fig. 1C). This gene is composed of six coding exons. In the ΔC1 strain, a 1065 bp fragment that includes coding exon 1 (ex1) is replaced with a Hprt(5)loxP-neo cassette. This cassette is left behind in the targeted mutation in the mouse strain. In the ΔC1-GFP strain, a 230 bp fragment from the initiation ATG codon to the fourth nt before the end of coding exon 1 is replaced in frame with a tauGFP-pA cassette, followed by a self-excising neo selectable marker cassette (ACNF). Following the removal of ACNF, one loxP site is left behind in the targeted mutation in the mouse strain, after tauGFP-pA. The black triangles indicate a loxP site. H, HindIII. (C,D) IHC with anti-C1 antibody (C) and anti-C2 antibody (D) on coronal sections of the VNO from wild-type (WT) and homozygous (ΔC1) mice of the ΔC1 strain. Mice were male and 10 weeks old. There is no C1 immunoreactivity in ΔC1 mice (C), and no obvious difference in C2 immunoreactivity between WT and ΔC1 mice (D). Scale bar, 100 µm. (E,F) IHC with anti-C1 antibody (E) and anti-GFP antibody (F) on coronal sections of the VNO from WT and homozygous (ΔC1-GFP) mice of the ΔC1-GFP strain. Mice were male and 4 weeks old. There is no C1 immunoreactivity in ΔC1-GFP mice (E). Sparse GFP+ cells are detected in ΔC1-GFP mice (F). Scale bar, 100 µm.

In the second strain (ΔC1-GFP), we replaced a 230 bp sequence of exon 1 with a tauGFP-pA cassette. Here too, there is no C1 immunoreactivity in homozygous mice (ΔC1-GFP), consistent with the null design of the targeted mutation. The tauGFP marker fuses to the initiation codon should enable us to detect VSNs in which the mutant C1 locus is transcribed, but no functional C1 receptor is expressed. However, we observed only sparse GFP+ VSNs in ΔC1-GFP mice. The intrinsic GFP fluorescence in VSNs of ΔC1-GFP mice was weak and needed to be enhanced by IHC with anti-GFP antibody (Fig. 1F). The number of GFP+ VSNs in ΔC1-GFP mice and in mice heterozygous for this mutation (data not available).
shown) is much less than the number of VSNs detected by anti-C1 antibody in WT mice. The mutant allele may yield unstable transcripts; alternatively, transcription of the mutant allele may extinguish with time.

We established the ΔC1 and ΔC1-GFP strains in a mixed (129P2/OlaHsd × C57BL/6J) background. We maintained the strains by mating heterozygous mice, such that WT and homozygous mice were obtained from the same litters. ΔC1 mice and ΔC1-GFP mice are viable and fertile and show no obvious differences in appearance compared to their WT littermates. These strains are publicly available from The Jackson Laboratory.

**Differential and graded decrease in the numbers of VSNs that express C1 type of V2R genes**

We examined the effects of the ΔC1 mutation on the numbers of various subpopulations of V2R+ VSNs in the VNO.

First, we crossed the ΔC1 strain with the gene-targeted strains V2r1b-IRES-tauGFP (abbreviated as V2r1b-GFP; Del Punta et al., 2002) or V2rf2-IRES-tauGFP (abbreviated as V2rf2-GFP; Ishii & Mombaerts, 2011). In these strains, VSNs that express the V2r1b/ Vmn2r26 gene (family B, C1 type) or the V2rf2/Vmn2r81 gene (family A9, C1 type), respectively, coexpress tauGFP by virtue of IRES-mediated bicistronic translation. We have previously shown that >90% of V2r1b-GFP+ VSNs and V2rf2-GFP+ VSNs are colabeled with anti-C1 antibody, and rarely with anti-C2 antibody (Ishii & Mombaerts, 2011). We counted GFP+ VSNs on VNO sections of ΔC1 mice and their WT littermates in crosses of ΔC1 with V2r1b-GFP or V2rf2-GFP. We observed a marked decrease in the number of V2r1b-GFP+ VSNs in ΔC1 mice (Fig. 2A). At 4 week, the number of V2r1b-GFP+ VSNs was decreased to 21.5% of WT (mean ± SEM in ΔC1, 185 ± 16 compared to WT, 860 ± 76; n = 4 per genotype; Mann–Whitney test, P = 0.0286) (Fig. 2E). At 10 week, this number was decreased further to 9.5% of WT (ΔC1, 285 ± 32 compared to WT, 803 ± 67; n = 4 per genotype; P = 0.0286) (Fig. 3C). In ΔC1-GFP mice at 10 weeks, the number of V2rf1-GFP+ VSNs was decreased to 35.8% of WT (ΔC1-GFP, 263 ± 17 compared to WT, 733 ± 117; n = 4 per genotype; P = 0.0286) (Fig. 3D). Thus, the two mutations in Vmn2r1 had the same effect on the subpopulation of V2r1b-GFP+ VSNs (Fig. 3E).

In summary, the Vmn2r1 mutations affect differentially the four populations of C1 type of V2R+ VSNs that we examined with gene-targeted strains of the V2R-IRES-marker design. At 10 weeks, V2r1b-GFP+ VSNs and V2rf4-Venus+ VSNs were strongly affected (decreased to 9.5 and 15.5% of WT, respectively), and V2rf2-GFP+ VSNs and V2rf1-mCherry+ VSNs were less affected (decreased to 63.4 and 35.5% of WT, respectively).

**No compensatory expression of other family-C V2R genes**

Conceivably, other members of family-C V2Rs may become expressed in the C1 type of V2R+ VSNs that remain in ΔC1 and ΔC1-GFP mice. Such expression might occur to compensate for the loss of C1 receptor function and as a result of the absence of a hypothetical negative feedback, analogous to what we observed in V2rf2 knockout mice (Ishii & Mombaerts, 2011).

To test this possibility, we examined coexpression of other family-C V2Rs in C1 type of V2R+ VSNs by immunohistochemistry with anti-C2 antibody. Representative images of VNO sections from 10-week-old mice with intrinsic signal of the fluorescent marker and C2 immunoreactivity are shown for the crosses ΔC1 × V2r2-GFP (Fig. 4A) and ΔC1 × V2r1b-mCherry (Fig. 4B). Only a few V2r2-GFP+ VSNs (4 of 369, 1%) and V2r1b-mCherry+ VSNs (5 of 321, 2%) were colabeled with anti-C2 antibody in WT mice, consistent with the preferential colabeling with anti-C1 antibody. In ΔC1 mice, the colabeling percentages with anti-C2 antibody in V2r2-GFP+ VSNs (2 of 324, 1%) and V2r1b-mCherry+ VSNs (3 of 114, 3%) were equally low. Likewise, in the cross ΔC1-GFP × V2r1b-mCherry (Fig. 4C), V2r1b-mCherry+ VSNs were rarely colabeled with the anti-C2 antibody in WT mice (2 of 293, 1%) and in ΔC1-GFP mice (4 of 105, 4%). Figure 4D shows the summary of colabeling analysis in 10-week-old mice (data from 4 mice per genotype for each cross) including the results for V2r1b-GFP+ and V2rf4-Venus+ VSNs. We found no compensatory expression of Vmn2r2 and Vmn2r5 (as detected with anti-C2 antibody) in these VSNs in the absence of C1 expression.

The anti-C2 antibody does not detect Vmn2r3, Vmn2r6, and Vmn2r7 (Silvotti et al., 2011). We further examined the possibility
of compensatory expression of other members of family-C V2Rs using an anti-panC antibody (Fig. 4E–H), which recognizes all seven family-C V2Rs (Martini et al., 2001; Silvotti et al., 2007). In WT mice, large fractions of V2r1b-GFP+, V2rf2-GFP+, V2rf4-Venus+, and V2rf1-mCherry+ VSNs were colabeled with panC antibody at 10 weeks, reflecting the preferential coexpression of Vmm2r1 in these VSNs, respectively, 87.8, 79.1, 75.2, and 74.6 or 72.7%. In sharp contrast, these subpopulations of VSNs were rarely colabeled with panC antibody in ΔC1 and ΔC1-GFP mice, respectively, 3, 1, 2, 5, or 4% (data from 4 mice per genotype for each cross).

Taken together, we found no evidence for compensatory expression of other family-C V2Rs in the absence of Vmm2r1. The subpopulations of C1 type of V2R+ VSNs that we examined do not appear to express any functional family-C receptor.

Axonal projections of subpopulations of VSNs in ΔC1 mice

V2R+ VSNs project their axons to the pAOB, where they coalesce into multiple glomeruli. V2r1b-GFP+ glomeruli are located in the anterior part of the pAOB (Del Punta et al., 2002; Ishii & Mombaerts, 2008), and V2rf2-GFP+ glomeruli in the middle part of the pAOB (Ishii & Mombaerts, 2011; Leinders-Zufall et al., 2014). Deletion of the V2rf2 gene results in a diffuse distribution of axons across the pAOB (Ishii & Mombaerts, 2011).

As a first step in elucidating possible biological functions of Vmm2r1, we asked if it is required for axonal wiring of VSNs that express C1 type of V2Rs. We examined the intrinsic GFP fluorescence in AOB sections of 4-week-old mice from the crosses ΔC1 × V2r1b-GFP and ΔC1 × V2rf2-GFP. We found that ΔC1 mice had only a few and small V2r1b-GFP+ glomeruli.
consistent with a decrease in the number of V2r1b-GFP+ VSNs at 4 weeks to 21.5% of WT (Fig. 2E). The small glomeruli in ΔC1 mice resided in the anterior part of pAOB, where V2r1b-GFP+ axons normally coalesce. We then visualized the glomerular layer of AOB, where VSN axons terminate and form synaptic connections, by IHC for a presynaptic marker using an anti-VGLUT2 antibody. We found that the few and small V2r1b-GFP+ glomeruli in ΔC1 mice were located in the VGLUT2+ glomerular layer, as was the case in WT mice (Fig. 5B). But for V2rf2-GFP+ VSNs, which were not affected in ΔC1 mice at 4 weeks (97% of WT, Fig. 2E), we observed multiple glomeruli with strong GFP intensity in the middle part of the pAOB in WT and ΔC1 mice, and without obvious difference in the pattern (Fig. 5C).

Thus, Vmn2r1 appear not to be required for the guidance of axons of V2rf2-GFP+ VSNs from the VNO to the appropriate region of the AOB, and for the formation of V2rf2 glomeruli. This finding excludes a major biological function of Vmn2r1 in axonal wiring and coalescence.

Gene expression profiling by NanoString multiplex analysis

The NanoString platform enables multiplex analysis of RNA levels of large gene repertoires such as ORs and VRs with high specificity and sensitivity (Khan et al., 2011, 2013; Leinders-Zufall et al., 2014). We designed a custom CodeSet Pao consisting of probes to analyze V2R gene expression in ΔC1 and ΔC1-GFP mice. Specific probes against the coding sequences were designed for 45 V2R genes, which is one-third of the repertoire of 120 V2R genes. To obtain higher coverage, we designed additional probes against 30 UTR sequences for 24 V2R genes, based on the sequence data from a transcriptome study by RNA-seq in the mouse VNO (Ibarra-Soria et al., 2014). We were unable to design specific NanoString probes for family-A4 V2Rs due to the high sequence homology among the family members. Of 69 V2R probes in CodeSet Pao, 10 probes gave very low counts in 10-week-old WT mice. Genes with a median count < 100 in WT samples were considered as not expressed in whole VNO mucosa at the age of analysis, and deleted from data. Of the probes designed against 30 UTR sequences, two gave extraordinary high counts; they were considered as non-specific, and these data were also deleted from the analysis. CodeSet Pao thus enabled us to examine expression of 57 V2R genes, covering 48% of the V2R gene repertoire and all V2R families except for family A4.

We examined total RNA samples from whole VNO mucosae of individual 10-week-old mice from the ΔC1 and ΔC1-GFP strains.
Fig. 4. No compensatory expression of Vmn2r2 through Vmn2r7 in the absence of Vmn2r1. (A,B) IHC with anti-C2 antibody on coronal VNO sections of WT and ΔC1 mice from the crosses ΔC1 × V2r2-GFP (A) and ΔC1 × V2r1-mCherry (B). In (A) intrinsic GFP fluorescence (indicated with an asterisk) is shown in green, and C2 immunoreactivity in red. In (B) intrinsic mCherry fluorescence is shown in red, and C2 immunoreactivity in blue. Mice were male and 10 weeks old. Scale bar, 50 μm. (C) IHC with anti-C2 antibody (blue) on coronal VNO sections of WT and ΔC1-GFP mice from the cross ΔC1-GFP × V2r2-mCherry at 10 weeks. Intrinsic mCherry fluorescence is shown in red. Scale bar, 50 μm. (D) Summary of the percentage of colabeling with anti-C2 antibody in VSNs that express a fluorescence marker from a gene-targeted locus. Four mice per genotype were analyzed for each cross. Samples were from the 10-week-old male mice that were used for VSN counting in Figs 2 and 3. Above each bar, the number of double-labeled VSNs/number of fluorescence marker-positive VSNs is given. (E,F) IHC with anti-panC antibody on coronal VNO sections of WT and ΔC1 mice from the crosses ΔC1 × V2r2-GFP (E) and ΔC1 × V2r1-mCherry (F) at 10 weeks. In (E) intrinsic GFP fluorescence is shown in green, and panC immunoreactivity in red. In (F) intrinsic mCherry fluorescence is shown in red, and panC immunoreactivity in blue. Scale bar, 50 μm. (G) IHC with anti-panC antibody (blue) on coronal VNO sections of WT and ΔC1-GFP mice from the cross ΔC1-GFP × V2r1-mCherry at 10 weeks. Intrinsic mCherry fluorescence is shown in red. Scale bar, 50 μm. (H) Summary of the percentage of colabeling with panC antibody in fluorescence marker-expressing VSNs. Four 10-week-old male mice per genotype were analyzed for each cross.
Six WT mice and six homozygous (MUT) mice were analyzed for each strain. Differential expression between WT and MUT mice was evaluated by the false discovery rates (FDR) based on the P-value of the Student’s t-test for each gene. If FDR is < 0.05, a gene was considered differentially expressed (DE). Counts for Vmn2r1 were at background level in MUT mice of the ΔC1 and ΔC1-GFP strains, confirming that these gene-targeted mutations are null mutations.

Figure 6A plots the log2 of the fold change (FC) of the mean count in MUT mice over the mean count in WT mice for the 57 V2R genes, arranged in order of gene families from C, A1–10, B, to D. Comparison of the results from ΔC1 and ΔC1-GFP strains revealed that there were no significant differences between the two strains: Gene expression profiles of V2R genes were very similar between the strains, as shown by close log2 FC values and overlap of the 99% confidence intervals (CI) for each gene. Only a few genes showed a discrepancy: Vmn2r94 and Vmn2r104 are non-DE genes in the data from the ΔC1 strain, whereas these genes were markedly decreased in the data from the ΔC1-GFP strain. An explanation is that for both Vmn2r94 and Vmn2r104, counts in the corresponding WT samples were substantially lower in the ΔC1 strain than in the ΔC1-GFP strain, resulting in differences of log2 FC values between the strains. The differences in counts in WT samples for Vmn2r94 and Vmn2r104 may reflect the mixed genetic background of the ΔC1 and ΔC1-GFP strains.

Family-ABD V2Rs are classified into two groups according to the colabeling patterns with antibodies against family-C V2Rs (Silvotti et al., 2011). V2Rs of family-A1 to A6 are preferentially colabeled with anti-C2 antibody, and appear to coexpress Vmn2r2 through Vmn2r7 (C2 type of V2Rs). V2Rs of family-A8, A9, A10, B, and D are preferentially colabeled with anti-C1 antibody and coexpress Vmn2r1 (C1 type of V2Rs). Of the 26 C1 type of V2Rs analyzed with CodeSet Pao (the right side of the diagram in Fig. 6A), 24 showed a significant decrease in MUT mice of the ΔC1 and ΔC1-GFP strains; of these 24, 19 were DE in both strains, and the remaining 5 were DE in one but not the other strain (Fig. 6A). In sharp contrast, there was no difference in expression of 25 of the 27 C2 type of V2R genes analyzed with CodeSet Pao (the left side of the diagram in Fig. 6A). This analysis is consistent with the differential effects of the Vmn2r1 mutations on the various subpopulations of VSNs that express C1 type of V2Rs. We identified two exceptions: Vmn2r65 of family-A5 and Vmn2r120 of family-A6 were significantly decreased in MUT mice. Vmn2r5 of family-C is the only gene that was increased in MUT mice in both strains.

The NanoString results are highly consistent with the cell counts on VNO sections (Fig. 6B, data from 10-week-old mice were used for the comparison). Vmn2r81/V2rf2 gave lower NanoString counts in ΔC1 and ΔC1-GFP mice, but the FC values were not significantly different; interestingly, the log2 FC values in NanoString were close to the log2 values of the ratio of the number of V2rf2-GFP+ VSNs in MUT over WT mice. Vmn2r83/V2rf4 showed a significant decrease in NanoString analysis, and to the same extent as the decrease of the number of V2rf4-Venus+ VSNs in cell counts. There was no difference in Vmn2r116 expression or in the number of V2Rp5+ VSNs.

CodeSet Pao includes probes for the nine non-classical major histocompatibility H2-Mv genes that are expressed in V2R+ VSNs (Ishii et al., 2003; Loconto et al., 2003; Ishii & Mombaerts, 2008; Leinders-Zufall et al., 2014). We found that H2-M1 and H2-M9 counts were decreased significantly in ΔC1 and ΔC1-GFP mice; H2-M9 was DE in both strains, H2-M1 was DE in one strain (Fig. 6C). The other H2-Mv genes were not affected. The decrease in H2-Mv gene expression level is consistent with the preferential expression of H2-M10 genes in VSNs colabeled with anti-C2 antibody (Silvotti et al., 2007), the coexpression of H2-M9 with V2rf1-3 genes (Ishii et al., 2003; Ishii & Mombaerts, 2008), and the decreased number of V2rf2-GFP+ VSNs and V2rf1-mCherry+ VSNs.

Reclassification of Vmn2r65 and Vmn2r120 as C1 type of V2R genes

Studies with family-specific antibodies have classified families A5 and A6 as C2 type of V2Rs (Silvotti et al., 2007, 2011). Our
NanoString results, however, indicate that counts for \textit{Vmn2r65} (family A5) and \textit{Vmn2r120} (family A6) were decreased significantly in ΔC1 and ΔC1-GFP mice. To follow up on these unexpected NanoString results, we performed ISH analyses using gene-specific probes for \textit{Vmn2r65} and \textit{Vmn2r120}, and counted the numbers of labeled VSNs on VNO sections from 10-week-old mice of the ΔC1 strain (Fig. 7A,B). In ΔC1-GFP mice, the number of \textit{Vmn2r65}+ VSNs was decreased to 5.7% of WT (ΔC1-GFP, 87 ± 8 compared to WT, 1515 ± 108; \(n = 4\) per genotype; Mann–Whitney test, \(P = 0.0294\)), and the number of \textit{Vmn2r120}+ VSNs was decreased to 37.0% of WT (ΔC1-GFP, 615 ± 82 compared to WT, 1662 ± 153; \(n = 4\) per genotype; \(P = 0.0284\)). As a control experiment, we tested \textit{Vmn2r118} (family A2) and \textit{Vmn2r76} (family A5). These are non-DE genes in NanoString analysis, and they also showed no changes in ISH cell counts in ΔC1 and ΔC1-GFP mice: \textit{Vmn2r118} in ΔC1-GFP, 816 ± 47 compared to WT, 744 ± 27 (\(n = 4\) per genotype; \(P = 0.2454\)), and \textit{Vmn2r76} in ΔC1-GFP, 1545 ± 129 compared to WT, 1650 ± 108 (\(n = 4\) per genotype; \(P = 0.6857\)). A comparison of NanoString and ISH results validates the concordance in the differences for these four \textit{V2R} genes (Fig. 7C).

Finally, we examined the coexpression patterns by ISH using gene-specific probes in combination with IHC using anti-C1 and anti-C2 antibodies, in three 10-week-old WT C57BL/6J mice (Fig. 7D–G). We found that the majority of \textit{Vmn2r65}+ VSNs...
family A5) were colabeled with anti-C1 antibody (438 of 543, 81%), and none were colabeled with the anti-C2 antibody (0 of 513, 0%). In contrast, a large fraction of Vmn2r76+ VSNs (also family A5) was colabeled with anti-C2 antibody (301 of 453, 66%), and a few were colabeled with anti-C1 antibody (3 of 492, 1%). The NanoString CodeSet Pao contains probes for 12 of the 15 family-A5 V2R genes, and of these 12, only Vmn2r65 was affected by the Vmn2r1 mutation. This phenotype is readily explained by the coexpression patterns: family-A5 V2Rs are colabeled with anti-C2 antibody, but Vmn2r65 is an exception. As Vmn2r120 is the sole gene in family-A6, the ISH probe is likely to be specific to Vmn2r120. We found that the majority of Vmn2r120+ VSNs were colabeled with anti-C1 antibody (305 of 528, 74.8%), and few Vmn2r120+ VSNs were colabeled with anti-C2 antibody (5 of 549, 1%).

Taken together, the histological analyses validate the apparent exceptions in the NanoString results, and lead us to reclassify Vmn2r65 and Vmn2r120 firmly as C1 type of V2Rs, in discordance with their phylogenetic classification as C2 type of V2Rs.

Discussion

Sequential and dependent model of coordinated coexpression of V2R genes

We have here described mouse strains with a knockout mutation in the Vmn2r1 gene, twenty years after mouse V2R genes were reported (Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). The anti-C1 and anti-C2 antibodies (Silvotti et al., 2007, 2011) were critical reagents for our study, as gene-specific ISH probes for family-C genes have proved difficult to design.

A first insight into the regulatory mechanisms of the coordinated expression of a single family-ABD gene with one or more family-C genes came from our analysis of a mouse strain with a gene-targeted deletion of the coding sequence of V2rf2 (Ishii & Mombaerts, 2011). We found that ~25% of VSNs that express the mutant V2rf2 allele coexpress another family-ABD gene, consistent with the absence of a hypothetical negative feedback from the mutant V2rf2 allele. Interestingly, 9.5% of VSNs expressing the mutant V2rf2 allele were colabeled with anti-C2 antibody, vs. 0% of V2rf2-GFP-
VSNs. We have proposed a sequential and dependent model for the coordinated expression of two V2R genes: a family-ABD gene is expressed first during differentiation of a VSN, and then the appropriate family-C gene(s) is/are expressed next, in a dependent manner (Ishii & Mombaerts, 2011). The lack of compensatory expression of family-C genes in VSNs expressing C1 type of V2Rs in the absence of Vmn2r1 is consistent with our sequential and dependent model.

Differential and graded dependence of V2R+ VSN subpopulations on Vmn2r1

We analyzed the impact of a Vmn2r1 knockout mutation on various subpopulations of VSNs that express C1 type of V2Rs in three complementary ways: by cell counts in crosses of ΔC1 and ΔC1-GFP with gene-targeted strains carrying V2R mutations of the IRES-marker design, by cell counts using ISH with V2R gene-specific probes, and by RNA counts with the NanoString multiplex gene expression platform. The four available gene-targeted strains carrying and by RNA counts with the NanoString multiplex gene expression platform. The four available gene-targeted strains carrying V2R mutations of the IRES-marker design, by cell counts using ISH with V2R gene-specific probes, and by RNA counts with the NanoString multiplex gene expression platform. The four available gene-targeted strains carrying V2R mutations of the IRES-marker design, by cell counts using ISH with V2R gene-specific probes, and by RNA counts with the NanoString multiplex gene expression platform.

As not all VSNs of a subpopulation expressing a C1 type of V2R are colabeled with anti-C1 antibody in WT mice, and the percentage of colabeling varies among C1 type of V2Rs, it is not surprising that the reductions of C1 type of V2Rs in the ΔC1 or ΔC1-GFP background were not complete, and varied. But, there is no correlation between the percentage of colabeling in WT mice and the percentage of reduction in the ΔC1 or ΔC1-GFP background. Therefore, the simple explanation cannot be offered that there would be two subsets for each C1 type of V2R+ VSN subpopulation: a subset of VSNs that normally express Vmn2r1 and do not survive in the absence of Vmn2r1, and a subset of VSNs that never express Vmn2r1 and survive in the absence of Vmn2r1.

Possible functions of Vmn2r1

The observation of few and small glomeruli formed by axons of V2r1b+ VSNs in the absence of Vmn2r1 is consistent with the high reduction in cell number. A minimal number of olfactory sensory neurons expressing a given odorant receptor is required to maintain glomeruli in the olfactory bulb (Ebrahimi & Chess, 2000). Nonetheless, some axons of V2r1b+ VSNs reach the glomerular layer. Importantly, axons of V2r1b+ VSNs formed normal glomeruli in the appropriate region of the AOB, consistent with the lower reduction in cell number compared to V2r1b+ VSNs. These findings exclude a major biological function for Vmn2r1 in axon guidance from the VNO to the AOB, and in the coalescence of axons into glomeruli.

Results from a heterologous HEK293 expression system suggest that Vmn2r1 is a calcium-dependent, low-sensitivity receptor for isoleucine, leucine, and valine (DeMaria et al., 2013). If these responses can be extended in vivo or ex vivo to VSNs of WT mice and can be shown to be reduced or abolished in the ΔC1 or ΔC1-GFP background, the reduced cell number could be interpreted in terms of reduced VSN survival due to absent or aberrant physiological responses. Vmn2r1 may respond to its own ligands, such as amino acids, independently of the family-ABD receptor responding to, for instance, peptides (Leinders-Zufall et al., 2009, 2014). Vmn2r1 may promote surface expression of a family-ABD receptor responding to native VSNs, as suggested by studies in the heterologous HEK293 system (DeMaria et al., 2013).

Reclassification of Vmn2r65 and Vmn2r120 as C1 type of V2R genes

An unexpected observation in the NanoString analysis was that Vmn2r65 and Vmn2r120 were DE, although they were thought to
be C2 type of V2Rs based on their phylogenetic classification in families A5 and A6, respectively. This analysis turned out to have predictive power. We found that the numbers of VSNs expressing \( \text{Vmn}2\text{r}65 \) and \( \text{Vmn}2\text{r}120 \) were reduced in \( \Delta C1\)-GFP mice, and to the same extent as the reduction in NanoString counts. We then showed that VSNs expressing \( \text{Vmn}2\text{r}65 \) and \( \text{Vmn}2\text{r}120 \) were colabeled preferentially with anti-C1 antibody but rarely with anti-C2 antibody in C57BL/6J mice. We have thus reclassified \( \text{Vmn}2\text{r}65 \) and \( \text{Vmn}2\text{r}120 \) firmly as C1 type of V2Rs. Application of RNA-seq may result in the reclassification of more V2Rs.

**Conclusion**

Subpopulations of VSNs expressing C2 type of V2Rs are not upregulated in the absence of \( \text{Vmn}2\text{r}1 \); they do not appear to fill the void created by the decrease in VSN subpopulations expressing C1 type of V2Rs. There thus appears to be a fixed probability of gene choice for a given C2 type of V2R, resulting in subpopulations of C2 type of V2R+ VSNs that are tightly regulated in terms of cell numbers.

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\text{tauVenus} \) were generated by T.I. in the laboratory of P.M. at The Rockefeller University, New York, NY.

**Conflict of interest**

The authors have no financial or other relationships to report that might lead to a conflict of interest.

**Data accessibility**

Original data are available upon request from the corresponding author. The NanoString probe sequences and data have been deposited in the Gene Expression Omnibus (NCBI) and are accessible by GEO accession number GSE104703.

**Author contributions**

S.A. and P.M. designed research, reviewed data, and wrote the manuscript. S.A., T.I., and Z.B. performed research.

**Abbreviations**

AOB, accessory olfactory bulb; CI, confidence intervals; DE, differentially expressed; DIG, digoxigenin; ES, embryonic stem; FC, fold change; FDR, false discovery rate; IHC, immunohistochemistry; IRES, internal ribosome entry site; ISH, in situ hybridization; NGS, normal goat serum; PBS, phosphate-buffered saline; VNO, vomeronasal organ; VSNs, vomeronasal sensory neurons.

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