# Calreticulin chaperones regulate functional expression of vomeronasal type 2 pheromone receptors

Sandeepa Dey[a,b,1] and Hiroaki Matsunami[a,c,d,2]

Departments of [a]Molecular Genetics and Microbiology, [b]Biochemistry, and [c]Neurobiology, and [d]Duke Institute for Brain Sciences, Duke University Medical Center, Durham, NC 27710

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved June 1, 2011 (received for review December 16, 2010)

**A variety of social behaviors like intermale aggression, fear, and mating rituals are important for sustenance of a species. In mice, these behaviors have been implicated to be mediated by peptide pheromones that are sensed by a class of G protein-coupled receptors, vomeronasal receptor type 2 (V2Rs), expressed in the pheromone detecting vomeronasal organ. Matching V2Rs with their cognate ligands is required to learn what receptors the biologically relevant pheromones are acting on. However, this feat has been greatly limited by the unavailability of appropriate heterologous tools commonly used to study ligand receptor specificity, because this family of receptors fails to traffic to the surface of heterologous cells. Here we show that calreticulin, a housekeeping chaperone commonly expressed in most eukaryotic cells, is sparsely expressed in the vomeronasal sensory neurons (VSNs). Correspondingly, knockdown of calreticulin in commonly available cell lines enables V2Rs to efficiently target to the cell membrane. Using this knowledge, we have now been able to successfully surface express receptors and functionally identify cognate ligands. Additionally, calreticulin4, a homolog of calreticulin shows restricted and enriched expression in the VSNs. Interestingly, in heterologous cells, calreticulin4 does not inhibit surface expression of V2Rs and can in part carry out functions of calreticulin. On the basis of our data, we postulate that V2Rs may use a unique trafficking mechanism whereby an important and more commonly expressed chaperone is deleterious for membrane export and is replaced by a functionally equivalent homolog that does not inhibit export while carrying out its functions.**

olfactory | olfaction | endoplasmic reticulum | pseudogene

Vertebrates detect many biologically relevant chemosignals through the accessory olfactory organ, vomeronasal organ (VNO). The vomeronasal sensory neurons (VSNs) express three unrelated families of G protein-coupled receptors (GPCRs), vomeronasal receptor type 1 (V1Rs), vomeronasal receptor type 2 (V2Rs), and formyl peptide receptors in molecularly and spatially distinct regions (1–6). V2Rs have been implicated in detecting urinary peptides that elicit intermale aggression, lachrymal gland secretions that promote female responsiveness to mating, and MHC peptides that may encode individuality (7–10). To understand how pheromones are detected by the vomeronasal receptors, it is essential to know their ligand specificity. However, only two V2Rs were matched with ligands and verified using transgenic mice: VSNs that express V2Rp5 are activated by exocrine gland secreting peptide 1 (ESP1), and knocking out V2Rp5 gene abolishes VSN responses to ESP1, indicating that V2Rp5 functions as the ESP1 receptor (8); additionally, some VSNs activated by MHC peptides express Vmn2R26 (10). Identifying cognate ligands for the V2Rs in vitro has been challenging, partly because they are poorly localized to the surface of heterologous cells. Here we demonstrate a unique export mechanism for V2Rs in VSNs: calreticulin, a cellular chaperone vital for survival of eukaryotic cells is detrimental for the surface targeting of V2Rs in heterologous lines. Correspondingly, its expression is down-regulated in V2R-expressing neurons in vivo. VSNs instead express calreticulin4, which does not inhibit V2R export and seems to perform the essential functions of calreticulin relevant for maintenance of the neuron.

## Results

***Calreticulin4* Is Pseudogenized in Humans but Intact in Mice.** Many genes expressed specifically in the mouse VNO, such as *V1Rs*, *V2Rs*, *H2-Mv*, and *TRPC2* genes, are pseudogenes or absent in the human genome (11). We hypothesized that a VSN chaperone would also share this characteristic. We searched the literature for human lineage-specific pseudogenes with functional orthologs in mice and found 34 genes that were intact in mice but were pseudogenized in humans (12). We tested for VNO-specific expression of these genes using RT-PCR and found that a calreticulin homolog, *calreticulin4*, was highly expressed in the VNO (Fig. 1*A*); expressed sequence tag data mining also supports the idea that calreticulin4 expression is limited.

Additional database searches retrieved *calreticulin4* gene sequences from 10 vertebrates (Fig. 1*B*). In each genome, *calreticulin4* is in a syntenic region flanked by the loci encoding oxysterol binding protein-like 9 and epidermal growth factor receptor pathway substrate 15 on the 5′ and 3′ sides, respectively. Like humans, *calreticulin4* gene is also pseudogenized in chimpanzee and marmoset (*SI Text, Calreticulin4 Alignment*). Data mining of a draft genome sequence suggests that orangutan, rhesus macaque, dog, and cow *calreticulin4* also seem to be inactivated. Inactivating mutations are interspersed in the ORF. Interestingly, although no intact *V2Rs* have been found in these species (13), rat, mouse, and opossum have a functional VNO expressing a large repertoire of *V2Rs* and seem to have a functional calreticulin4 encoding sequence spanning nine exons as well. Functional *calreticulin4* therefore seems to exist in genomes of animals that have functional *V2Rs*. These data raise the possibility that calreticulin4 has a role in the VNO that may be related to V2R function.

**Calreticulin4 Is a VNO-Specific Chaperone Homolog.** Calreticulin4 is 67% identical to calreticulin (Fig. 1*C*), a lectin-like, calcium-binding endoplasmic reticulum (ER) resident chaperone found in a diverse range of species and eukaryotic cell types (14, 15). Calreticulin, calnexin, and ERp57 constitute the calreticulin/calnexin cycle, which functions in the quality control of transmembrane and secreted glycoproteins that are translocated into

Author contributions: S.D. and H.M. designed research, performed research, contributed new reagents/analytic tools, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. JN255799).

[1]Present address: Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037.

[2]To whom correspondence should be addressed. E-mail: matsu004@mc.duke.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018140108/-/DCSupplemental.

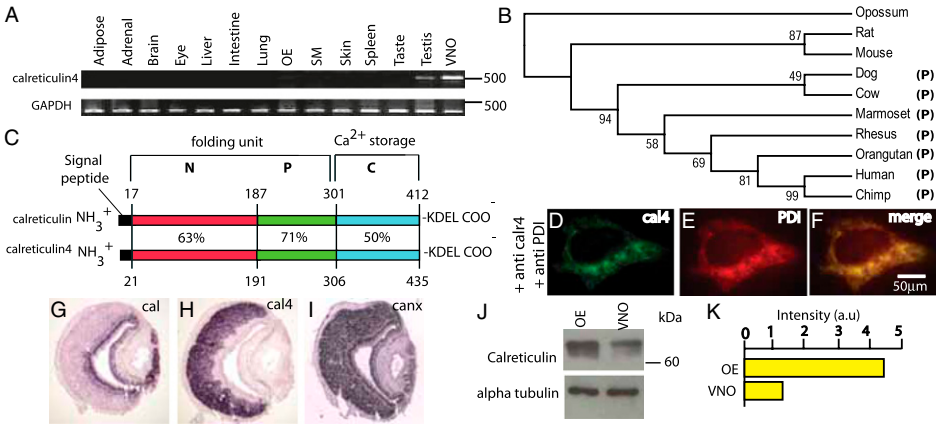


**Fig. 1.** Calreticulin4 is expressed in the VNO, whereas calreticulin expression is low in the VNO. (*A*) RT-PCR of calreticulin4 and GAPDH control from mouse tissues. SM, skeletal muscle. (*B*) Phylogenetic tree of calreticulin4 from different species. (P) denotes pseudogene. (*C*) Schematic representation of calreticulin and calreticulin4 domains and sequence similarity. (*D–F*) Intracellular staining for (*D*) calreticulin4, (*E*) PDI, and (*F*) merge in NIH 3T3 cells. (*G–I*) C57/BL6 adult VNO coronal section in situ hybridization for (*G*) calreticulin, (*H*) calreticulin4, and (*I*) calnexin. (*J*) Western blot carried out with anti-calreticulin (*Upper*) and anti-tubulin (*Lower*) antibodies on extracts from VNO and OE. (*K*) Quantification of calreticulin band intensities from *J*, normalized to tubulin loading control. a.u., arbitrary units.

the ER, ensuring that only correctly folded proteins exit the ER (14–16).

To determine the cellular localization of calreticulin4, we compared its localization with protein disulfide isomerase (PDI), an ER resident protein: we found that calreticulin4 signals overlapped with PDI (Fig. 1 *D–F*), indicating localization of calreticulin4 in the ER, as would be expected for an ER chaperone. No cross-reactivity was found in secondary antibodies (Fig. S1 *A–C*).

We then examined the expression of calreticulin-related ER chaperone family members in the VNO by in situ hybridization (Fig. 1 *G–I*). We found calreticulin4 to be highly expressed in the VNO and undetectable in the main olfactory epithelium (Fig. S1). Intriguingly, we found that calreticulin shows only low levels of expression in the VSNs (Fig. 1*G*) but was more prominently expressed in the olfactory epithelium (OE) (Fig. S1 *D–L*), whereas calreticulin4 showed robust expression in the VSNs (Fig. 1*H*) specifically. Calnexin, known to form a complex with calreticulin, was expressed in both the VNO (Fig. 1*I*) and other tissues (Fig. S1 *H* and *I*). Quantification of calreticulin protein levels in VNO and OE extracts showed the protein to be threefold higher in the OE compared with VNO (Fig. 1 *J* and *K*).

**Calreticulin4 Suppresses Cellular Stress Caused by Calreticulin Depletion.** Depletion of calreticulin has been shown to induce the unfolded protein response (UPR), whereas restoration of calreticulin expression alleviates it (17, 18). UPR is a cellular response to ER stress due to the accumulation of excess unfolded/misfolded proteins. This triggers a signal cascade that restores homeostasis in the ER (19–21). Does calreticulin4 functionally compensate for calreticulin? To address this question we stably expressed a shRNA that targets calreticulin mRNA in HEK293T cells. Using a Western blot assay, we confirmed the reduced expression of calreticulin in one stable line, which we called R24 cells (Fig. 2*A*).

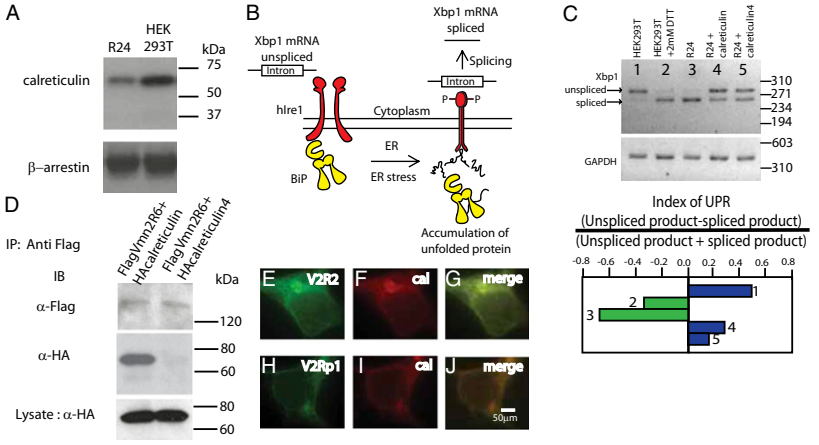


**Fig. 2.** Calreticulin vs. calreticulin4. (*A*) Western blot for calreticulin in R24 and HEK293T. (*B*) Schematic representation of the UPR. (*C*) *Top*: RT-PCR amplification of spliced or unspliced Xbp1 mRNA from HEK293T (lane 1), 2 mM DTT-treated HEK293T (lane 2), R24 (lane 3), R24 transfected with calreticulin for 24 h (lane 4), and R24 transfected with calreticulin4 (lane 5) for 24 h. *Middle*: Control GAPDH for RT-PCR. *Bottom*: Index of UPR in each lane as defined by formula. (*D*) Flag-directed immunoprecipitation (IP) from R24 lysate transfected with Flag-tagged receptor (Vmn2R6) and HA-tagged calreticulin or calreticulin4. *Top*: Anti-flag immunoblotting (IB) for precipitated receptor. *Middle*: Anti-HA IB for coprecipitated calreticulin or calreticulin4. *Bottom*: Anti-HA IB for calreticulin/calreticulin4 in cell lysate. (*E–G*) Intracellular staining in HEK293T cells for (*E*) V2R2, (*F*) calreticulin, and (*G*) merge. (*H–J*) Intracellular staining in HEK293T cells for (*H*) V2Rp1, (*I*) calreticulin, and (*J*) merge.

To investigate the consequences of depleting calreticulin we assayed UPR by ER stress (Fig. 2*B*). ER stress-induced unfolded proteins activate Ire1, which acts on transcription factor Xbp1 pre-mRNA to generate the mature *Xbp1* mRNA. This can then be produced as a protein and can trigger the transcription of genes required to alleviate ER stress (22, 23). We assayed for the amplification of unspliced and/or spliced Xbp1 mRNA using primers flanking the intron region and then segregated the two products, which were predicted to differ by 23 bases (Fig. 2*C*). The unspliced product was the predominant transcript (Fig. 2*C*, lane 1) in HEK293T cells, suggesting no detectable UPR. Addition of 2 mM DTT, known to break protein disulfide bridges, resulted in UPR and the generation of the spliced product predominantly (Fig. 2*C*, lane 2). Similar analysis of R24 cells, with depleted calreticulin, showed the spliced product indicative of the UPR (Fig. 2*C*, lane 3). Transfection of either calreticulin or calreticulin4 in R24 cells independently showed reduced accumulation of the spliced product and appearance of the unspliced form, indicating partial rescue of ER stress (Fig. 2*C*, lanes 4 and 5). These data suggest that calreticulin4 shares, at least in part, the function of calreticulin in R24 cells. These results are consistent with the idea that calreticulin4 may serve as a functional equivalent of calreticulin in the VSNs to regulate protein folding.

**V2Rs Interact Differentially with Calreticulin and Calreticulin4.** Why is calreticulin expressed at reduced levels in the VNO? Because calreticulin is known to interact with transmembrane proteins in the ER, we hypothesized that calreticulin and calreticulin4 might show different affinities with the V2Rs. To test this, we compared interaction of V2Rs with calreticulin and calreticulin4 by coimmunoprecipitation assays. A Flag-tagged V2R, Vmn2R6, was independently coexpressed with HA-tagged calreticulin or calreticulin4 in HEK293T cells; we immunoprecipitated the V2R and assayed for bound calreticulin or calreticulin4. We found that the V2R coprecipitated calreticulin, whereas calreticulin4 was not efficiently coprecipitated (Fig. 2*D*, *Middle*). Lysates showed comparable amounts of calreticulin and calreticulin4 expression (Fig. 2*D*, *Bottom*). Our data indicate that the V2Rs form more stable complexes with calreticulin than calreticulin4 in the ER.

**Depletion of Calreticulin Induces Family C V2Rs Cell Surface Expression.** V2Rs are retained in the ER when expressed in HEK293T cells. This is demonstrated by colocalization of V2Rs, N-terminally tagged with 20 amino acids of rhodopsin (rho-tag), with calreticulin (Fig. 2 *E–J*).

We initially examined the effects of calreticulin4 on the surface expression of the rho-tagged V2Rs in HEK293T cells, including V2R2 (Vmn2R1), which belongs to family C and V2Rp1 (Vmn2R112), which belongs to family A as representative V2Rs. However, we did not observe any cell surface expression of the V2Rs using live-cell immunostaining (Fig. S1 *M–P*), suggesting that calreticulin4 is not the factor that enables transport to the membrane for the V2Rs.

Because calreticulin is known to regulate protein trafficking and is not well expressed in the VSNs, we hypothesized that calreticulin itself may actually interfere with the export of V2Rs. We tested this hypothesis in the calreticulin-depleted cell line R24. Using this cell line, we first assessed the surface expression of the members of the family C V2Rs by comparing the surface expression of rho-tagged V2R2 and Vmn2R6 in R24 and HEK293T. Although Silvotti et al. (24) previously reported that V2R2 could be expressed without requiring special escort proteins in insect cells, V2R2 and Vmn2R6 were not efficiently trafficked in HEK293T cells when expressed alone. However, these receptors were robustly expressed on the surface of the R24 cells (Fig. 3 *A–D* and Fig. S2 *A–D*) compared with HEK 293T cells (4.2-fold and 4.8-fold surface labeling for Vmn2r1 and Vmn2R6, respectively; Fig. S2 *I* and *J*). Both cell lines showed comparable immunofluorescent signals when cells were permeabilized in staining, indicating no obvious differences in protein production or stability (Fig. 3 *A′–D′*).

To examine whether calreticulin expression levels in individual cells inversely correlate with the cell surface expression of the V2R2, we performed live-cell surface staining for rho-tagged V2R2, followed by permeabilized staining for calreticulin. Although R24 is a clonal cell line, significant differences in calreticulin expression in individual cells were observed. Importantly, V2R2 was only found on the surface of cells with low levels of calreticulin (Fig. 3 *E–G*); for single cells chosen over different fields, a plot of mean fluorescence intensity of surface V2R2 staining vs. mean fluorescence intensity of internal calreticulin staining showed an inverse correlation ($R^2$ = 0.6383), further supporting our hypothesis that the reduction of calreticulin expression promotes cell surface expression of the V2Rs (Fig. 3*H*).

**Depletion of Calreticulin and Coexpression of MHC Class 1b Induces Family A V2R Cell Surface Expression.** We next tested family A V2R members but found that the V2Rs remained poorly expressed at the cell surface when expressed alone in HEK293T cells and R24 cells. Some V2Rs form complexes with the H2-Mv family members of nonclassic MHC class 1b molecules and β2-microglobulin (β2m) (25–27). In addition, H2-Mv members and β2m promote the cell surface expression of EC2-V2R in a spermatogonia cell line (27). We therefore examined the H2-Mv members (H2M1, H2M9, H2M10.1, H2M10.3, H2M10.4, H2M10.5, and H2M10.6) with V2Rp1 and β2m to test the effect of the H2-Mv proteins on the surface export of V2R family A members in R24 cells and HEK293T cells. We stained cells fluorescently, normalized to GFP expression, and found that coexpression of H2M10.4 and β2m in R24 cells resulted in efficient surface trafficking of rho-tagged V2Rp1, whereas other H2-Mv family members had little or no effect in promoting the cell surface expression of V2Rp1 (Fig. 3*I* and Fig. S2 *K–Q*). Coexpression of unrelated control proteins $G_{\alpha i2}$ and RTP1S, known to promote the cell surface expression of odorant receptors (ORs) (28, 29), also had no effect on V2Rp1 surface expression (Fig. 3*I* and Fig. S2 *R* and *S*). To negate field bias, we carried out FACS and compared surface expression of V2Rp1 in large numbers of cells again coexpressing V2Rp1 and each of the H2-Mv members and controls. To accurately account for positively stained cells, we gated the histogram of a no-receptor control sample at a fluorescence intensity level that excluded >99% of the cells (Fig. 3*J*, *Inset*, gating indicated by dashed line). For each of the experimental runs this cutoff intensity was kept fixed, and all cells showing more fluorescence than this gated value were accounted as positively surface stained. We confirmed that H2-M10.4 was indeed the most efficient protein in trafficking V2Rp1 (Fig. 3*J*) in R24. A member of the V2Rp family was shown to be partially coexpressed with H2M10.4 in the VNO (30). Following the previous methodology, we tested the cell surface expression of a variety of rho-tagged V2Rs, including V2Rp2 (Vmn2R111), VR1 (Vmn2R123), VR4 (Vmn2R42), VR14 (Vmn2R37), and EC1-V2R (Vmn2R81). When these V2Rs were coexpressed with H2M10.4 and β2m, cell surface labeling averaged for all receptors (Fig. 3*K*) as well as individual ones (Fig. S2 *U–Z*) was enhanced fourfold in R24 cells compared with HEK293T cells. In contrast, when RTP1S was used instead of H2M10.4, no cell surface expression of the V2Rs was detected (Fig. S3). These results suggested that the depletion of calreticulin allowed V2Rs or V2R+H2-Mv complexes to be exported to the cell surface in heterologous cells.

**Restoration of Calreticulin Expression Represses V2R Surface Expression, Whereas Coexpression of Calreticulin4 Has No Detrimental Effects.** To confirm that reducing calreticulin expression directly enhances cell surface expression of the V2Rs in R24 cells, we examined the surface trafficking of V2R2 and V2Rp1 when ex-

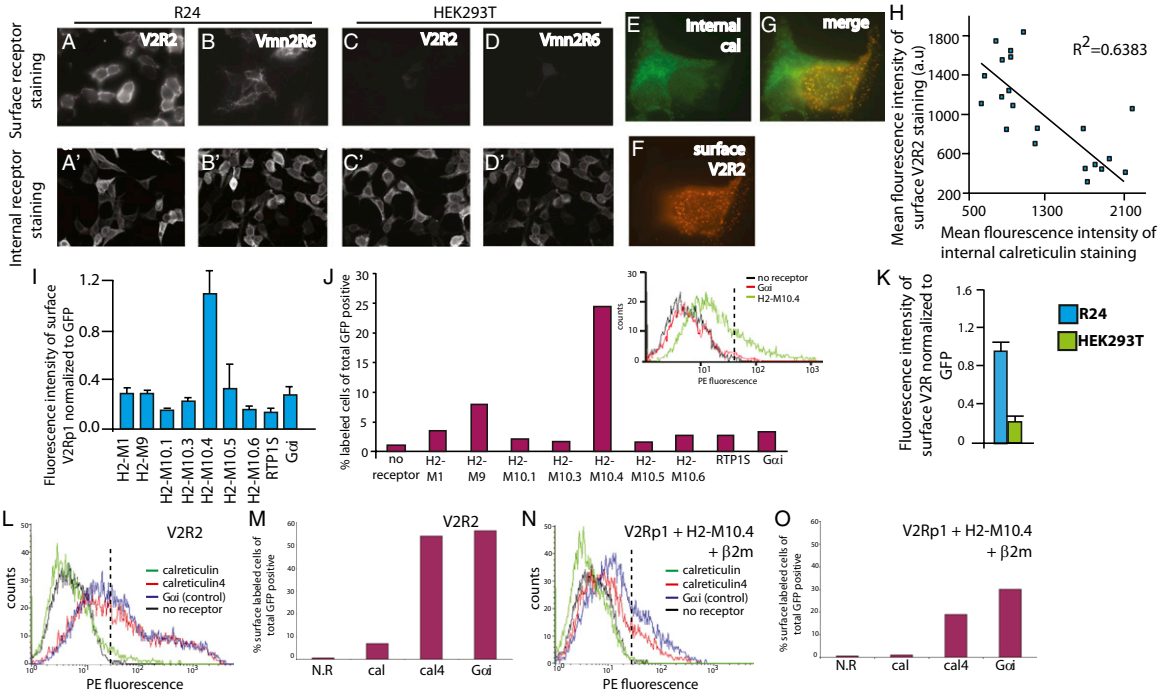


**Fig. 3.** Depleting calreticulin expression induces cell surface export of the V2Rs. (*A–D*) Surface staining for (*A*) V2R2 and (*B*) Vmn2R6 in R24 and (*C*) V2R2 and (*D*) Vmn2R6 in HEK293T. (*A′–D′*) Internal staining for (*A′*) V2R2 and (*B′*) Vmn2R6 in R24 and (*C′*) V2R2 and (*D′*) Vmn2R6 in HEK293T. (*E–G*) R24 cells. (*E*) Intracellular staining for calreticulin (green), (*F*) cell surface staining for V2R2 (red), and (*G*) merge. (*H*) Plot of mean fluorescence intensity of surface V2R2 staining vs. internal calreticulin staining per cell in 20 random cells from a population of doubly stained R24 cells ($R^2$ = 0.67), each point in the plot represents a cell. (*I*) Mean fluorescence intensities of live cell staining normalized to total GFP signals in the same field for surface expression of V2Rp1 cotransfected with indicated constructs in R24 cells. (*J*) Percentage surface stained of R24 cells expressing V2Rp1 in presence of cotransfections indicated, as quantified by FACS. FACS histogram overlay for surface staining of R24 cells expressing V2Rp1 cotransfected with Gαi (red) and H2-M10.4 (green) and no receptor control (black); *Inset*: dashed line indicates noise gating. (*K*) Averaged fluorescence intensities of surface staining in R24 or HEK293T for six V2Rs cotransfected individually with H2-M10.4+β2m, normalized to GFP. (*L*) FACS analysis of surface expression of V2R2 in presence of calreticulin (green), calreticulin4 (red), and Gαi (blue) and no-receptor control (black) in R24 cells; dashed line indicates noise gating. (*M*) Graphical representation of stained cells from FACS analysis of V2R2. (*N*) FACS analysis of surface expression of V2Rp1 in presence of calreticulin (green), calreticulin4 (red), and Gαi (blue) and no-receptor control (black) in R24 cells; dashed line indicates noise gating. (*O*) Graphical representation of stained cells from FACS analysis of V2Rp1+H2-M10.4+β2m.

ogenous calreticulin was expressed in R24 cells. To avoid the effect of the calreticulin shRNA being expressed in R24 cells, we introduced silent mutations corresponding to the shRNA target sequences within the calreticulin coding sequences. Both FACS analysis (Fig. 3 *L–O*) and live cell staining (Fig. S4 *A–F*) showed that restoration of calreticulin expression in R24 cells greatly reduced the surface expression of V2R2 and V2Rp1 compared with control protein $G_{\alpha i}$. Furthermore, expression of calreticulin4 in R24 cells had little or no effect in reducing cell surface expression of the V2Rs, suggesting that calreticulin plays a specific role in ER retention of V2Rs.

**Calreticulin Depletion Does Not Affect Export of Other GPCRs.** Does calreticulin depletion affect transport of other receptors to the cell membrane? The surface expression of a representative OR was similarly induced by RTP1S in HEK293T cells and R24 cells, whereas excluding the expression of RTP1S suppressed surface expression in both cell types (Fig. S4 *I–L*). The surface expression of V1Rs was not detected in either HEK293T or R24 cells, with or without RTP1S (Fig. S4 *M–P*). Among the GPCRs that show similarities to the V2Rs, mGluR4, a metabotropic glutamate receptor, showed comparable robust surface expression (Fig. S4 *Q* and *R*) in both cell lines. These results suggest that calreticulin depletion specifically enhances cell surface expression of V2Rs among the chemosensory and other GPCRs.

**Identification of ESP Family Members as V2R Ligands.** Last, we asked whether surface exported receptors in the calreticulin-depleted background are functionally responsive. The V2Rp family comprises seven predicted V2Rs. Kimoto and colleagues have shown that ESP1, a 7-kDa peptide secreted by the extraorbital lacrymal gland of male mice, induces c-fos expression in a subset of VSNs that express V2Rp5 (a receptor of the V2Rp subfamily) in female mice and induces lordosis behavior (8, 9). ESP1 is expressed in adult male mice, where ESP1 is a member of a gene family consisting of 38 related peptides, 15 of which have been shown to elicit electrical responses in the VNO (31). This raises the possibility that ESPs may activate one or more V2Rp family members, although one cannot exclude the possibility that other receptors coexpressed in the V2Rp-positive VSNs bind these. To address this, in an initial step we expressed ESP ligands in HEK293T cells as secreted proteins and tested for functional activity of the supernatant on R24 cells transfected with rho-tagged V2Rp1 together with H2-M10.4 and β2m, as well as $G_{\alpha 15}$, a Gq class G protein widely used in functional assays of GPCRs. Activation of $G_{\alpha 15}$, which couples to many but not all of the GPCRs, leads to a transient intracellular calcium release (29, 32, 33). Culture supernatant obtained from ESP6-expressing cells induced responses in the calcium imaging assays, whereas supernatant derived from ESP1-expressing cells did not (Fig. S5*A*), indicating that V2Rp1 may specifically respond to ESP6. We then endeavored to produce and purify large quantities of the ligands as bacterially expressed peptides as reported previously. We were able to produce five putative ligands, namely ESP3, ESP5, ESP6, ESP15, and ESP36, as soluble proteins and affinity-purified peptides. We transfected R24 cells with H2M10.4, β2m,

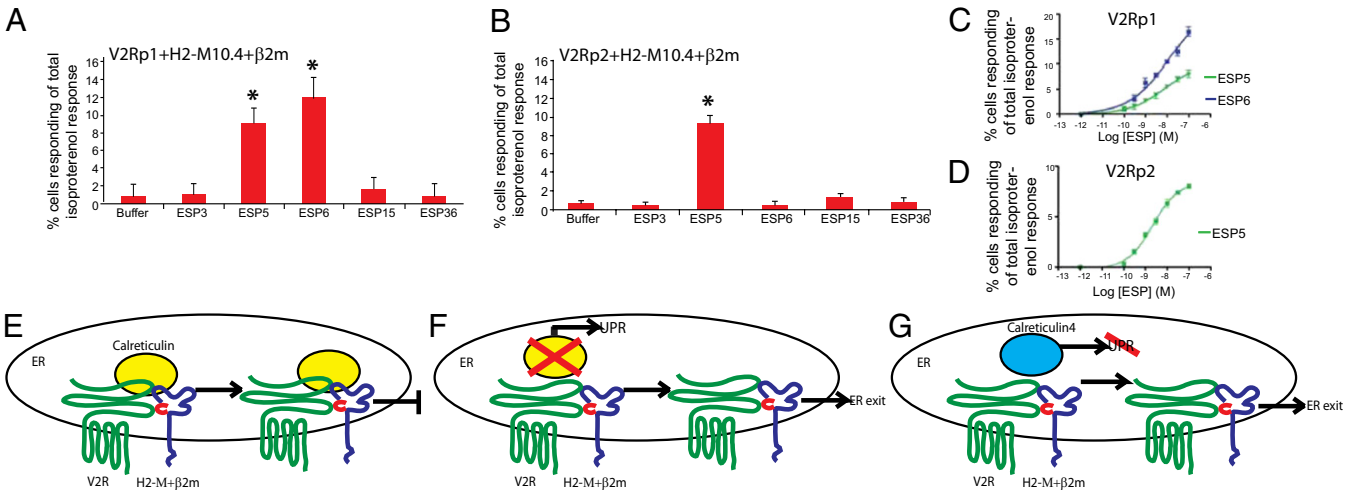


**Fig. 4.** V2Rp members are activated by ESPs. (*A* and *B*) Graphical representation of cells transfected with (*A*) V2Rp1+H2-M10.4+β2m or (*B*) V2Rp2+H2-M10.4 +β2m responding to buffer and ESP ligands as percentage of total isoproterenol response. Error bars represent SEM. *$P < 0.0001$. (*C* and *D*) Dose–response of R24 cells transfected with (*C*) V2Rp1or (*D*) V2Rp2+H2-M10.4+β2m with ESP5/6. (*E–G*) Model for interaction of calreticulin or calreticulin4 with V2Rs. (*E*) Calreticulin blocks ER exit. (*F*) In absence of calreticulin, V2Rs are able to exit ER and traffic to the cell surface, but there is onset of detrimental effects like the UPR. (*G*) In presence of calreticulin4, V2Rs can exit ER to reach the cell surface, and detrimental effects like the UPR are rescued.

$G_{\alpha 15}$, and one of six rho-tagged V2Rs (V2Rp1, V2Rp2, EC1-V2R, VR1, VR14, and VR4), sequentially applied the peptides at 100-nM concentration to transfected cells, and measured the calcium responses of these cells upon stimulation. Cells expressing V2Rp1 responded to ESP5 and ESP6 (Fig. 4*A*), whereas cells expressing V2Rp2 responded to ESP5 (Fig. 4*B*). Cells expressing either V2Rp1 or V2Rp2 did not show significant responses to ESP3, ESP15, or ESP36. We used 10 nM isoproterenol as a positive control to estimate efficiency of transfection and normalize ligand responses, because transfected $G_{\alpha 15}$ couples with the endogenous β2 adrenergic receptor on application of isoproterenol to produce a calcium influx in the cytoplasm. Dose–responses of V2Rp1 and V2Rp2 toward these compounds were measured, and these receptors were found to be activated by ESPs at nanomolar concentrations (Fig. 4 *C* and *D*). Cells transfected with the other V2Rs failed to show significant response to any of the tested peptides. Untagged V2Rp1 showed a similar response (Fig. S5*B*), suggesting that the N-terminal rho-tag does not affect its binding to the ligands; coexpression of untagged V2Rp1 with calreticulin greatly reduced or abolished the signals, and coexpression with calreticulin4 had little or no effect, consistent with the idea that calreticulin effects on inhibiting V2R trafficking do not depend on the tag (Fig. S5 *C* and *D*).

## Discussion

**Human Lineage-Specific Pseudogene Analysis Reveals a VNO-Specific Chaperone Homolog.** Recent expansions in mammalian genome sequence information enabled us to use an approach based on the screening of human lineage-specific pseudogenes to find functional mouse orthologs specifically expressed in the VNO, an organ that is functional in mouse but not in human. We find calreticulin4, a homolog of a widely expressed lectin chaperone calreticulin, to be specifically expressed in the VSNs. Comparative genome analysis revealed an intriguing correlation between intact *V2Rs* and *calreticulin4*, providing evolutionary evidence for the importance of the role of calreticulin4 in V2R function. In the future, more comprehensive genome analysis focusing on genes that are specifically absent or pseudogenized in species that lack a functional VNO might identify additional molecules important for VNO function.

**V2Rs Use a Unique Mechanism of Cell Surface Trafficking.** In contrast to most conventional GPCR trafficking mechanisms that require the addition of one or more factors, we demonstrate here that down-regulation of a widely expressed cellular chaperone, calreticulin, is essential for V2Rs to be effectively targeted to the cell membrane. The inhibitory effect of calreticulin seems to be specific to the V2Rs, because the surface expression of other GPCRs such as V1Rs and ORs are not significantly altered by calreticulin knockdown. The fact that an undisrupted *calreticulin4* ORF exists only in species that express a large repertoire of functional V2Rs points to the particular requirement of V2R export. Why are calreticulin4 and calreticulin present only in species that possess functional V2Rs? On the basis of our findings and previous reports, we postulate a model: calreticulin is an ER resident protein that blocks ER exit of V2Rs, potentially by stably binding to these receptors (Fig. 4*E*). The inability of V2Rs to exit the ER could be due to the inability of calreticulin to recognize the receptors as "correctly folded" units or simply due to a higher affinity in binding, although it is not clear whether the binding of calreticulin with the V2R directly blocks trafficking. Identification of calreticulin binding sites of V2Rs will clarify this point. Knockdown of calreticulin eliminates this barrier at the ER exit and allows the V2Rs to be trafficked to the cell surface. However, calreticulin has an important role in protein folding, and its removal triggers adverse consequences evidenced by the UPR (Fig. 4*F*). Calreticulin4 has ability function equivalent to calreticulin in assisting protein folding and suppressing the UPR but does not block the exit of V2Rs from the ER (Fig. 4*G*). One cannot discount the possibility that calreticulin4 is important for quality control or efficient maturation of the V2Rs in VSNs. Future experiments including analysis of calreticulin4 gene knockout animals will address whether calreticulin4 is required for normal VSN development, maintaining general physiology or function of VSNs and V2R trafficking.

Aberrant ER exit can cause diseases such as cystic fibrosis, which is caused by ER retention of a mutant form of cystic fibrosis transmembrane regulator (CFTR) chloride channel (34), which can otherwise function if allowed to traffic to the surface. Calreticulin was shown to promote ER retention of CFTR (35, 36), because depletion of calreticulin enhances CFTR to be trafficked to the plasma membrane. In the future it would be interesting to ask whether calreticulin4 could allow proper CFTR trafficking while suppressing the undesirable UPR. If so,

calreticulin depletion or inhibition in conjunction with calreticulin4 expression could be used for therapeutic purposes.

**Functional Expression of V2Rs Enables Identification of Cognate Ligands.** Finally, we used insights into the role of calreticulin family members in V2R trafficking to construct a heterologous system to study V2R–ligand matching. We have shown that V2Rs can be expressed on the cell surface and matched with potential ligands heterologously; we anticipate that our system will serve as a starting point for additional larger-scale studies of ligand–receptor interactions.

Matching the V2Rs to their cognate ligands and analyzing how different V2Rs are activated by different ligands are essential steps toward understanding how chemical information is encoded at the receptor level in the VNO. We have identified ESP5 and ESP6 as active ligands for V2Rp1 and ESP5 as an active ligand for V2Rp2 in the heterologous cell system. One cannot exclude the possibility that activation of the receptors in our system does not precisely reflect the VSN responses expressing the corresponding V2R, because receptor activation can differentially transduce signals to different G proteins (37). Nevertheless, our results raise the possibility that V2Rs may not necessarily follow strict labeled-cell coding, because V2Rp1 is activated by at least two ESPs, and ESP5 activates at least two V2Rs. Future identification of cognate ligands for additional V2Rs will reveal how peptide cues are coded for by V2Rs, as well as the role of V2R activation in the transmission of chemical information to the brain.

Although a given V2R-positive VSN expresses two types of V2Rs (one from family A/B and the other from family C), V2Rp receptors (family A) are sufficient to respond to ESP ligands in heterologous cells. We have not found family C receptors to enhance the responses in any significant way. Family C V2Rs might independently interact with unrelated ligands or may form heteromers with family A/B V2Rs for specific downstream signaling. Future studies are clearly required to reveal the role of family C V2Rs.

## Materials and Methods

For all VNO sections, adult C57BL/6J mice were used. Calreticulin knockdown cell line R24 was made by transfecting HEK293T with shRNA construct (Openbiosystems, V2HS15097). Ninety-six cell lines were screened for best expression of V2Rs, and R24 was selected. For detailed descriptions of the procedures used, see *SI Materials and Methods*. For detailed descriptions of the procedures used, see *SI Materials and Methods*.

**ACKNOWLEDGMENTS.** We thank A. Toyama, M. Kubota, W.L. Liu, and Q. Chi for their expert technical assistance; members of the Matsunami laboratory for discussions and critical reading of the manuscript; H. Amrein, R.J. Lefkowitz, R. Valdivia, F. Wang, H. Rockman, and D.A. Marchuk for generously sharing their equipment; M. Cook for help with FACS analysis; and L. Stowers, P. Cameron, and P. Sharma for critical discussions on the manuscript. This work was funded by the National Institutes of Health and the Human Frontier Science Program.

1. Ryba NJ, Tirindelli R (1997) A new multigene family of putative pheromone receptors. *Neuron* 19:371–379.
2. Matsunami H, Buck LB (1997) A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* 90:775–784.
3. Herrada G, Dulac C (1997) A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* 90: 763–773.
4. Dulac C, Axel R (1995) A novel family of genes encoding putative pheromone receptors in mammals. *Cell* 83:195–206.
5. Liberles SD, et al. (2009) Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. *Proc Natl Acad Sci USA* 106:9842–9847.
6. Rivière S, Challet L, Fluegge D, Spehr M, Rodriguez I (2009) Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. *Nature* 459:574–577.
7. Chamero P, et al. (2007) Identification of protein pheromones that promote aggressive behaviour. *Nature* 450:899–902.
8. Haga S, et al. (2010) The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature* 466:118–122.
9. Kimoto H, Haga S, Sato K, Touhara K (2005) Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* 437:898–901.
10. Leinders-Zufall T, Ishii T, Mombaerts P, Zufall F, Boehm T (2009) Structural requirements for the activation of vomeronasal sensory neurons by MHC peptides. *Nat Neurosci* 12:1551–1558.
11. Liman ER (2006) Use it or lose it: Molecular evolution of sensory signaling in primates. *Pflugers Arch* 453:125–131.
12. Consortium IHGS; International Human Genome Sequencing Consortium Finishing the euchromatic sequence of the human genome. *Nature* 431:931–945.
13. Young JM, Trask BJ (2007) V2R gene families degenerated in primates, dog and cow, but expanded in opossum. *Trends Genet* 23:212–215.
14. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M (1999) Calreticulin: One protein, one gene, many functions. *Biochem J* 344:281–292.
15. Michalak M, Groenendyk J, Szabo E, Gold LI, Opas M (2009) Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem J* 417: 651–666.
16. Michalak M, Robert Parker JM, Opas M (2002) Ca2+ signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium* 32:269–278.
17. Knee R, Ahsan I, Mesaeli N, Kaufman RJ, Michalak M (2003) Compromised calnexin function in calreticulin-deficient cells. *Biochem Biophys Res Commun* 304:661–666.
18. Christensen A, et al. (2008) Functional characterization of *Arabidopsis* calreticulin1a: A key alleviator of endoplasmic reticulum stress. *Plant Cell Physiol* 49:912–924.
19. Meusser B, Hirsch C, Jarosch E, Sommer T (2005) ERAD: The long road to destruction. *Nat Cell Biol* 7:766–772.
20. Schröder M, Kaufman RJ (2005) The mammalian unfolded protein response. *Annu Rev Biochem* 74:739–789.
21. Ma Y, Hendershot LM (2001) The unfolding tale of the unfolded protein response. *Cell* 107:827–830.
22. Calfon M, et al. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415:92–96.
23. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107:881–891.
24. Silvotti L, Giannini G, Tirindelli R (2005) The vomeronasal receptor V2R2 does not require escort molecules for expression in heterologous systems. *Chem Senses* 30:1–8.
25. Ishii T, Hirota J, Mombaerts P (2003) Combinatorial coexpression of neural and immune multigene families in mouse vomeronasal sensory neurons. *Curr Biol* 13: 394–400.
26. Ishii T, Mombaerts P (2008) Expression of nonclassical class I major histocompatibility genes defines a tripartite organization of the mouse vomeronasal system. *J Neurosci* 28:2332–2341.
27. Loconto J, et al. (2003) Functional expression of murine V2R pheromone receptors involves selective association with the M10 and M1 families of MHC class Ib molecules. *Cell* 112:607–618.
28. Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H (2004) RTP family members induce functional expression of mammalian odorant receptors. *Cell* 119:679–691.
29. Zhuang H, Matsunami H (2007) Synergism of accessory factors in functional expression of mammalian odorant receptors. *J Biol Chem* 282:15284–15293.
30. Haga S, Kimoto H, Touhara K (2007) Molecular charcterizatoin of vomeronasal sensory neurons resopnding to a male-specific peptide in tear fluid: Sexual communication in mice. *Pure Appl Chem* 79:775–783.
31. Kimoto H, et al. (2007) Sex- and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Curr Biol* 17:1879–1884.
32. Offermanns S, Simon MI (1995) G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. *J Biol Chem* 270:15175–15180.
33. Kostenis E, Waelbroeck M, Milligan G (2005) Techniques: Promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol Sci* 26:595–602.
34. Cheng SH, et al. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 63:827–834.
35. Harada K, et al. (2006) Calreticulin negatively regulates the cell surface expression of cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 281:12841–12848.
36. Harada K, et al. (2007) Curcumin enhances cystic fibrosis transmembrane regulator expression by down-regulating calreticulin. *Biochem Biophys Res Commun* 353: 351–356.
37. Shirokova E, et al. (2005) Identification of specific ligands for orphan olfactory receptors. G protein-dependent agonism and antagonism of odorants. *J Biol Chem* 280: 11807–11815.