

Gene cluster lock after pheromone receptor gene choice

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In mammals, perception of pheromones is based on the expression in each vomeronasal sensory neuron of a limited set of receptor genes, chosen among a large repertoire. Here, we report an extremely tight control of the monogenic and monoallelic transcription of the *V1rb2* receptor gene. Combining genetic and electrophysiological approaches, we show that the transcription of a non-functional V1r allele leads to the coexpression of another, functional V1r gene. The choice of this coexpressed gene surprisingly includes genes located on the cluster homologous to the one from which the mutant allele is transcribed. However, V1r genes located in *cis* relative to the transcribed mutant allele are excluded from the coexpression choice. Our observations strongly suggest a monogenic regulatory mechanism acting (a) at a general level, via the expression of the V1r receptor itself, and (b) at a more local level, defined by the V1r gene cluster.

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Introduction

In most mammalian species, inter-individual interactions related to reproduction are largely dependent on pheromonal communication. In the mouse, the vomeronasal organ (VNO), a chemosensory structure located in the nasal cavity (Figure 1A), represents a major tool for such exchanges (Halpern and Martinez-Marcos, 2003).

Vomeronasal sensory neurons (VSNs) are in contact with the outside environment, and express on their dendrites seven transmembrane receptors encoded by two different superfamilies, V2rs (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997) and V1rs (Dulac and Axel, 1995) (Figure 1B). These latter, expressed by VSNs located on the apical portion of the vomeronasal neuroepithelium, have been shown to encode pheromone receptors

(Boschat *et al*, 2002; Del Punta *et al*, 2002). The mouse genome contains over 150 potentially functional V1r genes (and an equal number of pseudogenes), which are classified into 12 families each containing between one and 30 members (Rodriguez *et al*, 2002; Zhang *et al*, 2004). To this already remarkable diversity, which is at least partly the result of positive Darwinian selection, one can add numerous polymorphic allelic variants.

V1r genes are present on most chromosomes, and members of a given family are usually found clustered in the genome. A single V1r cluster of 600 kb located on chromosome 6 has been analyzed in some detail, and contains all members (but one) of the V1ra and V1rb families, intermingled with no apparent specific organization (Del Punta *et al*, 2000; Lane *et al*, 2002). It is not known if the organization of V1rs in clusters plays a role in the regulation of their expression, or if each V1r gene behaves as a single independent unit.

A mouse possesses many VSN subpopulations, each composed of only a few hundred neurons expressing monoallelically a given V1r receptor or set of receptors (Rodriguez *et al*, 1999). Members of each subpopulation project their axons toward the brain, in a specific area of the olfactory bulb, termed the accessory olfactory bulb. There, fibers coalesce into multiple neuropil-rich spherical structures called glomeruli, which are usually exclusively innervated by a functionally identical subpopulation of VSNs (Belluscio *et al*, 1999; Rodriguez *et al*, 1999; Wagner *et al*, 2006). A surprising role is played by V1r receptors at this level: they are involved in the elaboration of the axonal projection map (Belluscio *et al*, 1999; Rodriguez *et al*, 1999). V1r receptors are thus both involved in chemodetection and axonal guidance processes.

As for many monoallelically expressed or imprinted genes, including those coding for odorant receptors or interleukins, it has been reported that V1r genes are characterized by asynchronous DNA replication (Singh *et al*, 2003). This peculiar characteristic is thought to reflect an early tag, of unknown nature, but related to the future expression or non-expression of one of the parental alleles.

We here report that the transcription of a non-functional V1r allele allows the expression of another, functional V1r gene. Unexpectedly, none of the V1r genes located in the V1r cluster containing the expressed non-functional allele is chosen for coexpression, while V1r genes present on the homologous cluster of the other parental chromosome can be chosen. These data strongly suggest that the organization of V1r genes in clusters does not simply reflect evolutionary proximity, but underlies mechanisms regulating monogenic V1r expression.

Results

Expression of V1r families in the VNO

About 150 putative functional V1r genes are present in the mouse genome, and are therefore potentially expressed by

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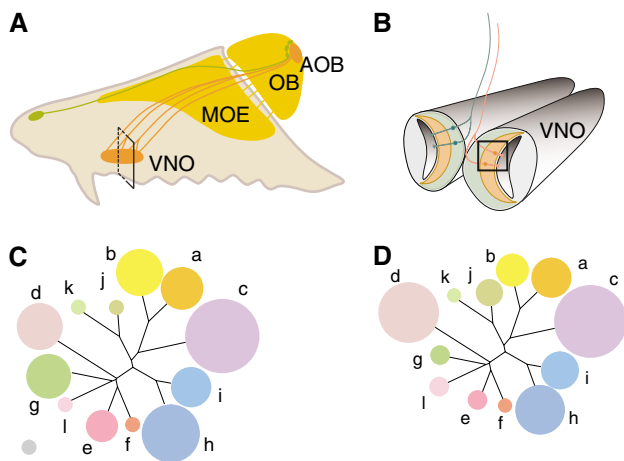


Figure 1 Size and expression variability of the V1r families in the VNO. **(A)** A schematic representation of a hemisection through the nasal cavity of a mouse. The main olfactory epithelium (MOE) and its projections to the olfactory bulb (OB) are shown in dark yellow. The Grunberg organ and its projections to the accessory olfactory bulb (AOB) are shown in orange. **(B)** Coronal section through a mouse VNO, corresponding to the plane indicated in panel A. Basal sensory neurons expressing V2r receptors are shown in green, while apical, V1r- and *Gxi2*-expressing VSNs are in orange. **(C)** A tree depicting the percentage of V1rs recognized by our set of *in situ* probes. The area occupied by the gray disk on the lower left corresponds to 1%. **(D)** A representation of the proportion of VSNs, which express V1rs from a given family.

VSNs. The transcribed fraction of these 150 genes is unknown.

To obtain a global view of the transcribed V1r repertoire, we opted for an *in situ* approach. Since V1r sequences are highly divergent between V1r families (Rodriguez *et al*, 2002), a situation which prevents a single cRNA probe to recognize all V1r transcripts, we selected one or two V1r genes for each of the 12 V1r families, based on intrafamily homologies. This allowed us to design probes that exclusively recognized members of the corresponding family, without binding to V1r genes outside the family (Supplementary Table 1). Sequences corresponding to the *V1ra3*, *V1ra9*, *V1rb2*, *V1rb9*, *V1rc32*, *V1rd16*, *V1re4*, *V1rf4*, *V1rg1*, *V1rh5*, *V1ri1*, *V1ri8*, *V1rj3*, *V1rk1* and *V1rl1* genes were used. Since V1r genes pertaining to families V1ra and V1rb are relatively closely related (up to 75% sequence homology), we tested the family specificity of the probes corresponding to these two families by hybridizing V1rb2-expressing VSNs with V1ra3/a9 probes. No cross-hybridization was observed (4000 VSNs analyzed). We also evaluated the potential cross-hybridization between V1ra3/a9 versus V1rg1/e4/f4 probes, and V1ra3/a9 versus V1ri1/i8 probes. Over 4500 VSNs were scored and none reacted with two probes. Control hybridizations using multiple probes at once showed a labeling of over 75% of the apically located, V1r- and *Gxi2*-expressing VSNs (data not shown).

With these tools in hand, we investigated the potential correlation between the size of a given V1r gene family and the fraction of VSNs expressing its members. We performed *in situ* hybridizations using family-specific probes on VNO sections, counted the mean number of VSNs recognized by the probe(s) and calculated, for each family, the fraction of the expressed V1r repertoire it represented (Figure 1D and

Supplementary Table 1). We then compared this diagram with the V1r repertoire potentially recognized by our probe sets (i.e., V1r genes sharing over 80% of the probe sequences; Figure 1C and Supplementary Table 1).

This approach showed a remarkably good correlation between the size of the V1r gene families and the number of VSNs expressing their members, except for members of families V1re and V1rg, which appear to be rarely chosen for transcription, and members of family V1rj, which are on the contrary often transcribed, relative to the fraction of the repertoire they represent (Supplementary Table 1).

Our probes are able to recognize several members of a given V1r family. But do the *in situ* signals reflect expression of different V1r genes pertaining to a given family or the frequent choice of a single or a very limited number of genes? To answer this question, we evaluated the transcriptional status of all V1r genes from the V1ra and V1rb families. Taking advantage of the high V1r intrafamily sequence identity, we designed PCR primer pairs specific and exclusive for each of these two V1r families. We then amplified the corresponding cDNAs from adult vomeronasal neuroepithelium and subcloned the amplicons. The sequencing of 35 random clones indicated that the *V1ra6*, *V1ra11*, *V1ra3*, *V1ra4*, *V1ra1*, *V1ra7*, *V1rb4*, *V1rb8*, *V1rb2* and *V1rb1* genes were transcribed, which corresponds to over 60% of the potential V1ra/b repertoire. The relative representation of each of the V1r sequences varied from 1 to 4 (the potential bias of the primers for any given V1r was tested on genomic DNA and was found negligible, data not shown). No single or small group of V1r gene transcripts were drastically over-represented, indicating that a majority of the potential V1ra and V1rb genes was transcribed by VSNs.

Expression of a single V1r gene per sensory neuron

Monogenic expression of odorant and vomeronasal receptor genes is thought to be the rule in the olfactory system. However, a systematic investigation of this dogma is still lacking, and reports suggest that olfactory receptors may express multiple chemosensory receptor genes. For example, previous data indicate that probes recognizing about 35% of the complete V1r repertoire (families V1ra, b and d) react with most of the *Gxi2*-expressing VSNs, suggesting a possible coexpression of different V1rs in single sensory neurons (Pantages and Dulac, 2000).

We investigated the monogenic expression of *V1rb2*, a V1r gene that we studied previously, and for which we generated gene-targeted animals bearing genetic tags (Supplementary Figure S1) (Rodriguez *et al*, 1999). We used an *in situ* approach, taking advantage of these tags, which permit the discrimination between neurons expressing *V1rb2* and highly related V1rb genes. We tested the possible coexpression of *V1rb2* with any member of the V1r superfamily, excluding the family to which *V1rb2* belongs (families a, c–l) (Figures 2A–D). A total of 29 240 VSNs hybridizing with the non-V1rb family probes and 6220 reacting with the *V1rb2* probe were analyzed, but none exhibited hybridization for both probe sets (Figure 2E). This analysis included 2241 VSNs expressing V1ra genes, genes that are part of the large V1r cluster containing *V1rb2*.

We performed a similar analysis with a *V1rb2*-specific probe and a probe recognizing multiple members of the V1rb family (*V1rb9*, which also recognizes *V1rb2*), and

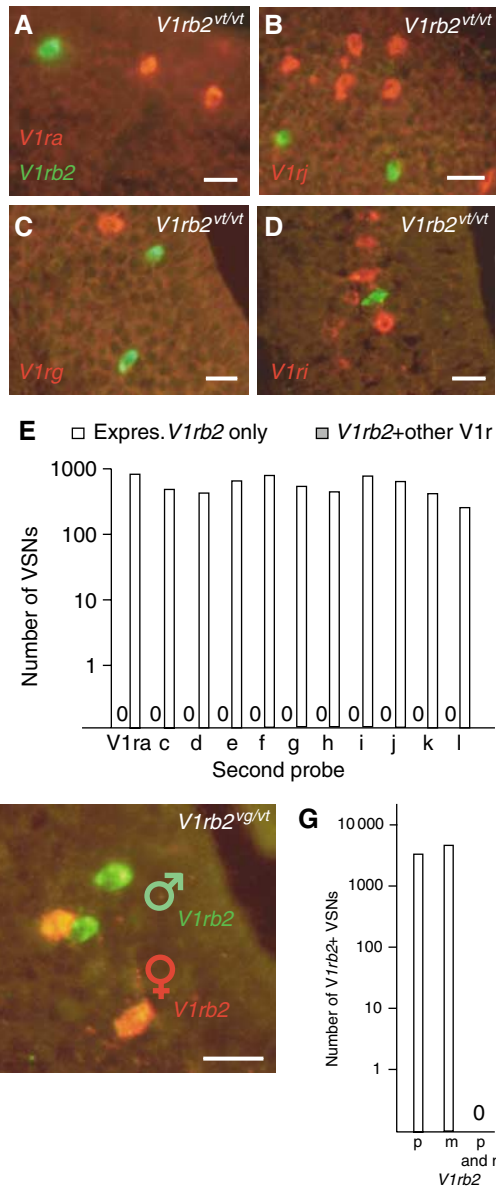


Figure 2 Monogenic expression of V1r genes. (A–D) Representative two-color *in situ* hybridizations using a probe specific for *V1rb2* (green), and probes specific for a given V1r family (red). (E) Empty bars represent the number of VSNs reacting with the *V1rb2*-specific probe, and potentially with the V1ra–l probes. Dark bars (invisible since no coexpression was found) indicate the number of these *V1rb2*-expressing VSNs cotranscribing a member of any of the V1r families. (F, G) Monoallelic expression of *V1rb2*. (F) An *in situ* hybridization with allele-specific probes for *V1rb2* (green and red represent paternal and maternal alleles, respectively) (G) Graph showing the number of VSNs reacting with allele-specific probes for *V1rb2* (empty bars), and those coexpressing both alleles (dark bar). Genotypes are shown, in white, in the upper right corner. Scale bar, 30 μ m.

found multiple VSNs hybridized exclusively with the *V1rb9* probe (data not shown), showing that not all members of a given family are expressed in a given VSN.

The monoallelic expression of the *V1rb2* gene was previously reported (Rodriguez *et al*, 1999). This study however only analyzed the translated products of two tagged *V1rb2* alleles (*V1rb2*^{vt}, Tau::LacZ and *V1rb2*^{vg}, Tau::GFP; Supplementary Figure S1), and not the transcription of the

corresponding mRNAs, leaving post-transcriptional allele-specific silencing as an alternative explanation for the observation. To address this issue, we performed *in situ* hybridizations with probes specific for either of the two *V1rb2* parental alleles on vomeronasal sections from compound heterozygotes, bearing different tags (Tau::LacZ and Tau::GFP) on each of the *V1rb2* alleles. We observed 1251 and 1498 VSNs expressing respectively the paternal or the maternal *V1rb2* allele, and none expressing both alleles ($n = 15$, seven males and eight females analyzed), confirming the monoallelic transcription of *V1rb2* (Figures 2F and G).

Our data thus strongly support a remarkably strict monoallelic and monogenic expression of V1rs in the VNO.

Expression of a null V1r allele allows the coexpression of another V1r gene

VSNs expressing the same V1r gene coalesce into 10–30 glomeruli in the accessory olfactory bulb (Figure 3A) (Belluscio *et al*, 1999; Rodriguez *et al*, 1999; Wagner *et al*, 2006). It was reported that in two mouse lines, carrying non-functional alleles of the *V1rb2* and of the *V1ra1* receptor genes, the transcription of either of the two nonfunctional genes in a given VSN affected the position of their axonal projection targets, and resulted in a non-convergence of axons from VSNs expressing the deletion alleles (Belluscio *et al*, 1999; Rodriguez *et al*, 1999). These observations were suggested to be the result of a lack of targeting information for the misrouted VSNs (information carried by the expressed receptor itself). We analyzed in detail the targeting defect of the *V1rb2* deletion line (*V1rb2*^{dv}), in which the endogenous *V1rb2* CDS is replaced by the GFP CDS, leaving all *V1rb2* non-coding sequences intact (including introns) (Rodriguez *et al*, 1999). In this mutant line, we observed that multiple axonal projections emanating from neurons expressing the *V1rb2*^{dv} allele did enter and arborize into apparently random glomeruli (Figure 3B), similar to what fibers pertaining to VSNs expressing other V1rs would do. This observation and parallel data related to the regulation of odorant receptors suggest a potential coexpression of the mutant *V1rb2* allele and of another, possibly functional V1r gene in the same VSN.

To test this hypothesis, we performed *in situ* hybridizations on vomeronasal sections from *V1rb2*^{dv/dv} animals, with probes specific for most of the V1r families (families a–j) (Figures 3C–E). VSNs expressing the *V1rb2* deletion allele were visualized (2567 VSNs), and hybridized with probes representing most V1r families. We found VSNs coexpressing the *V1rb2* mutant allele and members of families V1ra, V1rb, V1rc, V1rd, V1rg, V1rh, V1ri and V1rj (Figure 3F). The percentage of neurons cotranscribing the *V1rb2* mutant allele and another V1r ranged from 0 to 7.4% (V1ra: 1.1%, V1rb: 0.4%, V1rc: 7.4%, V1rd: 1.6%, V1rg: 0.9%, V1rh: 5.3%, V1ri: 1.5%, V1rj: 1.8%, V1re/f: 0%), showing no strong bias for a V1r family to be more likely chosen for coexpression with the *V1rb2* mutant allele (after normalization of the frequencies with the number of VSNs recognized by the probes).

We did not detect V1r coexpression in about 80% of the *V1rb2*^{dv}-expressing VSNs, possibly pointing to a limited time window during which sensory neurons can select a V1r gene, or alternatively to the cotranscription of non-V1r chemoreceptor genes. We tested this latter possibility by investigating the potential coexpression of members of the V2r family, a

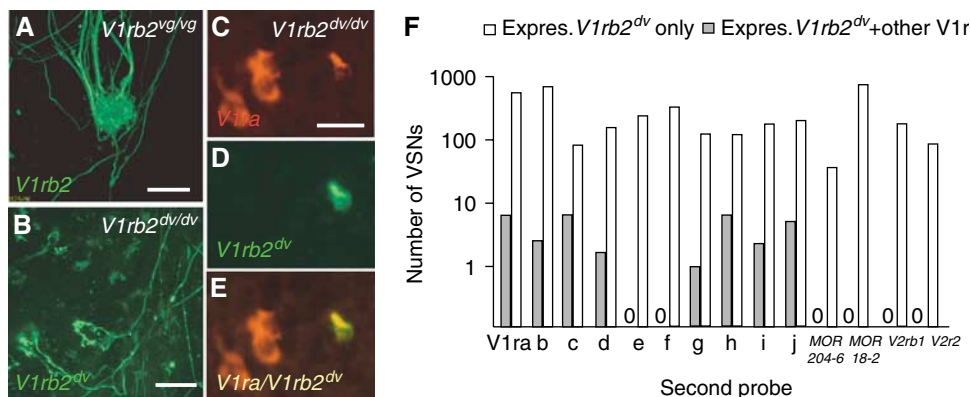


Figure 3 Expression of a deletion *V1rb2* allele allows for coexpression of another V1r. **(A)** Two-photon imaging of a wt glomerulus innervated by *V1rb2*-expressing axons. **(B)** Similar imaging of fibers emanating from VSNs expressing the *V1rb2^{dv}* allele, which enter the accessory olfactory bulb glomerular layer, and apparently innervate random glomeruli. **(C–E)** *In situ* hybridizations of VSNs coexpressing the *V1rb2^{dv}* allele (green) and a member of the V1ra family (red). **(F)** The number of VSNs coexpressing the *V1rb2^{dv}* allele together with a member of another receptor family (dark bars). Genotypes are shown, in white, in the upper right corner. Scale bar, 20 μ m.

receptor family also expressed in the vomeronasal neuroepithelium, but in the basal, *G α o*-expressing zone. We took advantage of the fact that all *V2r*-expressing VSNs coexpress the *V2r2* gene (Martini *et al*, 2001), and used a corresponding probe to visualize potential VSNs coexpressing the *V1rb2^{dv}* allele together with a member of the *V2r* family. In addition, and independently, we used a *V2r* probe specific for the *V2rb1* receptor gene. A total of 276 VSNs expressing the *V1rb2^{dv}* allele were scored, and none was found to coexpress *V2r2* or *V2rb1* (Figure 3F).

We then investigated the possibility that odorant receptors could be expressed subsequently to the transcription of the *V1rb2^{dv}* allele. First, a probe recognizing the *MOR204-6* odorant receptor gene (which is part of a subfamily of 28 members) was used. A total of 52 VSNs expressing the deletion allele were scored, none of them coexpressed *MOR204-6* or related odorant receptor genes (Figure 3F). Second, we tested the potential coexpression of the odorant receptor gene *MOR18-2*, a receptor gene known to be transcribed by VSNs (Levai *et al*, 2006). A total of 1727 *MOR18-2* and 713 *V1rb2^{dv}*-expressing VSNs were scored, none coexpressing the deletion allele and the odorant receptor gene.

Taken together, our data show that expression of the *V1rb2^{dv}* allele leads to the expression of other V1rs, and strongly support a role played by the V1r protein as a main player in the monogenic/monoallelic V1r regulation.

Functional chemoreceptors are coexpressed with the deletion allele

Do the newly chosen V1r genes modify the chemosensory capacities of VSNs expressing them? We previously showed that VSNs transcribing a functional *V1rb2* gene respond to 2-heptanone, while those expressing a *V1rb2* non-functional allele (*V1rb2^{dv}*) are insensitive to this pheromone (Boschat *et al*, 2002). We applied multiple chemical compounds (known to activate VSNs) to neurons expressing the *V1rb2^{dv}* allele, and measured their electrical responses using whole-cell patch-clamp recordings. We analyzed 12 VSNs expressing the non-functional allele, among which one responded with reversible inward currents to α and β -farnesenes (26 pA; holding potential -60 mV) and one to pentyl acetate (72 pA; holding potential -60 mV) (Figures 4B and C). The 10 remaining VSNs did not respond to the

potential agonists (Figure 4A). Among 15 VSNs that expressed the wild-type *V1rb2* allele, none responded to the compounds, except as expected, to 2-heptanone (Figure 4D), which triggered a response in all VSNs tested.

Neurons expressing the *V1rb2^{dv}* allele are therefore able to acquire chemosensory capacities not shared by VSNs, which express a functional V1RB2 receptor, a situation, which very likely reflects the acquisition of novel V1r receptors.

Cluster lock after gene choice and coexpression of genes on homologous clusters

We showed an apparent random coexpression of a V1r gene together with the *V1rb2* deletion allele, including V1rs from the V1ra or V1rb families. But are the parental chromosomes containing the newly transcribed V1rs randomly chosen? Previous reports related to the regulation of monoallelically expressed genes suggest that (a) a particular chromatin state surrounding an expressed odorant receptor gene may favor the choice of another gene located closely, in *cis*, on the same chromosome (Lewcock and Reed, 2004), and that (b) a transcriptionally active gene is, relative to its homologue located on the other parental chromosome, marked early during development, marking that reflects its future expression or non-expression (Simon *et al*, 1999; Singh *et al*, 2003).

A priori, based on the publications mentioned earlier, our results showing the coexpression of the *V1rb2^{dv}* allele and another V1ra or V1rb gene likely reflect the coactivation of V1r genes located in *cis* relative to the active *V1rb2^{dv}* allele. To test this apparently logical conclusion, we opted for the combination of an *in situ* and a genetic approach, allowing the visualization of V1ra and V1rb genes transcribed from a known parental chromosome.

The entire functional V1ra and V1rb repertoires include nine V1ra and eight V1rb genes, located in a 600 kb V1r cluster on chromosome 6, except for one V1rb member, *V1rb9*, which is present on the X chromosome. We took advantage of a previously generated mouse line (Supplementary Figure S1, *V1rab^A*) which lacks the entire V1ra/b cluster (Del Punta *et al*, 2002). To identify the parental origin of the V1ra/V1rb genes coexpressed with the *V1rb2^{dv}* allele, we crossed the *V1rab^A* with the *V1rb2^{dv}* line, generating *V1rab^A;V1rb2^{dv}* compound heterozygotes (Figure 5A). To perform hybridizations revealing exclusively V1ra and V1rb

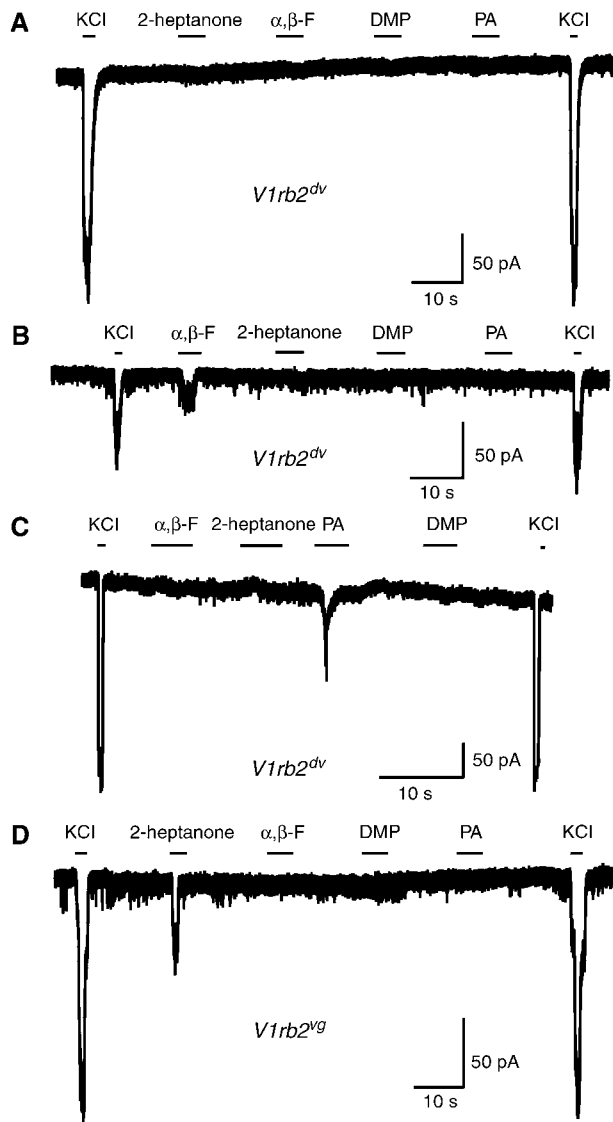


Figure 4 Coexpressed V1rs are functional receptors. (A–C) Representative whole-cell patch-clamp recordings of VSNs expressing the *V1rb2^{dv}* allele to 5'' pulses of 2-heptanone, α and β -farnesenes, 2,5-dimethylpyrazine or pentyl-acetate (10^{-6} M; holding potential, -60 mV). VSNs did not respond to these agonists (A), or responded to α and β -farnesenes (B), or to pentyl-acetate (C). (D) Only 2-heptanone (10^{-8} M) induced an inward current in VSNs expressing *V1rb2* (*V1rb2^{vg}* line). Cell viability was assessed by KCl (100 mM) pulses before and after the perfusion with agonists.

transcripts produced by genes surrounding the active *V1rb2^{dv}* allele, we had to test the following caveats: (a) a potential transcription of the X-linked *V1rb9* gene. This turned out not to represent a problem since hybridization of *V1rab^{Δ/Δ}* VNO sections with a *V1rb9/V1rb2* probe set showed no *V1rb9* transcripts in mice whose age matched the one chosen for the present study (Figure 5D); (b) a potential general *trans*-acting V1r control element lying inside the *V1ra/b* cluster and therefore absent in mice carrying the *V1rab^Δ* deletion; its absence could thus affect the expression of all V1rs. We tested this hypothesis by hybridizing *V1rab^{Δ/Δ}* VNOs with *V1ri1/V1ri8* probes, and observed an unaltered expression profile of this gene family, thus rejecting this hypothesis (Figure 5E); (c) a similar unknown *trans*-acting control element specific for *V1ra* and *V1rb* genes and lying inside the *V1rab^Δ* deletion

could, if removed, prevent expression of all members of the *V1ra/b* cluster present on the other parental chromosome. This was tested by hybridizing *V1rab^{Δ/+}* VNOs with *V1rb2/V1rb9* probes. *V1rb* expression was observed, rejecting the hypothesis (Figure 5C). We thus disposed of compound *V1rab^Δ;V1rb2^{dv}* heterozygous mice that lacked a complete set of parental *V1ra/V1rb* genes, and possessed a functional *V1ra/b* repertoire limited to the *V1ra/V1rb* genes surrounding the *V1rb2^{dv}* allele.

In situ hybridizations on these compound *V1rab^Δ;V1rb2^{dv}* heterozygous mice were performed with *V1ri1/V1ri8*, *V1ra3/V1ra9* or *V1rb2/V1rb9* probes. First, using the *V1ri* probes we confirmed that the non-functional *V1rb2* allele in the *V1rab^Δ;V1rb2^{dv}* configuration could be coexpressed with members of the family I (Figure 5F and G). We then analyzed 2513 *V1rb2^{dv}*-expressing VSNs hybridized with the *V1ra3/V1ra9* probes and 2542 *V1rb2^{dv}*-expressing VSNs hybridized with the *V1rb2/V1rb9* probes (Figure 5G). Unexpectedly, we did not find a single VSN coexpressing the *V1rb2^{dv}* allele and any of the *V1ra/V1rb* genes surrounding this latter. A comparison of these results with the corresponding hybridizations on *V1rb2^{dv/dv}* sections (6/637 for *V1ra3/V1ra9* and 3/733 for *V1rb2/V1rb9*, Figure 3F), showed a clear unequal propensity (Fisher exact test, $P < 0.001$), of the *V1r* genes physically associated with the deletion allele to be co-opted for expression (Figure 5G).

These surprising results show that (a) the co-opted *V1r* genes can include those located on the homologous cluster present on the other parental chromosome and that (b) *V1r* genes located in the cluster from which the deletion allele is expressed are not available for coexpression.

Discussion

Tight control of pheromone receptor gene expression

Our results strongly support the fact that a single functional *V1r* gene and allele is transcribed in each VSN, and show that the expression of a non-functional *V1r* allele allows neurons to choose another, functional *V1r* gene. Why express in each VSN a single *V1r* receptor gene, moreover from a single allele? Monogenic, but also monoallelic expression, are tools to achieve the expression of a single *V1r* chemodetector 'morph', since allelic polymorphic variants are common in *V1r* genes (Rodriguez *et al*, 1999). In terms of information coding, the use of multiple and narrowly tuned parallel coding lines allows the vomeronasal system to avoid a too complex decoding strategy. But the monogenic expression does not only affect peripheral chemodetection: *V1r* receptors are indeed involved in axon guidance mechanisms (although their role in this function remains elusive). The coexpression of multiple *V1r* receptor genes, even highly related, may thus simply be incompatible with the formation of functional topographical maps in the accessory olfactory bulb. This explanation is supported by the fact that, in the main olfactory system, very minor odorant receptor sequence modifications lead to different axonal responses to guidance cues in the olfactory bulb (Feinstein and Mombaerts, 2004). A dual pressure may therefore act on the mechanisms leading to the expression of a single *V1r* 'morph' per VSN: on one side to avoid a too complex decoding expense, and on the other side, to facilitate axonal wiring processes.

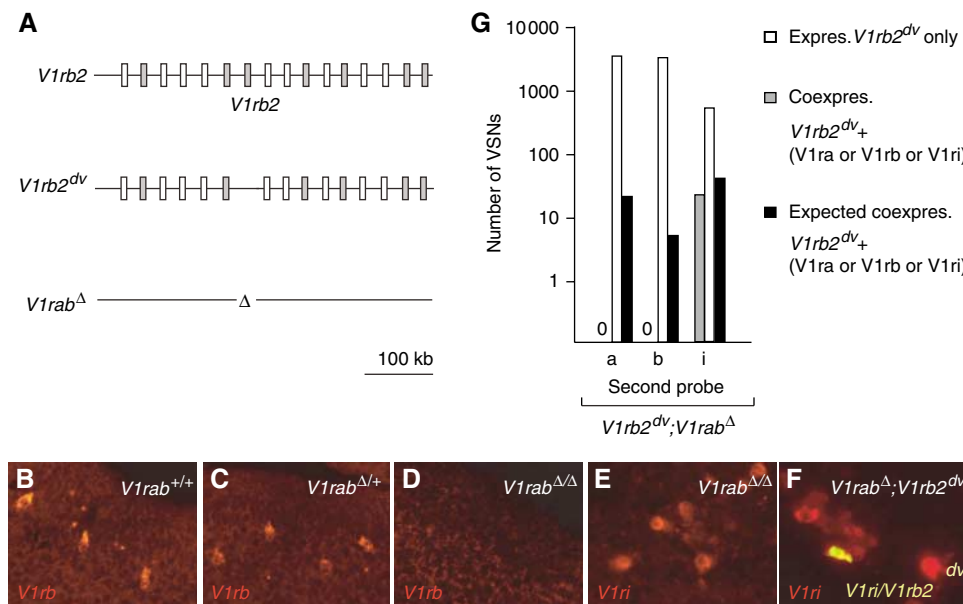


Figure 5 Cluster lock associated with V1r gene expression. **(A)** A schematic representation of the V1ra/V1rb alleles used. **(B–D)** *In situ* hybridizations of *V1rab*^{+/+}, *V1rab*^{Δ/+} and *V1rab*^{Δ/Δ} VNO sections using *V1rb9-V1rb2* probes. V1rb transcripts were identified in *V1rab*^{+/+} and *V1rab*^{Δ/+} animals, while no expression of V1rb genes was observed in *V1rab*^{Δ/Δ} mice. **(E)** *In situ* hybridization of *V1rab*^{Δ/Δ} VNO sections using the *V1ri1-V1ri8* probe set; expression of V1ri genes was observed in *V1rab*^{Δ/Δ} mice. **(F)** *V1rab*^Δ/*V1rb2*^{dv} VNO sections hybridized with *V1ri1-V1ri8* probes. A single VSN (yellow) coexpresses the *V1rb2*^{dv} allele and a V1ri gene. **(G)** Graph corresponding to *in situ* hybridizations, indicating an absence of coexpression between the *V1rb2*^{dv} allele and V1ra/V1rb genes surrounding it. Expected values of coexpressing VSNs were based on the observed frequencies, for each family, of coexpressing VSNs in *V1rb2*^{dv/dv} mice. Genotypes are shown, in white, in the upper right corner.

In the main olfactory system, cotranscription of a pseudo- and a functional odorant receptor gene is compatible in a single odorant sensory neuron, while the coexpression of more than one functional odorant receptor gene is found, although rarely (Rawson *et al*, 2000). The mechanisms responsible for a single receptor gene per neuron are unknown, but are thought to be based on negative feedback mediated by the expressed chemoreceptor, and/or on negative selection allowing only sensory neurons expressing a single chemoreceptor morph to survive (Serizawa *et al*, 2003; Feinstein *et al*, 2004; Lewcock and Reed, 2004; Shykind *et al*, 2004).

Despite the remarkable tightness of V1r regulation we report here (at least of the *V1rb2* gene), which somehow contrasts with an apparently looser regulation of some odorant receptor genes (Rawson *et al*, 2000), and despite a complete lack of sequence homology between V1rs and odorant receptors, parallels between the two olfactory sub-systems are striking. It is therefore likely that a common mechanism, in which the receptor itself is an essential regulator of the process leading to the expression of a single monomorphic chemoreceptor gene per neuron, is at work in the main olfactory and vomeronasal systems.

Coactivation of two homologous V1r gene clusters

We show here that the choice of a non-functional V1r gene accompanies the expression of a functional V1r. Unexpectedly, this latter can originate from V1r genes located in trans, from the cluster homologous to the one from which the nonfunctional gene is transcribed. This observation was surprising for the following reason: asynchronous chromosome replication during S phase is known to characterize

pheromone receptor gene clusters (a characteristic also shared by odorant receptor gene clusters and by many imprinted genes) (Mostoslavsky *et al*, 2001; Singh *et al*, 2003; Gimelbrant and Chess, 2006). The identity of the asynchronously replicated chromosome is established early during development, apparently randomly, and is clonally inherited. Although the significance of the unequal treatment of these chemosensory gene clusters is not understood, it is thought to reflect a functional difference between different parental chromosomes, and the availability or non-availability for expression of the genes subject to this asynchronous replication. Our data, which indicate that two allelic V1r clusters can be chosen for expression and cotranscribed in a single VSN, thus contradict the idea that a tag labels the parental chromosomes for V1r expression, or non-expression.

Cluster lock after gene choice

To date, no experimental evidence supporting a mechanism involved in V1r regulation is available. An obvious hint to the regulatory option possibly taken by the pheromone receptor gene repertoire may be represented by the very peculiar genomic organization of V1r genes, which are almost invariably found in clusters of variable sizes, usually as an uninterrupted string of genes. Does this arrangement underlie regulatory mechanisms taking place at the cluster level, or does it simply reflect a physical proximity resulting from the multiple duplication events, which led to the emergence of the V1r clusters?

The observation of the cluster lock naturally argues in favor of a global regulatory mechanism. Different explanations can be proposed. We could, for example, consider each V1r gene, irrespective to the chromosome on which it is

located, as a single and independent unit, chosen randomly among all other members of the V1r repertoire. The potential repertoire would in this case be composed of twice the number of V1r genes, that is, 300 members. The unavailability of the V1r genes surrounding the expressed allele could in this case represent a hypothetical physical or epigenetic constraint on these latter, negative constraint and side effect mediated by the transcription environment responsible for the expression of the first chosen and expressed allele.

However, data related to the regulation of other seven transmembrane receptor gene clusters, such as the X-linked opsin gene cluster (Pichaud *et al*, 1999) or those containing odorant receptor genes (Serizawa *et al*, 2003; Lomvardas *et al*, 2006), point at least partly to cluster-mediated or non-gene autonomous regulatory mechanisms.

One of the simplest models to explain a cluster-mediated type of regulation for V1r genes, could involve an activator sequence unique to each cluster, and responsible for the transcription of any member of the cluster. This random interaction would naturally be at the expense of the other members of the cluster, and lead to a single V1r gene expressed in the cluster.

We thus favor a model in which the expressed pheromone receptor is an essential and global tool in the establishment of monogenic and monoallelic V1r expression, but is only part of a more complex diagram, involving regulation at the V1r gene cluster level.

Materials and methods

Animals

Animals were housed and handled in accordance with the guidelines and regulations of the institution and of the state of Geneva.

In situ hybridizations

Cryostat slices (14 μ m) of adult mouse VNOs were arranged on SuperFrost+ slides (Menzel-Glaser), dried for 40', fixed for 20' at 4°C with 4% paraformaldehyde, and hybridized overnight at 65°C in the following buffer: 1 \times salt buffer (10 mM NaCl, 5 mM NaH₂PO₄ · H₂O, 5 mM Na₂HPO₄ · 2H₂O, 5 mM EDTA, pH 7.5), 50% formamide, 10% dextran sulfate, 1 μ g/ μ l tRNA, 1 \times Denhardt's, with 20 ng/ μ l cRNA probe(s). Following hybridizations, slides were washed twice at 65°C and once at RT with 1 \times SSC, 50% formamide and 0.1% Tween20. Slides were preincubated in 1 \times MABT, 2% blocking reagent (Roche) for 30' followed by 1 h incubation with alkaline phosphatase-anti-digoxigenin antibody (1:500, Roche) or/and peroxidase-anti-fluorescein antibody (1:200, Roche). Peroxidase activity was detected by washing slides three times with TNT (Tris 150 mM, NaCl 150 mM, Tween20 0.05%, pH 7.5), incubation for 30' with a biotinyl-tyramide solution (PerkinElmer), 3 \times washes with TNT and incubation for 30' with streptavidin-alexa488 (Molecular Probes). Alkaline phosphatase activity was detected by incubating slides with Fast Red substrate (DAKO) for 30'. Sections were mounted in DABCO mounting medium (Sigma). Fluorescein- and digoxigenin-labeled RNA probes were prepared

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using the DIG RNA labeling kit (Roche) following the manufacturer's instructions. The following sequences were used: *V1ra3*, nt 249–839; *V1ra9*, nt 193–750; *V1rb2*, nt 1–933; *V1rb9*, nt 196–581; *V1rc32*, nt 103–467; *V1rd16*, nt 417–619; *V1re4*, nt 178–754; *V1rf4*, nt 397–920; *V1rg1*, nt 311–833; *V1rh5*, nt 163–740; *V1ri8*, nt 496–933; *V1ril*, nt 313–750; *V1rj3*, nt 334–775; *V1rk1*, nt 332–804; *V1rl1*, nt 433–876; *V2R2*, nt 1898–2733; *MOR204-6*, nt 158–881; *MOR18-2*, nt 257–937; *Gxi2*, nt 248–1138; *LacZ* (1.8 kb *HindIII* fragment) or *EGFP* (entire ORF) probes were used to specifically identify VSNs expressing wt or mutant *V1rb2* alleles.

Microscopy

For two-photon imaging (Figures 3A and B), GFP was excited at 900 nm with a pulsed Ti-sapphire laser (Spectraphysics Mai Tai). The image was reconstructed from the fluorescence collected by descanned photomultipliers as maximum intensity projections of a z-stack of 20 slices (Olympus Fluoview 3.0).

Electrophysiology

Isolated mouse VSNs were studied at room temperature by whole-cell patch-clamp using an Axopatch 200B amplifier (Axon Instruments, USA). The softwares Pulse (Instrutech, USA) and Igor (Wavemetrics, USA) were used to analyze the signals. Patch pipettes were made from borosilicate glass microcapillaries (TW150F-6, World Precision Instruments, USA) and fire polished with a microforge (MF-830, Narishige, Japan) to have a resistance of 8–10 M Ω . The pipette solution contained (in mM) the following: 120 KCl, 10 Hepes, 1 CaCl₂, 2 MgCl₂, 11 EGTA and 2 ATP (pH adjusted to 7.3 with KOH). The bath and perfusion solutions contained (in mM) the following: 140 NaCl, 5 KCl, 10 Hepes, 2 CaCl₂, 1 MgCl₂ and 10 Glucose (pH adjusted to 7.6 with NaOH). Solutions were perfused with a fast perfusion apparatus (SF-70, Warner Instruments, USA). VSNs were exposed to 5'' pulses of α , β -farnesenes, 2,5-dimethylpyrazine, pentyl-acetate and 2-heptanone added to the perfusion solution at 10⁻⁶, or otherwise mentioned. The holding potential was maintained at –60 mV. KCl pulses (100 mM) were used as a test of neuronal viability.

RT-PCRs

mRNAs were extracted from 6-week-old male mouse VNOs. For the amplification of V1ra cDNAs, the following primers were used: fw GGG AAG TCT TTC TTA TTA GTC TCA TGG TCC TCT C, rev CAA TGC TGT CAA AGA TGG ACA TCA GCA CAA AGA AG; for V1rb: fw TCA CCC TTT GTG CTA CCT GTC TGC TGA AT, rev GTA ACT CAT GGG TAG AAG TGA GCA GGA CT. Amplifications were performed under the following conditions: 5' at 95°C, followed by 34 cycles of 1' at 95°C, 1' at 60°C and 2' at 72°C, and a final extension of 10' at 72°C. For V1ra amplicons, 17 cDNA and 18 genomic clones were sequenced. For V1rb amplicons, 18 cDNA and 21 genomic clones were sequenced.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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