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First Immunohistochemical Demonstration of the Expression of a type-2 Vomeronasal Receptor, V2R, in Wild Canids

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Abstract: The vomeronasal system serves as a pivotal element in mammalian sensory biology, allowing the perception of chemical signals essential for social communication via the activation of two main receptor families, V1R and V2R. The expression of V1Rs and V2Rs is linked with that of the G-protein alpha-subunits, Gαi2 and Gαo, respectively. The exploration of the evolutionary pathways of V1Rs and V2Rs across all mammalian species remains a main challenge, juxtaposing available genomic data against emerging immunohistochemical evidence. However, recent investigations, in contrast to what is predicted by the currently annotated genomic sequences, have revealed the expression of Gαo in the vomeronasal neuroepithelium of wild canids, including wolves and foxes. In the present study, the employment of a specific antibody raised against the mouse V2R2, member of the C-family of vomeronasal receptors, V2Rs, has confirmed the expression of this receptor in the fox and wolf, but it has revealed the lack of expression in the dog. This may reflect the impact of domestication on the regression of the VNS in this species, in contrast to their wild counterparts, and underscores the effects of artificial selection on sensory functions. Thus, these findings suggest a more refined chemical detection capability in wild species.

Keywords: vomeronasal organ; V2R; G-proteins; Canidae; immunohistochemistry

1. Introduction

The ability of organisms to adapt to their environment is crucial for the survival of both individual members and the species as a whole. Evolutionary processes have endowed mammals with complex sensory systems, specialized organs crucial for sensing environmental cues and enabling continuous adaptation. Among these, the chemical senses, particularly olfactory subsystems, are key to managing essential survival functions, including communication among members [1], social interactions [2,3], and mating behaviors [4,5]. Chemical communication between individuals depends on chemical signals, called semiochemicals [6,7], produced by individuals, released into the environment through secretions [8,9] , and recognised by the olfactory systems of animals [10,11].

The principal mechanisms for odor and pheromone detection comprise the main olfactory system (MOS) and the vomeronasal system (VNS). The former operates through the main olfactory epithelium and the olfactory bulb and has a significant linkage to the limbic system, playing the main role in both memory retrieval and conscious perception [12]. On the other hand, the VNS, which

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comprises the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB), is implicated in non-conscious influences in sexual behaviour [13,14], maternal identification [15,16], and the detection of predators [17,18], and, therefore, it is specifically tuned to sensing of pheromones [19], kairomones [10], steroids [20], and major histocompatibility complex (MHC) molecules [21,22].

Despite their proximity, these two systems maintain anatomical independence, exhibiting significant morphological and functional disparities, which suggest that they likely evolved separately [23–26]. However, functional dichotomy has been questioned, based on the presence of vomeronasal receptors (VR) in the olfactory mucosa and odorant receptors in the VNO [27–29].

Rodent species represent the primary model for studies concerning the VNS in mammals [30,31]. Shinohara and colleagues [32] first revealed the presence of Gαi2 and Gαo into non overlapping regions of the AOB, showing that the rostral part expressed Gαi2 whereas the caudal one, Gαo. Since the vomeronasal sensory neurons (VSNs) send their axonal projections to the AOB, it was possible to demonstrate that also the VNO is provided with a similar anatomical organization in several rodent species, in that, Gαi2 is specifically expressed in the apical neurons of the VNO whereas Gαo is expressed in the basal ones [33–35]. Unraveling the involvement of both G-proteins within the signal transduction sequence in VSNs proved essential to the discovery of the two main VR families. In fact, in the mouse VNO, Dulac and Axel [36] identified a set of receptors, named V1Rs, whose expression pattern specifically matched the Gαi2 expression profile.

The identification of a second VR family, type-2 V2R, in rat and mouse occurred in 1997 through simultaneous studies [37–39], cementing the notion of dual independent sensory pathways. In fact, V2Rs were shown to exclusively co-express with Gαo in the basal neurons of the VNO. Thus, the immunohistochemical profiling of Gαi2 and Gαo since has become a key method for indicating V1R and V2R receptor expression in the VNO, respectively. However, later studies, in the VNO of other mammals, such as goat [40], dog [41,42], horse and marmoset [41], sheep [43], hyrax [44], cat [45], hippopotami [46], meerkat [47], and cow and pig [48], demonstrated the expression of Gαi2 but not of Gαo. Unexpectedly, the wallaby (genus *Notamacropus*) was initially proposed as an alternative model in the organization of G-protein expression, only showing Gαo immunostaining, which was localized in the basal neurons of the VNO, but, unexpectedly, scattered throughout the AOB without any evident compartmentalization [49]; however, it has recently been demonstrated that this marsupial genus possesses canonical immunohistochemical labelling of Gαo and Gαi2 in both VNO and AOB [50].

The differentiation of two segregated pathways for pheromonal information processing, as demonstrated by immunohistochemistry, has received further support from genomic studies in different species [51–54]. However, more recent immunohistochemical observations have revealed the expression of Gαo in the VNO of species that, based on the available genomic data, were assumed to lack V2Rs, specifically in wild canids such as the fox and wolf [55,56], suggesting caution when dealing with incompletely annotated genomes, as is often the case of wild species.

In this context, a phenotypic characterization of the expression of vomeronasal receptors, especially of V1Rs and V2Rs in the VNO, would be desirable. For this reason, we have characterized the expression, through an immunohistochemical approach, of V2Rs in the VNO of wolf and fox, in which Gαo is abundantly expressed [55,56]. In this immunohistochemical study, we have also included, the VNO of the dog since, in this species, there is no definitive information to date [42,57].

2. Results

In our study, we have applied immunohistochemical techniques to investigate the expression pattern of V2R2 in the vomeronasal epithelium of three canid species: two wild, the wolf and the fox, and one domestic, the dog.

We have taken advantage of the availability of anti-V2R2, an antibody that recognizes all functional receptors of family-C V2Rs [58]. Anti-V2R2 is a polyclonal antibody that has been exhaustively characterized and validated in the mouse and rat VNO [59,60]. We used this antibody as family-C V2R are highly conserved throughout species.

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The results of the immunolabeling with anti-V2R2 in the wolf VNO are presented in Figure. 1. The analysis revealed extensive labeling throughout the entire extent of the vomeronasal neuroepithelium, with pronounced intensity in its basal part. Moreover, the apical portion of the epithelium displayed a more diffuse labeling, in the dendritic processes of the VSNs. This pattern suggests a significant expression of V2R2 in the wolf VNO (Figure 1A).

Counterstaining with hematoxylin further confirmed the observed labeling pattern and facilitated the delineation of the structural characteristics of the cellular strata and the underlying lamina propria as well as the localization of V2R2 within specific cellular compartments (Figure 1B). Higher magnification of both non-counterstained (Figure 1C) and counterstained (Figure 1D,E) images provided a clearer insight into the cellular specificity of the V2R2 expression. The labeling corresponded predominantly to cells identified as VSNs, based on their spatial placement and distinctive morphological features. These cells exhibited a clean and sharply defined pattern of labeling, primarily concentrated in the soma. Additionally, we observed a diffuse labeling pattern in the apical region of the epithelium (Figure 1C). The counterstained slides showed that the labeling in the VSNs was concentrated in the apical portion of their somata (Figure 1D). Dendrites originating from these somata extended towards the apical surface, culminating in the formation of immunopositive dendritic knobs (Figure 1D).

Interestingly, not all the receptor cells displayed immunopositivity for V2R2, with some cellular somata in various locations remaining unstained (Figure 1D, E). Additionally, our observations confirmed the absence of immunopositivity in the diverse basal stem cells (Figure 1E). These findings underscore the specific localization and expression patterns of V2R2 within the wolf VNO, highlighting the receptor potential role in pheromonal signaling and its implications in the sensory functions of this species.

In the immunohistochemical study of V2R2 expression in the sensory epithelium of the fox VNO, we have as well obtained consistent labeling throughout the vomeronasal sensory neuroepithelium, especially pronounced in the basal area (Figure 2). Additionally, the apical zone shows a diffuse pattern of labeling (Figure 2A). Hematoxylin counterstaining of the neuroepithelium facilitates the visualization of the structural features of the cellular layers and the lamina propria (Figure 2B). No counterstained higher magnification images confirm the established labeling pattern. This labeling displays a distinct arrangement, mainly concentrated in the soma. Additionally, the apical part of the epithelium exhibits a more widespread staining pattern (Figure 2C). Counterstaining at higher magnification shows the labeling mostly concentrated at the somata apical end. The dendritic ends are also immunopositive. As expected, not every VSN cell is immunopositive for V2R2. The basal stem cells display no immunopositivity (Figure 2D,E). These findings highlight the expression of V2R2 within the VNO sensory epithelium of the fox.

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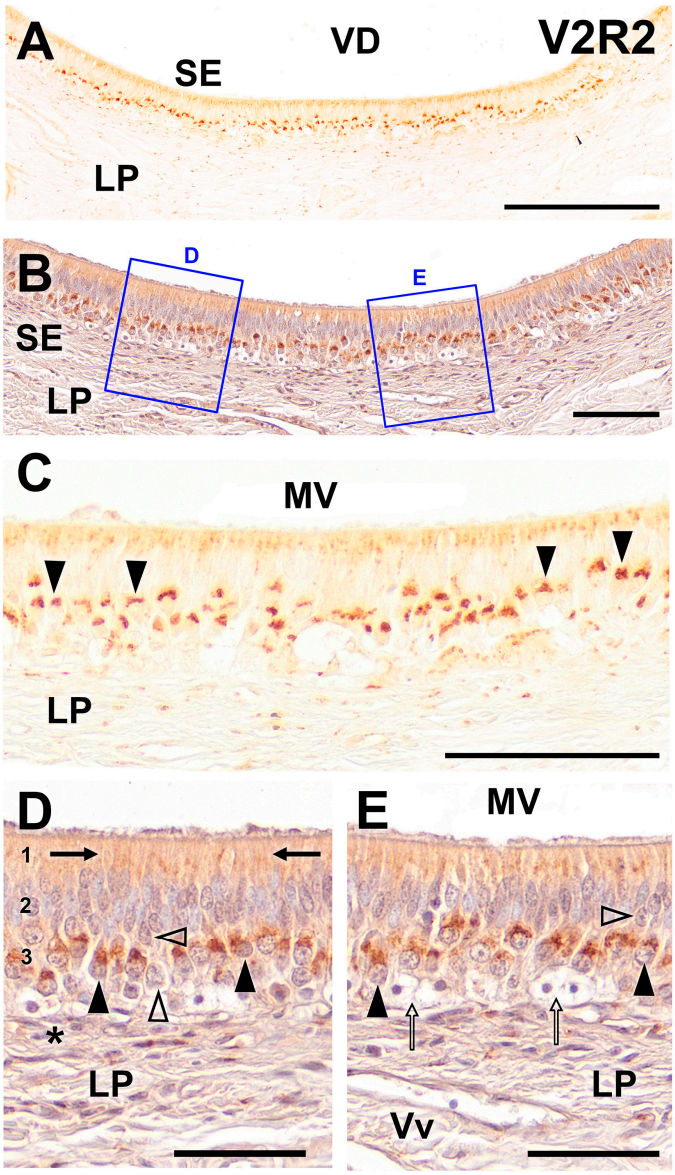

Figure 1. Immunohistochemical labelling of the wolf VNO using anti-V2R2 antibody. **(A)** Labeling is observed throughout the entire strip of the vomeronasal sensory neuroepithelium (SE), particularly in its basal part. Additionally, the apical portion of the epithelium exhibits a more diffuse labeling. **(B)** Counterstaining with hematoxylin of the neuroepithelium confirms this labeling pattern and allows for the identification of the structural characteristics of the cellular strata and lamina propria (LP). **(C)** At higher magnification, the labeling is observed to correspond to cells that, because of their spatial placement and morphological characteristics, are identified as neuroreceptor cells (arrowheads). This labeling exhibits a clean and well-defined pattern, predominantly localized to the soma. In addition, a more diffuse pattern of labeling is evident in the apical region of the epithelium. **(D-E)** Higher magnification of the counterstained areas shown in 1B allows for the determination of the morphological features of the immunopositive structures. It is noticeable that in the immunopositive cells (arrowhead), the labeling is concentrated in the apical portion of the somas. From this portion, dendrites emerge and project towards the apical surface to form the immunopositive dendritic knobs (arrows). Not all receptor cells are immunopositive for V2R2, with cellular somas in different locations being immunonegative (open arrowhead). Finally, no immunopositivity is observed in the various basal cells (asterisk and open arrows). (1) Apical

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processes; (2) Sustentacular cells layer; (3) Neuroreceptor cells layer. MV, Mucomicrovillar complex; VD, Vomeronasal duct; Vv, Blood vessels. Scale bars: **(A)**: 250 µm; **(B-E)**: 100 µm.

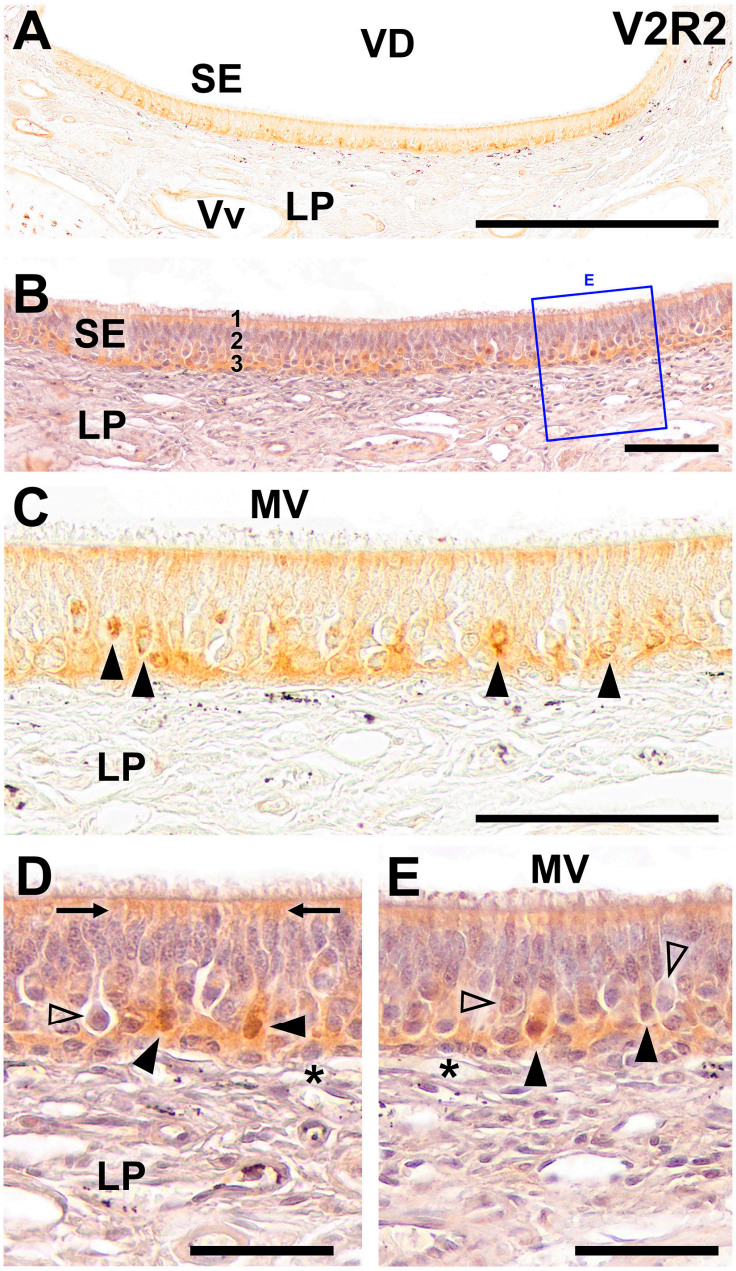

Figure 2. Immunohistochemical labelling of the fox VNO using anti-V2R2 antibody. **(A)** Throughout the vomeronasal sensory neuroepithelium (SE), consistent labeling is evident, especially pronounced in the basal area. Additionally, the apical zone shows a diffuse pattern of labeling. **(B)** Hematoxylin counterstaining of the neuroepithelium facilitates the visualization of the structural features of the cellular layers and lamina propria (LP). **(C)** Higher magnification corroborates the established labeling pattern and also enables detailed recognition of the cellular strata and the underlying lamina propria. This labeling displays a distinct arrangement, mainly concentrated in the soma. Additionally, the apical part of the epithelium exhibits a more widespread staining pattern. **(D-E)** Detailed imaging of the counterstained SE, including the section shown in 2B, permits the assessment of the morphological features of the immunopositive structures. The immunopositive cells (arrowhead) have labeling concentrated at the somata apical end. The dendritic ends are as well immunopositive

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(arrows). Not every vomeronasal neuron is immunopositive for V2R2, with some cell bodies in different locations showing immunonegativity (open arrowhead). The basal cells display no immunopositivity (asterisk). Scale bars: **(A)**: 500 µm; **(B-E)**: 100 µm.

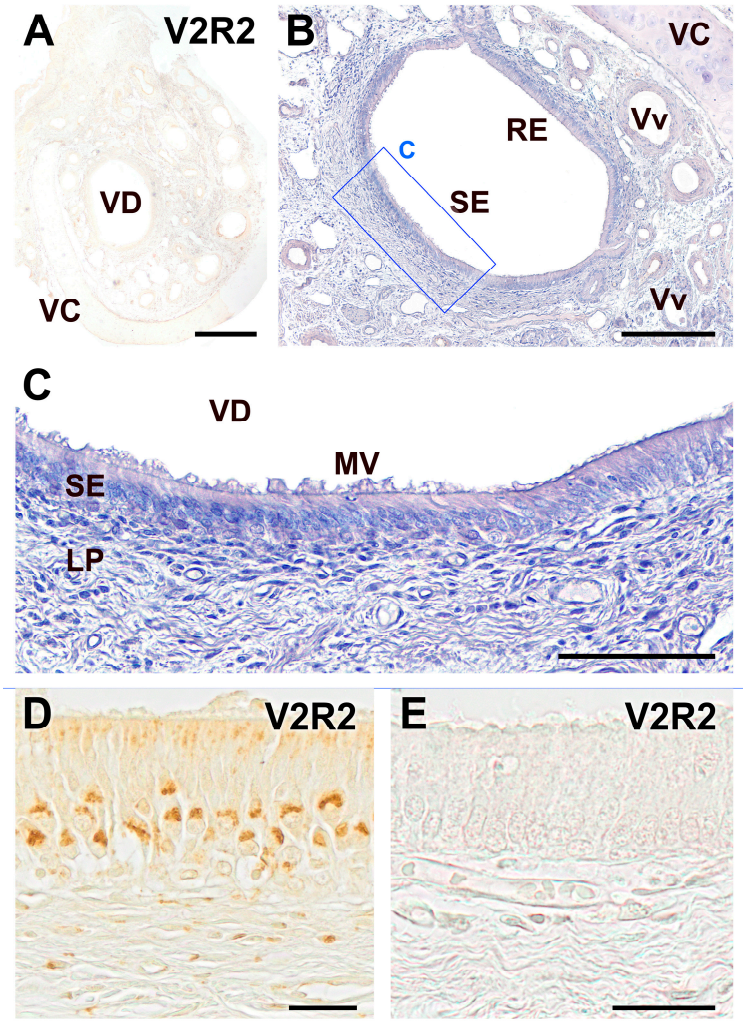

Figure 3. Immunohistochemical labelling of the dog VNO using anti-V2R2 antibody**. (A)** The immunohistochemical study of the dog VNO using antibodies against V2R2 did not produce any immunostaining in any of the structural components that constitute it. An immunonegative section is shown. **(B)** The same section hematoxylin counterstained reveals the differentiation between the sensory epithelium (SE) and the non-sensory or respiratory epithelium (RE). **(C)** An image of the SE at higher magnifications, in which the neuroepithelium cellular strata, the mucomicrovillar complex, and the underlying lamina propria can be seen, all of them immunonegative. **(D-E)** High magnification images of the VNO sensory epithelium. Comparison of the wolf anti-V2R2 immunopositive labelling (D), with the dog immunolabelling (E), with produces a complete lack of immunostaining. Scale bars: **(A)**: 500 µm; **(B)**: 250 µm; **(C)**: 100 µm; **(D,E)**: 25 µm.

The immunohistochemical labeling of the dog VNO using the anti-V2R2 antibody did not result in immunostaining of any structural components of the organ in all the examined sections (Figure 3A,E), which contrasts with the clear labeling obtained in the vomeronasal sensory epithelium of the wolf (Figure 3D). Counterstaining of the immunostained sections allowed for the clear distinction between the sensory epithelium and the non-sensory or respiratory epithelium, highlighting the structural organization of the dog VNO (Figure 3B,C). The sensory epithelium displayed the neuronal

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layers, the mucomicrovillar complex on its surface, and the underlying lamina propria. All of which were found to be immunonegative, confirming the absence of V2R2 immunoreactivity in these structures (Figure 3E).

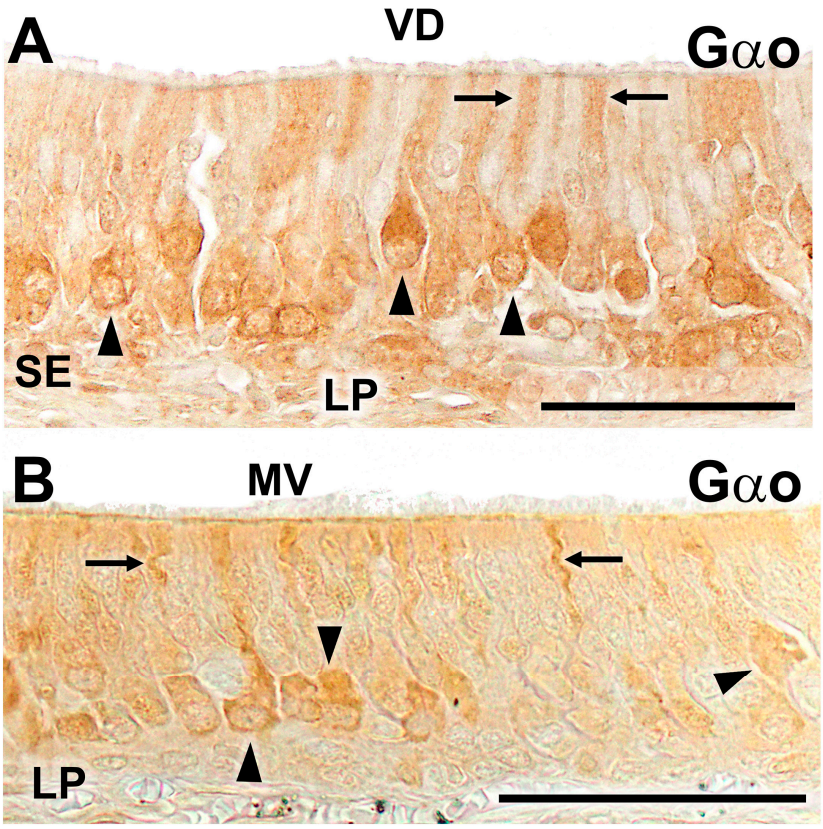

Figure 4. Immunohistochemical labelling of the wolf and fox VNO using anti-Gαo antibody**. (A)** Immunohistochemical labeling against Gαo in the sensory epithelium of the wolf VNO results in positive staining in a subpopulation of sensory neurons, which are predominantly located in the basal position. The labeling concentrates on the cellular somata (arrowheads) and the dendritic processes (arrows). **(B)** The study with the same antibody in the fox VNO produces a similar staining pattern, with immunopositive cellular somata (arrowheads) and dendrites (arrows). Scale bars: **(A-B)**: 50 µm.

Finally, given that the expression of V2Rs is tightly linked to that of Gao, we conducted an immunohistochemical study on VNO sections of the wolf and fox vomeronasal organ to assess whether this transduction protein was also detectable in these samples. The results are presented in Figure 4. In both species, immunopositivity for Gao was observed in the sensory epithelium of the VNO. The immunolabeling comprised a subpopulation of non-receptor cells and extended to both the cellular somas and the dendritic processes present on the luminal surface (Figure 4A,B).

3. Discussion

The vomeronasal system in mammals has undergone highly sophisticated and complex evolution, leading to significant diversity within vomeronasal receptor families that are characterized by extensive gene diversity and gene inactivation across species [25,61]. Understanding how this diversity arose requires a phylogenetic perspective, in which the system is characterized from a morphological, biochemical, and comparative genomic viewpoint, avoiding making extrapolations between groups, even those that appear to be very closely related, and taking into account the interaction between the main and accessory olfactory systems [26,62].

For decades, it has been widely accepted that there are two models of expression for the two main families of vomeronasal receptors, V1R and V2R: a segregated model (expressing both families)

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and a uniform model (expressing only V1R). The segregated model would include Rodents [24,63], Lagomorphs [64], Marsupials [33], and Strepsirrhine Primates [65,66], while the rest of the investigated mammals would fall under the uniform model [19,41]. The distinction between these models was based on information originally derived from the expression of the Gαi2 and Gαo, as they are considered relevant markers for the expression of V1Rs and V2Rs, respectively. The development of genomics in recent decades has helped to clarify the enormous disparity and diversity of morphological data available on the vomeronasal system. However, there are still some mismatches between genetic and morphological information in numerous mammalian groups [67,68]. Current genomic evidence sometimes clashes with histochemical data, and vice versa as immunological features challenge the information provided by genomics.

In our study of the vomeronasal system in wild canids, specifically wolves and foxes, we encountered the latter situation. While the current genomic information seems to exclude the presence of V2Rs in these species, thus assigning them to the uniform model, our immunohistochemical data demonstrate the expression of both Gαo and V2Rs at the protein level, thereby aligning the VNO of fox and wolf with the segregated model.

To our knowledge, this study represents the first demonstration of the expression of a V2R beyond a laboratory rodent model, namely rat and mouse. This has also allowed the confirmation of the likely role in pheromone transduction of G◎o positive cells found in both species, fox and wolf. As V2R2 belongs to family-C V2Rs, this finding has additional implications. Studies on the expression of the V2R2 have demonstrated that genes of family-C are expressed in all basal neurons of the VNO and that are highly conserved throughout species making its antibody useful for detecting Gαo pathways in many mammals.

The results obtained in the current study regarding immunostaining with V2R2 in the VNO of both wolf and fox are clearly consistent with the expression of this receptor in the vomeronasal neuroepithelium. Specifically, the staining is clear both in the somas and in the dendritic processes that reach the mucomicrovillar complex covering the luminal surface of the epithelium. It is noteworthy that there is no staining in the axonal terminals that leave the somas at their basal part, with staining concentrated on the apical part of the somas. This absence of axonal staining coincides with observations made by Martini and colleagues [58] in mouse and rat.

Although previous studies on the expression of Gαo in the VNO of fox and wolf were already available [55,56], for this study, we have confirmed these results using antibodies against Gαo from two different commercial brands. In both cases, the staining pattern is comparable to that observed with the V2R2 antibody. It is noteworthy that, while in the case of laboratory rodents there is a basal-apical zonation of the VNO neuroepithelium in terms of G-protein and receptor expression, in canids, the reduced size of the organ does not allow to highlight this differentiation.

A second notable aspect of our study is the absence of immunoreactivity for V2R2 in the VNO of the domestic dog. This finding, however, aligns with the accepted absence of Gαo expression found in the vomeronasal epithelium of this species [42]. The contrast in the expression patterns of Gαo and V2R2 between dogs and the closely related wild canids, like wolves and foxes, offers valuable evidence that complements existing morphological analyses in the VNS of these species [55,69,70,56], which show remarkable differences in the degree of differentiation of both the VNO and the AOB. These features may suggest a regression of the system due to the effects of domestication. This issue has also been postulated in the study of the main olfactory system of the dog through different approaches [71–73], therefore, our study may impact the understanding of some of the effects of domestication.

Finally, regarding the discrepancy between the available genomic information on the absence of V2R receptors in the genome of canids and the phenotypic results provided by G proteins, a probable explanation might lie in the absence of exhaustively annotated complete genomes in species such as the fox and the wolf. It is feasible that genomic studies may be overlooking the presence of V2R receptors. For example, in the recent attempt to sequence and assemble the red fox genome, intact V2R genes were not reported [74].

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In conclusion, this study reinforces the existence of a segregated model in the development of the VNS in wild canids, with the expression of the two vomeronasal receptor families, V1R and V2R, in species previously thought to exhibit deterioration of the latter. Additionally, it supports the notion that domestication may have produced a dramatic effect on the capacity of detecting pheromonal information.

4. Materials and Methods

In this research, we employed samples from three adult male wolves (*Canis lupus signatus*), three adult male foxes (*Vulpes vulpes*), and three adult male mixed-breed dogs (*Canis lupus familiaris*). The wolves were sourced from wildlife rehabilitation centers located in the Galicia provinces and had succumbed to fatal accidents. We selected only those individuals that had recently passed away and exhibited no signs of external or internal injuries to the head for inclusion in our study. The foxes came from activities organized by the Galician Hunting Federation. They were obtained in the field, the same day of their shooting, with a maximum of two hours delay. The procurement of wolves and fox samples was conducted in compliance with the necessary authorizations from the Galician Environment, Territory, and Housing Department, under the approval codes EB-009/2020 and EB-007/2021. The dogs subjected to necropsy were adult mesocephalic canines, sourced solely from the Department of Clinical Sciences at our institution, where they had died for various clinical conditions. Their heads were intact, displaying neither clinical nor postmortem signs of neurological disorders.

Upon their arrival to the Unit of Anatomy of the Faculty of Veterinary in Lugo, all heads were promptly dissected. The VNOs were exposed by removing the nasal bones and the lateral walls of the nasal cavity. The bone tissue encasing the VNO on the ventral and medial sides was meticulously dissected away from each sample, allowing for processing without the necessity for decalcification. Subsequently, the samples were immersed in Bouin's fluid (BF) for a 24-hour period, then transferred to 70% ethanol, embedded in paraffin, and sectioned using a microtome. They were systematically sectioned in a transverse plane along their entire length, from the caudal end to the rostral end, at a thickness of 6-7 μm.

Immunohistochemistry methodology

After deparaffinizing and rehydrating the samples, they were processed without performing antigen retrieval. This approach was chosen to avoid unmasking epitopes that could result in non-specific binding. The first step involved treating all samples with a 3% H_2O_2 solution to inhibit endogenous peroxidase. Subsequently, samples were immersed in a 2.5% horse serum solution, compatible with the ImmPRESS Anti- Rabbit IgG reagent kit (Vector Laboratories, Burlingame, CA, USA) to preclude non-specific binding. Samples were incubated overnight at 4ºC with the primary antibodies.

The next day, samples were incubated for 30 min with the ImmPRESS VR Polymer HRP Anti-Rabbit IgG. Prior to the visualization stage, all samples were rinsed for 10 minutes in 0.2 M Tris-HCl buffer at pH 7.61. DAB chromogen was used for visualizing. A 0.05% 3,3'-diaminobenzidine (DAB) chromogen solution and a 0.003% H_2O_2 solution, in 0.2 M Tris-HCl buffer were used. The DAB reagent develops into a brown precipitate in the presence of the hydrogen peroxide solution, which enables the visualization of the reaction.

Negative controls omitted the primary antibodies. As positive control we employed sections from tissues known to express the protein of interest.

To carry out the counterstaining procedure, after taking the necessary microphotographs for the immunohistochemical study, the slides were immersed in xylene for 24 hours to facilitate the removal of the cover slips. Once this was accomplished, they were cleared and rehydrated, to stain them with hematoxylin for 5 minutes. Finally, the samples were dehydrated, cleared, and mounted once more, and microphotographs were taken of them.

Primary Antibodies

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The antibody against V2R2 was developed by Prof. Tirindelli (University of Parma, Italy). It is a polyclonal antibody raised in rabbit [60]. For the immunohistochemical study of the expression of the G protein alpha subunit Gαo, two commercial polyclonal antibodies were used. The reason for employing two different antibodies was to confirm the immunolabeling obtained with this protein, which in both cases was analogous. The antibodies used were from Santa Cruz Biotechnology (sc-387) and Medical & Biological Laboratories (MBL-551).

Image Capture

Images were digitally captured using a Karl Zeiss Axiocam MRc5 camera coupled with a Zeiss Axiophot microscope. Adobe Photoshop CS4 was employed for brightness, contrast and balance adjustment; however, no enhancements, additions, or relocations of the image features were made. Additionally, an image-stitching software (PTGuiPro) was used for low magnification images composed of several photographs.

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Institutional Review Board Statement: Not applicable, as all the animals employed in this studydied by natural causes..

Informed Consent Statement: Not applicable, as this research did not involve any humans.

Data Availability Statement: All relevant data are within the manuscript, and are fully available without restriction.

Conflicts of Interest: The authors declare no conflicts of interest.

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