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Functional Characterization of Olfactory Binding Proteins for Appeasing Compounds and Molecular Cloning in the Vomeronasal Organ of Pre-pubertal Pigs

Gaëlle Guiraudie[1], Patrick Pageat[1], Anne-Hélène Cain[2], Iltud Madec[1] and Patricia Nagnan-Le Meillour[2]

[1]Phérosynthèse SA, Le Rieu Neuf, F-84490 Saint-Saturnin-Les-Apt and [2]INRA, Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint-Cyr, F-78026 Versailles Cedex, France

Correspondence to be sent to: Patricia Nagnan-Le Meillour, UMR CNRS/USTL 8576, Glycobiologie Structurale et Fonctionnelle, Bâtiment C9, Université des Sciences et Technologies de Lille1, F-59655 Villeneueve d'Ascq Cedex, France. e-mail: patricia.le-meillour@univ-lille1.fr

Abstract

The appeasing behaviour of pre-pubertal pigs appears to result from the perception of maternal odours (fatty acids) and of steroids coming from the male. We have used a ligand-oriented approach to functionally characterize olfactory binding proteins involved in the detection of appeasing compounds in the nasal mucosa (NM) and the vomeronasal organ (VNO) of pre-pubertal pigs. Several proteins were identified, combining binding assay, immunodetection and protein sequencing. Their sites of expression in nasal and vomeronasal tissues were studied by reverse transcription polymerase chain reaction (RT-PCR). The proteins belong to the lipocalin superfamily: Alpha-1-acid glycoprotein (AGP), odorant-binding protein (OBP), salivary lipocalin (SAL) and Von Ebner's gland protein (VEG), and displayed different binding capacities for the appeasing compounds. RT-PCR experiments showed that OBP and VEG are expressed not only in the NM, but also in the VNO and that SAL is only expressed in the VNO. This is the first report of the expression of these lipocalins in the VNO. Different binding affinities between lipocalins and appeasing compounds, together with their different localizations in the olfactory systems, suggest multiple possibilities for the peripheral coding of appeasing signals.

Key words: pig maternal pheromone, odorant-binding protein, Von Ebner's gland protein, salivary lipocalin, steroid

Introduction

Lipocalins constitute a heterogeneous family of small, secreted proteins that share amino acid motifs, a common structure and the ability to bind a remarkable array of small hydrophobic molecules (Akerstrom *et al.*, 2000). Among them, olfactory binding proteins mediate the reception of olfactory signals in several biological fluids and organs implicated in the chemical communication of mammals (Tegoni *et al.*, 2000). Their precise physiological role is partially understood and that led to an arbitrary classification, based on their known (or unknown) binding properties towards different classes of ligands: pheromone-binding proteins (PBPs) and odorant-binding proteins (OBPs) differ in their localizations (Pelosi, 2001). They are evolutionary and structurally unrelated to insect PBPs and OBPs, despite their common function of odorant binding (Pelosi, 1994).

Mammalian PBPs are secreted in diverse biological fluids involved in social and sexual behaviours mediated by pheromones (urine, vaginal discharge or saliva) such as rodent major urinary protein (MUP) (Finlayson *et al.*, 1965) and aphrodisin (Singer *et al.*, 1986), or the salivary lipocalin (SAL) characterized in pig (Marchese *et al.*, 1998).

The physiological role of OBPs is less documented. They are secreted in the mucus lining the nasal cavity and, contrary to PBPs, bind a broad array of hydrophobic ligands with dissociation constants in the micromolecular range (Tegoni *et al.*, 2000). This apparent lack of binding specificity led authors to confer on OBPs the role of solubilization and transport of odorant molecules to their target receptors located in the membrane of olfactory receptor neurons (Pelosi, 2001). OBPs are also assumed to concentrate odorants and/or to scavenge them from receptors in a deactivation process (Pelosi, 2001). The poor binding specificity observed for OBPs could be explained by the fact that none of the ligands commonly used in published binding assays are relevant to the animal, i.e. their perception does not evoke any specific behaviour. This point is meanwhile of critical importance to study the involvement of OBPs in odour discrimination.

Olfactory cues are crucial in the recognition and acceptance of conspecifics, as well as in the establishment of a social hierarchy (Kristensen *et al.*, 2001). In pigs, maternal pheromones are involved in the regulation of nursing pig behaviours (Morrow-Tesch and McGlone, 1990). In particular, odours isolated from the skin of milking sows (Pageat, 2001) have been shown to reduce agonistic behaviours in piglets (Pageat and Teyssier, 1998). The commercial synthetic analogue had similar effects when tested in industrial husbandries: applied once at weaning, it reduced agonistic behaviours, stimulated feeding behaviour and led to an increase in daily weight gain (McGlone and Anderson, 2002). This putative maternal pheromone is composed of six fatty acids in different proportions (Pageat, 2001): hexadecanoic acid (palmitic acid, PA; 35%), *cis*-9-octadecenoic acid (oleic acid, OA, 26%), (*cis*,*cis*)-9,12-octadecadienoic acid (linoleic acid, LiA, 22%), dodecanoic acid (lauric acid, LaA, 8%), tetradecanoic acid (myristic acid, MA, 7%) and decanoic acid (capric acid, CA, 2%). In addition, McGlone and Morrow (1988) demonstrated that the use of the pheromonal component androstenone (5α-androst-16-en-3-one) reduces agonistic behaviour and transiently improves performance in growing, especially in newly regrouped pre-pubertal pigs (McGlone *et al.*, 1986). The appeasing behaviour of pre-pubertal pigs appears to result from the perception of maternal odours (fatty acids) and of steroids coming from the male.

The availability of components evoking appeasing behaviour was an opportunity to understand the molecular mechanisms involved in their detection, in particular the role of olfactory binding proteins (PBPs and/or OBPs) in their early coding. This process takes place at the peripheral level of the olfactory system, in the mucus lining the cavity of the target organ. Two distinct olfactory systems are described in pigs as in most mammals, the main olfactory system (MOS), whose target organ is the snout and the vomeronasal system, whose target organ is the vomeronasal organ (VNO). Their respective involvement in the detection of odours and pheromones is unclear. The prevailing view is that odours are detected only in the MOS (Mombaerts, 1999), whereas pheromones are generally detected via the VNO (Buck, 2000; Holy *et al.*, 2000; Leinders-Zufall *et al.*, 2000). However, recent studies in mammals demonstrate that VNO neurons can detect both odours and pheromones (Sam *et al.*, 2001).

The objective of this study was to characterize proteins involved in the detection of appeasing compounds, in the nasal mucosa (MOS) and/or in the VNO mucosa, using a ligand-oriented approach.

Materials and methods

Animals and dissections

Pre-pubertal male pigs (*Sus scrofa*, Large White × Landrace) of ~35 kg were bought from an industrial husbandry. No food was delivered 24 h before the animals were killed. Twenty-nine pigs were used for sample collection. Methods of breeding and collection were performed according to the 95/29/CE European convention.

Pigs were first anaesthetized using xylazine (Rompun®, 10 mg/kg of live weight). After 10 min, a solution of sodium pentobarbital (Doletal®, 25 mg/kg of live weight) was injected. The animal was then transported to the surgical room to be killed by bleeding.

Nasal mucosa (NM) and VNOs were dissected from anaesthetized animals immediately after death to preserve the mRNA. The dissection took place in sterile conditions, using single use materials. Extraction of the VNO (two per animal) began by removing the bone palate and the soft palate. VNOs appeared each side of the nasal septum. After collection of the mucosa, VNOs were extracted. The respiratory mucosa was directly collected from the opened nasal cavity. Each sample was put in an individual Eppendorf tube and immediately stored at –80°C until use.

Analogues of pig appeasing compounds

Tritiated 9-octadecenoic acid (oleic acid [9,10-^{3}H], [^{3}H]OA), hexadecanoic acid (palmitic acid [9,10-^{3}H], [^{3}H]PA), tetradecanoic acid (myristic acid [9,10-^{3}H], [^{3}H] MA), and 4-pregene-3,20-dione (progesterone (1,2,6,7-^{3}H[N]), [^{3}H]Pro) were from Sigma-Aldrich. Radiolabelled analogues of 9,12-octadecadienoic acid (linoleic acid [9,10,12,13-^{3}H], [^{3}H]LiA), dodecanoic acid (lauric acid [11,12–^{3}H], [^{3}H]LaA) and decanoic acid (capric acid [1-^{14}C], [^{14}C]CA) were purchased from American Radiolabeled Chemicals Inc. (St Louis, MO). The specific activity was 1.2 TBq/mmol for [^{3}H]OA, 1.8 TBq/mmol for [^{3}H]PA, 2 TBq/mmol for [^{3}H]MA, 3.4 TBq/mmol for [^{3}H]Pro, 1.4 TBq/mmol for [^{3}H]LiA, 2.2 TBq/mmol for [^{3}H]LaA, 1.9 GBq/mmol for [^{14}C]CA. For simplicity, the radiolabelled analogues are referred to as the cold molecules in this paper (e.g. OA refers to [^{3}H]OA).

Preparation of protein samples

The proteins were extracted from pig tissues by phase partition using chloroform/methanol (2:1) on ice. The resulting samples were centrifuged (15 000 *g* for 15 min at 4°C) and the methanol phase was collected, then evaporated in a Speed-vac concentrator and stored at –80°C until use.

Electrophoresis, protein sequencing, Western blot and binding assay

Native polyacrylamide gel electrophoresis (16.8%) was carried out at 150 V (constant voltage) and room temperature. The gels were stained overnight in a colloidal Coomassie blue R solution (12% trichloroacetic acid, 5% ethanolic solution of 0.035% Serva blue R 250) and rinsed with distilled water. For immunodetection, proteins were electrophorized as above then electroblotted (400 mA constant current) onto a poly(vinylidene) difluoride membrane (Immobilon P, Millipore) using a Bio-Rad Trans-Blott Cell System.

For blocking unspecific sites, membranes were soaked overnight at 4°C in TBS-T (Tris 20 mM, NaCl 137 mM, pH 7.6, 0.1% Tween 20) containing 5% non-fat milk. After three brief rinsings in TBS-T, membranes were incubated for 1 h 15 min at room temperature with primary antibodies at a 1/5000 dilution (anti-OBP or anti-VEG or anti-SAL, crude sera provided by P. Pelosi). After three rinsings in TBS-T, membranes were incubated 15 min with secondary antibodies (anti-rabbit Ig horseradish peroxidase-linked whole antibodies, Amersham). Immunoreactivity was detected with the Enhanced ChemiLuminescence kit (ECL, Amersham), following the manufacturer's instructions. For the binding assay, each sample of olfactory tissues was incubated with 1 µCi of a radiolabelled ligand. The samples were treated as above for electrophoresis and blotting (ProBlott membranes, Perkin-Elmer). For fluorography, membranes were successively dipped 30 min in formaldehyde and 1 h in salicylic acid without soaking. After drying on the bench, they were exposed to Hyperfilm MP (Amersham) for 7 days at –20°C. The films were developed and the membranes were stained with a Ponceau Red S solution (Sigma Chimie; 0.2% in 1% acetic acid) and destained in distilled water. Bands giving a radioactive signal on the corresponding film were carefully cut off the membrane for N-terminal sequencing. N-terminal sequences, when unblocked (SAL), were obtained by gas-phase microsequencing (J. d'Alayer, Institut Pasteur, France). In case of N-terminal blocking (OBP, VEG), the protein identification was obtained by internal sequencing. Briefly, samples were loaded in 16.8% preparative non-denaturing gels, and the proteic bands of interest were cut after staining (0.3% amidoblack in 45% methanol/10% acetic acid). Proteins were digested with trypsin and the resulting peptides were sequenced by Edman degradation (J. d'Alayer). Sequences were compared with those of the NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Molecular cloning of pig OBP, SAL and VEG in the VNO

Total RNA was extracted from one male pig VNO with the Tri-Reagent (Euromedex) and subjected to reverse transcription with the Advantage™ RT-for-PCR kit (Clontech), using 200 units of reverse transcriptase, 20 pmol of oligo $(dT)_{18}$ primer, 0.5 mM of each dNTP, 0.5 unit of RNase inhibitor, 50 mM Tris–HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$ in 20 µl total volume. The reaction mixture was incubated at 42°C for 1 h, and for 5 min at 94°C, and the products were directly used for polymerase chain reaction (PCR) amplification or stored at –20°C.

Degenerated oligonucleotides OBP sense: 5′-CAYGARGARATGGAYA AAAC-3′, were designed from protein sequence (SwissProt P81245). PCR was performed on a PCR express Thermal Cycler (Hybaid), using 1.25 units of DNA polymerase (Promega), 200 µM of each dNTP (Promega), 1 µM of the primer and the oligo $(dT)_{18}$, 3 mM $MgCl_2$, 50 mM Tris HCl (pH 9.0), 50 mM NaCl, 10 µg of activated calf thymus DNA and 0.1 mg/ml BSA in a final volume of 25 µl. After a denaturation step at 94°C for 5 min, the reaction was performed for 40 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min), followed by a final step of 10 min at 72°C. The amplified cDNA was analysed by electrophoresis on a 1.5% agarose gel and purified using GenElute microcolumns (Supelco) and ligated into the plasmid PCR®II-TOPO® using the TOPO TA cloning kit (Invitrogen). After transformation, positive clones were digested with *Eco*R1 (Promega) to screen the presence of insert. Recombinant plasmids were isolated using a Plasmid Midi kit (Qiagen) and subjected to automated sequencing with vector primers (T7 and M13 promoters) by ESGS (Evry, France). Signal peptide sequence of OBP was obtained by using 5′-SMART™ RACE cDNA Amplification kit (Clontech) with the following antisense primer: 5′-TGGAATCCCGTTCTC TCTTGTCACCTC-3′. Touchdown PCR was carried out with a first cycle of 1 min at 94°C, followed by five cycles of 30 s at 94°C, 3 min at 72°C, followed by five cycles of 30 s at 94°C, 30 s at 70°C and 3 min at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 68°C and 3 min at 72°C, completed by a final step at 72°C for 5 min. The amplified cDNA was analysed by electrophoresis on a 1.5% agarose gel and purified using GenElute microcolumns (Supelco), then subjected to automated sequencing in both senses by ESGS (Evry, France).

PCR amplifications were carried out with 200 ng of cDNA in a solution containing 2.5 units of ProofStart DNA polymerase (Qiagen), 300 µM of each dNTP, 1 µM of each PCR primer, 1 × of ProofStart manufacturer buffer, 1 × of manufacturer Q-Solution and 1.5 mM of Mg^{2+} for SAL and OBP, and 3 mM of Mg^{2+} for VEG. Oligonucleotides were designed from the nucleotide sequences described in GenBank and a Kozak sequence was included in 5′ ends: SAL specific primer sense: 5′-AGGATGAAGCTGCTGCTC-3′ and antisense: 5′-TCACTCAGCACTGGACTC-3′, VEG specific primer sense: 5′-AGGATGATGAGGGCTCTGCTCCTGGCC-3′ and antisense: 5′-CTAGTTCCCTCCTGGAGAGCAGGTTTCGCTTT-3′; OBP specific primer sense: 5′-AGGATGAAGAGTCTGCTGCTG-3′ and antisense: 5′-TCACTTGGCAGGACAGTCATC-3′. The reaction cycles were performed as follows: 95°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 51°C, 65°C and 52°C respectively for SAL, VEG and OBP, and 1 min at 72°C. After purification and 3′-end A-tailing, the DNA amplification products were ligated into the expression vector pcDNA3.1/V5-His-TOPO© using the pcDNA3.1/V5-His© TOPO® TA expression kit (Invitrogen). The plasmids DNA were transformed into One Shot™ INVαF′ competent cells. Positive clones were subjected to automated sequencing (Genome express, Meylan, France) in both senses.

Reverse transcription-polymerase chain reaction

For RT-PCR, total RNAs were extracted from olfactory tissues (NM and VNO) of male pig with the Tri-Reagent (Euromedex). They were subjected to 'One tube RT-PCR'

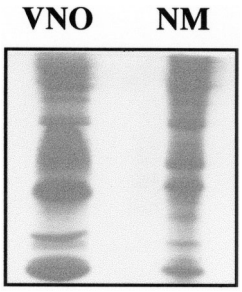

Figure 1 Comparison of protein patterns in pig olfactory tissues. Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) of vomeronasal organ (VNO) and nasal mucosa (NM) extracts (90 mg of each tissue) stained with Coomassie blue.

with the sensiscript™ reverse transcriptase (Qiagen) and the DNA *Taq* polymerase (Promega). The following combinations of gene-specific primers were used to detect the transcripts coding for the binding proteins functionally characterized by binding assay: the 5′-AGP: 5′-CCGCTGTGCGCCAACTTG-3′ and the 3′-AGP: 5′-CTAGGACCCCTCCTTCTC-3′; the 5′-SAL: 5′-AGGATGAAGCTGCTGCTC-3′ and the 3′-SAL: 5′-TCACTCAGCACTGGACTC-3′; the 5′-VEG: 5′-AGGATGATGAGGGCTCTGCTCCTGGCC-3′ and the 3′-VEG: 5′-CTAGTTCCCTCCTGGAGAGCAGGTTTCGCTTT-3′; the 5′-OBP: 5′-AGGATGAAGAGTCTGCTGCTG-3′ and the 3′-OBP: 5′-TCACTTGGCAGGACAGTCATC-3′. For detection of the 60S ribosomal protein L35 (control), two primers were used: the 5′-RIBO: 5′-ATGGCCAAGATTAAGCTC-3′ and the 3′-RIBO: 5′-TCAGGCCTTGACGGCAAAC-3′. After a reverse transcription step of 60 min at 37°C, and an activation step of 10 min at 94°C, the reactions were performed for 40 cycles (1 min at 94°C, 1 min at 56°C, 51°C, 65°C and 52°C respectively for AGP, SAL, VEG and OBP, 1.5 min at 72°C). Ribosomal cDNA was amplified by 40 cycles of a stepwise program (1 min at 94°C, 1 min at 58°C, 1.5 min at 72°C). PCR products were separated in agarose gels and subjected to automated sequencing in both senses by Genome express (Meylan, France).

Results

Comparison of the protein patterns from NM and VNO

The two olfactory tissues coming from different pigs were compared to analyse the variability in protein content between mucosae and between animals. There was a quantitative difference between NM and VNO mucosae from the same animal (Figure 1), whereas profiles coming from the same tissue of different animals were similar (data not shown). To compare the protein content of the two mucosae, extracts equivalent to 90 mg of NM and VNO were loaded on a gel and stained with Coomassie blue (Figure 1). The profiles were qualitatively identical with bands that co-migrate, but the proteins were much more abundant in the VNO extract. All further experiments were performed on standardized extracts made from the same weight of each mucosa.

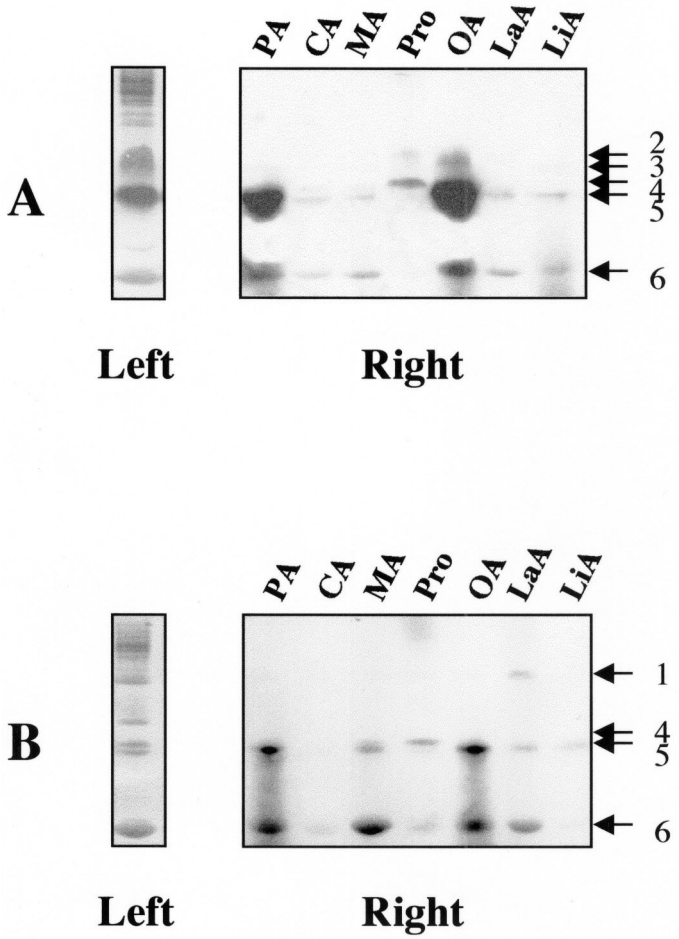

Figure 2 Binding experiments between VNO **(A)** and NM **(B)** extracts and radiolabelled analogues of the maternal pheromone components and progesterone. Left: membrane stained with Ponceau red after 1 week exposure at –20°C to Hyperfilm MP. Right: fluorography. Each well results from the incubation of 20 µl of each olfactory tissue extract with 1 µCi of each of the radiolabelled compounds (PA, palmitic acid; CA, capric acid; MA, myristic acid; Pro, progesterone; OA, oleic acid; LaA, lauric acid; LiA, linoleic acid). Protein bands binding the radiolabelled analogues are numbered according to increasing acidity (1–6).

Characterization of the binding proteins

The binding experiments were performed using the radiolabelled analogues of pig appeasing components in order to characterize eventual binding proteins in the two olfactory mucosae. In addition, we used radiolabelled progesterone, which is not known to be detected by the olfactory system but is a steroid hormone of this species, structurally related to the porcine sex steroid androstenone.

Binding with the two tissues revealed that six protein bands differently bound the radiolabelled compounds (numbered 1–6; Figure 2).

In the VNO, the five bands that were involved in binding (Figure 2A, right) were analysed by N-terminal sequencing: band 2 only bound progesterone and contained the pure N-Ter HKEAGQDVVTSNFDASKIAG with 100% identity (Blastp search) with that of the pig salivary lipocalin (SAL) previously described (Marchese *et al.*, 1998). Band 3 also contained a N-ter identical to the pig SAL, but bound oleic acid. Bands 2 and 3 that shared the same N-terminal sequence and migrated to different positions in native PAGE could have corresponded to two isoforms of the SAL, with different internal domains and binding capabilities. The N-terminal sequences of the three other bands were blocked. Internal sequences were obtained, and made it possible to identify the corresponding proteins by Blastp searches. Band 4 and band 5 both contained the internal sequence DPENNPE that corresponds to the pig Von Ebner's gland protein (VEG) (Garibotti *et al.*, 1995). Other internal peptides were sequenced (VVYILPS, WYLK), and they all corresponded to VEG domains. Even if the two bands were not well separated by 16.8% acrylamide gels, the binding data showed distinct binding properties for the components tested (clearly distinguishable when comparing wells MA and Pro): the lower migrating band (band 4) binding progesterone, and the faster migrating band 5 binding the analogues of pig appeasing compounds with different apparent affinities. Finally, band 6 gave the WITSYIGS internal sequence, which has 100% identity with the pig OBP (Paolini *et al.*, 1998). The porcine OBP contained in band 6 bound all the radiolabelled analogues, except progesterone.

In NM, four bands were involved in the binding with ligands (Figure 2B, right). Band 1 specifically bound lauric acid. As the protein was blocked at the N terminus, internal sequencing was performed. The peptide FFDPKPAE was obtained, and the Blastp search provided 100% identity with the porcine alpha-1-acid glycoprotein (AGP, GenBank M35990). Proteins contained in bands 4 and 5 were blocked at the N terminus. Internal sequences of peptides resulting from the trypsin digestion were identical to those obtained for bands 4 and 5 of the VNO. They corresponded to the porcine VEG. Again, the binding abilities of the two isoforms were different: band 4 bound progesterone, while band 5 bound the pig appeasing compound analogues with different apparent affinities. The two VEG isoforms had identical binding abilities in the VNO and in the NM. Band 6 bound all the radiolabelled ligands with different affinities. A strong binding was observed with palmitic acid, myristic acid, oleic acid and lauric acid. A faint binding was observed with capric acid, progesterone and linoleic acid. Internal sequencing allowed identification of the porcine OBP. It should be noted that the two isoforms of SAL were absent from the NM (Figure 2B, left).

To control for the identification of OBP, VEG and SAL in the two mucosae, NM and VNO extracts were treated for Western blot with specific antibodies (Figure 3). Each well contained the same weight-equivalent (1/4 aliquot of 90 mg of each mucosa). Anti-OBP serum strongly cross-reacted with the protein(s) contained in band 6 of both tissues. Anti-VEG serum cross-reacted with bands 4 and 5 in VNO and NM; this reaction was weaker for the latter. Anti-SAL serum cross-reacted with a broad band co-migrating with bands 2 and 3 in both tissues. As no binding was detected with the anti-SAL immunoreactive band in NM, we performed N-terminal sequencing to identify the protein(s). The N terminus was blocked and internal sequencing of a resulting peptide gave the LLELDQPPK sequence, 100% identical to an internal domain of the porcine catheline (SwissProt P80054), which exerts anti-microbial activity. This protein did not bind any ligand and was not studied further.

Molecular cloning of OBP, SAL and VEG in the VNO

The amino acid sequence of porcine OBP has been previously obtained by Edman degradation of a highly purified sample issued from one pig's nasal tissue (Paolini *et al.*, 1998), but the full-length nucleotide sequence of cDNA encoding for pOBP was unknown. Thus, combining PCR with degenerated primers, designed on the amino acid published sequence (Paolini *et al.*, 1998), and the 5′-RACE-PCR technique, we obtained the full-length cDNA encoding the porcine OBP in the VNO. This sequence, including signal peptide was deposited in GenBank, under accession

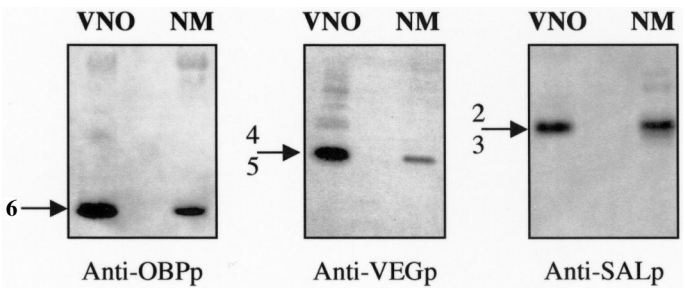

Figure 3 Immunodetection of odorant-binding protein (OBP), Von Ebner's gland protein (VEG) and salivary lipocalin (SAL) in pig olfactory tissues (VNO and NM) by Western-blot. Primary anti-sera raised against porcine OBP (anti-OBPp, 1:5000 dilution), porcine VEG (anti-VEGp, 1:5000 dilution) and porcine SAL (anti-SALp, 1:10 000 dilution). Enhanced ChemiLuminescence (ECL) detection (30 s exposure). The numbers to the left refer to the bands numbered in Figure 2.

#AF436848. The deduced amino-acid sequence showed 100% identity with the published protein sequence (Paolini *et al.*, 1998; SwissProt P81245). The 5′-end of the coding region contains a signal sequence of 15 amino acids, typical of secreted proteins (Table 1). The mature OBP consists of 173 amino acids, leading to a deduced molecular mass of 17 835 daltons and an isoelectric point of 4.43, consistent with previous data obtained from the purified protein (mass = 17 689 daltons and pI 4.2; Paolini *et al.*, 1998). The nucleotide sequence allowed designing primers for the pOBP expression study by RT-PCR.

The full-length nucleotide sequence encoding SAL in the VNO was obtained. The alignment of both nucleotide and deduced amino acid sequences with those of published SAL showed 100% identity (respectively GenBank AJ249974 and GenBank CAB93679).

We have cloned the cDNA sequence encoding the VEG in the VNO, which displays 100% identity with the published sequence (GenBank S77587).

Analysis of the tissue expression by RT-PCR

In order to identify the site(s) of expression of olfactory binding proteins characterized above and the presence of corresponding messenger RNAs, we performed RT-PCR in the two olfactory tissues, using specific primers deduced from the published sequences of AGP, SAL and VEG, and from the OBP sequence obtained above. As control, the mRNA encoding the 60S ribosomal protein L35 of *Sus scrofa* was reverse-transcribed and the cDNA encoding this protein was amplified in the two tissues (Figure 4). The sequencing of the purified RT-PCR products showed 100% sequence identity with the published sequence (AB055884).

One band was amplified only in the NM using specific primers for porcine AGP (Figure 4). The nucleotide sequence showed 100% identity with the published sequence of porcine AGP (GenBank M35990).

Table 1 Nucleotide and deduced amino acid sequence of pOBP clone from the pig VNO (GenBank accession no. AF436848). The peptide signal is underlined and the asterisk marks the translation-termination codon.

M K S L L L S L V L G L V C A Q E P		18
ATG AAG AGT CTG CTG CTG AGT CTG GTC CTT GGT CTG GTT TGT GCC CAG GAA CCT		54
Q P E Q D P F E L S G K W I T S Y I		36
CAA CCT GAA CAA GAT CCC TTT GAG CTT TCA GGA AAA TGG ATA ACC AGC TAC ATA		108
G S S D L E K I G E N A P F Q V F M		54
GGC TCT AGT GAC CTG GAG AAG ATT GGA GAA AAT GCA CCC TTC CAG GTT TTC ATG		162
R S I E F D D K E S K V Y L N F F S		72
CGT AGC ATT GAA TTT GAT GAC AAA GAG AGC AAA GTA TAC TTG AAC TTT TTT AGC		216
K E N G I C E E F S L I G T K Q E G		90
AAG GAA AAT GGA ATC TGT GAA GAA TTT TCG CTG ATC GGA ACC AAA CAA GAA GGC		270
N T Y D V N Y A G N N K F V V S Y A		108
AAT ACT TAC GAT GTT AAC TAC GCA GGT AAC AAC AAA TTT GTA GTT AGT TAT GCG		324
S E T A L I I S N I N V D E E G D K		126
TCC GAA ACT GCC CTG ATA ATC TCT AAC ATC AAT GTG GAT GAA GAA GGC GAC AAA		378
T I M T G L L G K G T D I E D Q D L		144
ACC ATA ATG ACG GGA CTG TTG GGC AAA GGA ACT GAC ATT GAA GAC CAA GAT TTG		432
E K F K E V T R E N G I P E E N I V		162
GAG AAG TTT AAA GAG GTG ACA AGA GAG AAC GGG ATT CCA GAA GAA AAT ATT GTG		486
N I I E R D D C P A K *		174
AAC ATC ATC GAA AGA GAT GAC TGT CCT GCC AAG TGA		522

RT-PCR performed with SAL-specific primers led to the amplification of one PCR product in the VNO and no amplification in the NM (Figure 4). The oligonucleotide sequence obtained after purification and sequencing showed 100% identity with the published sequence of pig SAL (GenBank CAB93679).

RT-PCR experiments using specific primers designed from VEG sequence enabled us to amplify a 531 pb nucleotide sequence in both tissues (Figure 4). The nucleotide sequence obtained from the VNO, compared to published sequence of VEG (GenBank S77587) showed two mutations: $T^{12}\rightarrow C$ and $T^{504}\rightarrow C$, which are silent as the deduced amino-acid sequence has 100% identity with the porcine VEG already published. In the NM, the alignment of the amino acid sequence deduced after sequencing with the porcine VEG (GenBank AAB34720) indicated 99.4% of sequence identity to be due to the presence of one substitution: Pro141→Leu. This variant has previously been described in mature pig nasal tissue, but no nucleotide sequence was published (Paolini *et al.*, 1998). We thus deposited the sequence in GenBank (AY177149). The VEG isoform expressed in the VNO corresponded to the isoform extracted from Von Ebner's glands, whereas the isoform expressed in NM exhibited the same amino acid substitution (Pro141→Leu) to the nasal mucus isoform (Garibotti *et al.*, 1995; Scaloni *et al.*, 2001).

In the VNO, two amplified RT-PCR products were obtained with the OBP-specific primers (Figure 4). The nucleotide sequence of the 522 pb product shared 100% identity with the OBP sequence isolated from the VNO (this paper), and the deduced amino acid sequence showed 100% identity with the porcine OBP sequence already published (SwissProt P81245). The 600 pb amplified product also showed 100% sequence identity with porcine OBP (GenBank AF436848). In the NM, two amplified products were obtained. The 433 pb RT-PCR product corresponded to a part of the *Sus scrofa* breed Landrace mitochondrion. The 522 pb product shared 100% sequence identity with the porcine OBP sequence obtained in this paper (GenBank AF436848).

Discussion

Functional characterization of proteins that differently bind appeasing compounds in pig olfactory mucosae

We have used a ligand-oriented approach to functionally characterize binding proteins that could be involved in the detection of appeasing compounds in different areas of the pig olfactory system: the NM that lines the nasal cavity of the main olfactory system, and the VNO that is part of the vomeronasal system (Takami, 2002). Four proteins have been identified, combining binding assays, immunodetection and protein sequencing, which belong to the lipocalin superfamily, whose members are known to bind and trans-

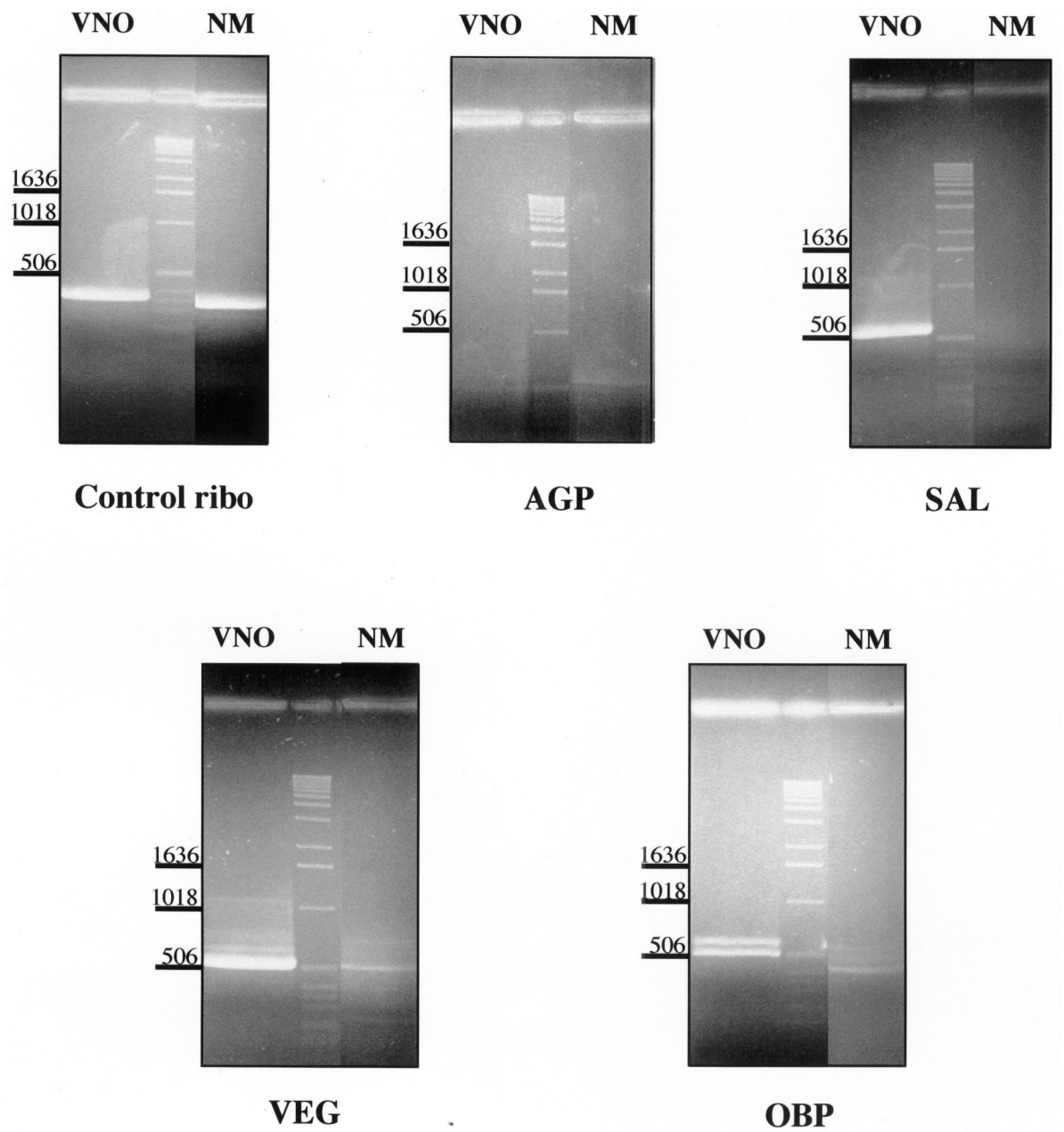

Figure 4 cDNA fragments amplified by RT-PCR with specific primers for porcine 60S ribosomal protein (control, 2% agarose gel), porcine AGP, porcine SAL, porcine VEG, porcine OBP (1.5% agarose gel), and RNA templates from VNO and NM. The DNA ladder is indicated on the left (in base pair).

port hydrophobic molecules with more or less specificity (Akerstrom *et al.*, 2000).

Alpha-1-acid glycoprotein (AGP)

This lipocalin is a plasmatic protein synthesized in the liver of pigs, and its plasma level varies according to age, sex and health status (Stone and Maurer, 1987). In particular, increases in AGP concentrations have been observed in swine with pleuritis, dysentery, abscesses and arthritis (Itoh *et al.*, 1992), Thus, AGP concentration, together with that of cortisol, is used as a haematological indicator of stress in piglets, related to daily weight gain (Stull *et al.*, 1999). This is the first report of the expression of AGP in the nasal mucosa and of a specific binding with a fatty acid (lauric acid). Until now, AGP binding has been reported with lipophilic molecules and drugs in plasma (lidocain, propanolol) (Son *et al.*, 1996).

Odorant-binding protein

The porcine OBP (pOBP) has been primarily identified by purification from pig nasal epithelium (Dal Monte *et al.*, 1991) and by its ability to bind 2-isobutyl-3-methoxypyra-

zine (IBMP). The X-ray structure of the purified pOBP has been determined (Spinelli *et al.*, 1998) and shows that this lipocalin is monomeric in solution and devoid of natural bound ligand, contrary to bovine OBP (Ramoni *et al.*, 2001). pOBP binds a large variety of odorants with affinity constants in the micromolar range, related to the molecular structure of ligands (Hérent *et al.*, 1995; Burova *et al.*, 1999; Paolini *et al.*, 1999; Vincent *et al.*, 2000).

Even though this protein has been structurally characterized, its sites of expression have not been investigated precisely until now. Our RT-PCR experiments demonstrate that this protein is expressed both in NM and VNO. Our binding assay shows that pOBP extracted from NM and VNO binds the ligands with different apparent affinities. The molecules used in this study were either fatty acids (appeasing compounds), saturated (capric, lauric, myristic, palmitic, C10→C16) or with one (oleic, C18:1) or two double bonds (linoleic, C18:2), or a steroid (progesterone, C21). The best binding was observed in both mucosae for palmitic and oleic acid, the major components of the maternal pheromone (36% and 25%, respectively). Progesterone, linoleic and capric acid bind the pOBP more weakly. The intensity of binding with lauric and myristic acid differ between VNO and NM extracts. This binding assay, set up to functionally characterize binding proteins for identified odorants is only qualitative and does not allow us to establish a quantitative relationship between the chemical structure of the ligands and the binding intensity. We can only observe that better binding is obtained when the alkyl chain length is >10 carbons, and that the functional group has less importance. These results are in accord with the data previously published on structure–activity relationships in pOBP binding with odorants (Vincent *et al.*, 2000).

Salivary lipocalin (SAL)

Salivary proteins were first described as proteins of the saliva secreted by the submaxillary glands of the boar (and not castrated males or females) and involved in binding with the steroid sex pheromones 5α-androst-16-en-3-one and 5α-androst-16-en-3-ol (Marchese *et al.*, 1998). SAL isoforms were further isolated from nasal mucosa of both sexes, but contrary to SAL extracted from boar saliva that contains endogenous androstenone and androstenol (Marchese *et al.*, 1998), the nasal SALs are free of ligand (Loebel *et al.*, 2000). Two isoforms of SAL were characterized both in submaxillary glands of the male pig (Marchese *et al.*, 1998) and nasal mucosa of males and females (Loebel *et al.*, 2000). We have also characterized two isoforms of SAL in the VNO, which can only be distinguished by their different binding properties. In our RT-PCR experiments, only one PCR product of ~600 bp was amplified in the VNO, corresponding to the sequence of SAL isoform A already published (Loebel *et al.*, 2000). We failed to obtain the sequence of isoform B but we cannot exclude the amplification of minor different sequences of identical length in the 600 bp band.

The expression of SAL in the VNO has not been previously reported, but is not entirely surprising as SAL is supposed to be involved in porcine sexual communication by transporting steroid pheromones to the sensory neurons of the VNO. The SAL isoforms of the VNO present different binding properties, as has been reported for saliva and nose isoforms (Marchese *et al.*, 1998; Scaloni *et al.*, 2001). Unlike pOBP, SAL has higher affinity for steroid sex pheromones than for any other odorant tested in binding assays (Marchese *et al.*, 1998; Loebel *et al.*, 2000; Scaloni *et al.*, 2001) and in docking experiments (Spinelli *et al.*, 2002). This specificity was recently explained by the fine structure resolution of SAL isoform A (Spinelli *et al.*, 2002). The internal cavity fits closely to the two steroids androstenone and androstenol, whereas smaller odorants do not establish as many favourable Van der Waals interactions with the residues forming the wall of the cavity. The SAL isoforms of the VNO only bind progesterone or oleic acid, which are the biggest ligands tested in this study, in term of steric hindrance. Progesterone is a steroid, the structure of which is closely related to androstenone and androstenol. Nevertheless its apparent affinity for SAL (band 3) seems weak. These data confirm the strong binding specificity of SAL for pig sex pheromones, and reinforce the hypothesis that SAL acts as a pheromone-binding protein in pig sexual communication, like MUPs and aphrodisin in rodents.

Von Ebner's gland protein

In pig, VEG protein isoforms have been first extracted from Von Ebner's gland of the tongue (saliva) and lacrymal glands (tears) (Garibotti *et al.*, 1995), as well as from nasal epithelium (Scaloni *et al.*, 2001). The high concentration of VEG protein in pig saliva led authors to propose a role in the transduction of taste stimuli. However, binding experiments with sapid substances, as well as with several odorants or retinol were negative (Garibotti *et al.*, 1995; Loebel *et al.*, 2000; Burova *et al.*, 2000). In humans, VEG protein and tear lipocalin are identical. The endogenous natural ligands of human tear lipocalin have been identified as fatty acids (palmitic, stearic and lauric), cholesterol, phospholipids and glycolipids (Glasgow *et al.*, 1995). They seem to be tightly bound to the protein *in vivo*. Such strong binding between VEG protein and its natural ligand has been reported for pig VEG, as attempts to extract the ligand from the protein have all failed (Garibotti *et al.*, 1995; Burova *et al.*, 2000). Our binding assay shows that the faster migrating isoform of VEG protein in VNO and NM binds fatty acids, with a much higher affinity for oleic and palmitic acids, the major components of the pig maternal pheromone, as well as the natural ligands of human VEG (Glasgow *et al.*, 1995). The second isoform of VEG protein that we characterized in VNO and NM extracts has a high affinity for progesterone, higher than SAL, but does not bind the fatty acids, components of the maternal pheromone. The binding preference of a VEG isoform for a steroid is extremely interesting, when

considering the composition of male pig saliva: the major components are the sex steroids, androstenone, androstenol (Booth, 1977) and testosterone (Booth, 1972), found not only in unusual high levels in boar saliva, but also in age-dependant levels in castrated or pre-pubertal male saliva. If androstenone and androstenol are naturally bound to the SAL, the existence of a binding protein for testosterone has never been investigated. Testosterone and progesterone structurally differ on carbon 17 where an alcohol for the former substitutes a methyl-ketone group for the latter. Besides, our data indicate that VEG protein is the most abundant protein expressed and secreted in the VNO, with two isoforms displaying different binding properties related to the chemical structure of the ligands (fatty acids versus steroids). These data strongly suggest that testosterone, in high quantities in the saliva, could be the natural ligand of one of the VEG protein isoforms and that VEG protein could act as a pheromone-binding protein.

How olfactory binding proteins could participate to the coding of odorant and pheromonal mixtures?

As already mentioned, the role of OBPs in odour discrimination is still a subject of discussion, mainly due to their apparent lack of binding specificity. Conversely, our study indicates that proteins exhibiting ligand specificity (SAL, VEG) can bind other ligands *in vitro*. In a recent review, Ma *et al.* (2002) have pointed out the increasing evidence showing that ligands with different shapes, sizes and composition may bind at a single binding site of protein with an equal or even higher affinity than the presumable specific ligand. This reflects the existence of populations of protein conformers in solution. Around the native state, the protein exists in a range of conformations. In the process of binding, the conformer that is selected is the one with a binding site that complements most favourably that of the incoming ligand (Ma *et al.*, 2001). These data support our findings of several isoforms for SAL and VEG in both main and vomeronasal mucosae, exhibiting different binding properties towards ligands. Hence, binding site shape and size are defined by the ligand. It is notably the case for pig SAL, whose binding pocket's shape and size are adapted by the presence of ligand. In the presence of androstenol or androstenone, two small cavities collapse with the main cavity to form a larger binding pocket. Smaller ligands (3,7-dimethyl-1-octanol, 2-phenyl ethanol, IBMP) can enter the binding pocket, but bind with much less affinity to the SAL (Spinelli *et al.*, 2002).

The occurrence of OBP, SAL and VEG in oral, nasal and vomeronasal areas has to be placed in a physiological and more generally, behavioural context. Previous data have demonstrated the presence of OBP in the nasal area (Dal Monte *et al.*, 1991), and of SAL and VEG in both the nasal and oral cavities (Scaloni *et al.*, 2001). This study assesses the occurrence of OBP, SAL and VEG in the VNO of pre-pubertal pigs (Figure 5). Furthermore, these proteins are much more abundant in the VNO, despite its small size, than in NM. It suggests an important role of the VNO in the detection of odours and/or pheromones in pre-pubertal pigs. Indeed, it was recently demonstrated that VNO neurons are stimulated by both odours and pheromones (Sam *et al.*, 2001). On the one hand, the binding between one isoform of SAL and VEG with fatty acids suggests their involvement in the detection of the pig maternal pheromone. On the other hand, the binding between the other VEG isoform and a steroid, as well as between another SAL isoform and sex steroids have to be based on behavioural data showing that the perception of androstenone induces appeasement behaviour in pre-pubertal pigs (McGlone *et al.*, 1986; McGlone and Morrow, 1988). The detection by growing pigs of steroids emitted by dominant males would participate in the establishment of the social hierarchy in the group.

The appeasing behaviour of pre-pubertal pigs seems to result from the perception of maternal odours (fatty acids) and/or odours coming from the dominant males (sex steroids). At the peripheral level, three lipocalins (at least) participate in the molecular coding of appeasing signals. In pre-pubertal pigs, OBP and VEG are expressed in the mucus of both MOS and VNO, whilst SAL is solely expressed in the VNO. These proteins are localized in oral, nasal and vomeronasal areas, which communicate with each other, either permanently between mouth and nose (Figure 5), or occasionally between VNO and other cavities during flehmen. In fact, the stimulation of the VNO is a dynamic process that can only occur when the incisive duct is opened

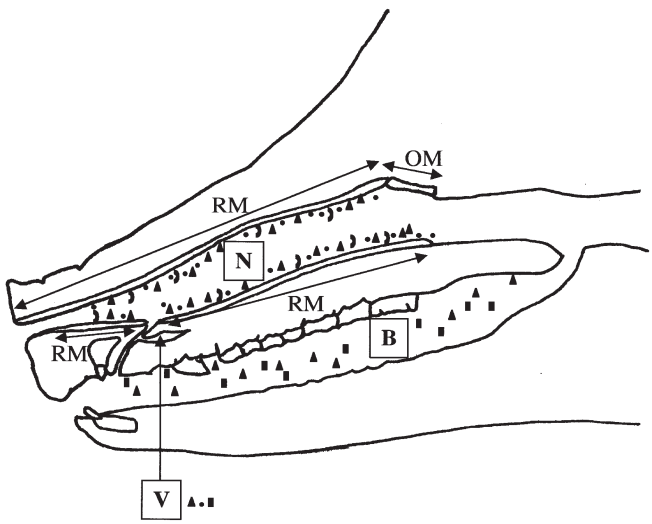

Figure 5 Schematic drawing of a pig head, showing oral, nasal and vomeronasal cavities, lined by their mucosae (from Barone, 1976). N, nasal cavity; B, buccal cavity; V, vomeronasal organ; RM, respiratory mucosa and OM, olfactory mucosa = NM, nasal mucosae; filled circle, OBP; filled triangle, VEG; open square, SAL; filled square AGP. The protein symbols refer to presumed location of each protein in the VNO mucus (this work), in the nasal mucus (Dal Monte *et al.*, 1991; Scaloni *et al.*, 2001; this work) and in the oral cavity (Garibotti *et al.*, 1995; Marchese *et al.*, 1998). AGP and OBP have not been identified in the oral cavity.

by an active mechanism (Takami, 2002). The stimulation of the main olfactory mucosa results from a passive inhalation of the ambient air. On the contrary, it seems that external signals are necessary to evoke flehmen. It has been shown that postures, such as lordosis in ovulating sows during the approach of the boar (Signoret, 1970), or the exposure to some body areas that are usually hidden (mammal area) are able to enhance flehmen. The perception of one odour among a mixture could be an olfactory signal enhancing flehmen, activating the muscle that controls the cartilage closing the incisive duct to which the VNO opens. During flehmen, vascular modifications induce the wash out of the VNO and the secretion of the mucus containing lipocalins in its lumen. In this hypothesis, the main olfactory mucosa should be stimulated first by a part of the putative appeasing pheromone, and the AGP that specifically binds lauric acid is a good candidate. Then, the VNO could be opened by the flehmen, leading to the stimulation of the VNO mucosa.

Different binding affinities between lipocalins and appeasing compounds, together with their different localizations in the olfactory systems (MOS and VNO) suggest multiple possibilities for the peripheral coding of appeasing signals. Even if the nature of the olfactory signal (odour or pheromone) is more likely determined by the central treatment of information coming from different peripheral sources, it is clear that lipocalins participate in their early coding. Moreover, the two olfactory systems can be stimulated at the same time or sequentially, increasing the capacities for coding and discrimination.

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