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Biochemical and Chemical Supports for a Transnatal Olfactory Continuity through Sow Maternal Fluids

Gaëlle Guiraudie-Capraz[1,2], Marie-Christine Slomianny[3], Patrick Pageat[1], Christian Malosse[4], Anne-Hélène Cain[4], Pierre Orgeur[5] and Patricia Nagnan-Le Meillour[3]

[1]Phérosynthèse SA, Le Rieu Neuf, F-84490 Saint-Saturnin-Les-Apt, France, [3]UMR CNRS/USTL 8576, Glycobiologie Structurale et Fonctionnelle, Bâtiment C9, F-59655 Villeneuve d'Ascq Cedex, France, [4]INRA, Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint-Cyr, F-78026 Versailles Cedex, France and [5]UMR INRA-CNRS-Université de Tours, Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France
[2]Present address: Laboratoire de Neuroglycobiologie, IFR 131 'Cerveau et cognition', GLM-CNRS, 31 chemin Joseph Aiguier, F-13402 Marseille Cedex 20, 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 Lille 1, F-59655 Villeneuve d'Ascq Cedex, France. e-mail: Patricia.Le-Meillour@univ-lille1.fr

Abstract

Recognition of the mother is of major importance for the survival of mammalian neonates. This recognition is based, immediately after birth, on the detection of odours that have been learned by the fetus *in utero*. If the ethological basis of a transnatal olfactory continuity is well established, little is known on the nature of its olfactory cues, and nothing about the presence of potential carrier proteins in the maternal fluids such as amniotic fluid, colostrum and milk. We have identified the components of the pig putative maternal pheromone in these fluids of the sow. We also used a ligand-oriented approach to functionally characterize carrier proteins for these compounds in the maternal fluids. Six proteins were identified, using binding assay, immunodetection and peptide mapping by mass spectrometry. These proteins are known to transport hydrophobic ligands in biological fluids. Among them, alpha-1 acid glycoprotein (AGP) and odorant-binding protein (OBP) have been described in the oral sphere of piglets as being involved in the detection of pig putative maternal pheromone components. These are the first chemical and biochemical data supporting a transnatal olfactory continuity between the fetal and the postnatal environments.

Key words: amniotic fluid, colostrum, milk, odorant binding protein, olfaction, pig

Introduction

Olfaction is of major importance for communication in mammals. Interactions with congeners are mediated by odours for individual recognition (Kristensen *et al.*, 2001), sexual (Signoret, 1974, 1996) or maternal (Signoret *et al.*, 1997) behaviours. In particular, the establishment of the mother–young link is largely due to olfactory cues mediated by maternal fluids such as milk, colostrum and amniotic fluid (Nowak *et al.*, 2000): females and newborns develop a positive orientation to those birth fluids (Lévy *et al.*, 1983; Marlier *et al.*, 1998; Schaal *et al.*, 1994). One-day-old rabbits are able to detect and discriminate abdominal odours emitted by adult conspecifics (Coureaud and Schaal, 2000; Coureaud *et al.*, 2000). Furthermore, they selectively respond to the major odour to which they have been exposed *in utero* (Coureaud *et al.*, 2002). Odour information gained in the amniotic medium constitutes familiarities bridging the fetal and the neonatal niches, suggesting that the degree of chemosensory similarity of amniotic fluid and colostrum should be high around birth (Marlier *et al.*, 1997). The positive orientation towards the maternal fluids suggests the existence of a transnatal olfactory continuity in mammals (Schaal *et al.*, 1994, 1995).

For piglets, it is much more important than for other mammals to rapidly find the nipple, essential for the survival of neonates, because sows do not take care of their litter (Orgeur *et al.*, 2002). The search of the nipple is based on olfactory information (Hrupka *et al.*, 2000) since piglets are attracted by odours from the maternal ventral skin, such as amniotic fluid and milk (Morrow-Tesch and McGlone, 1990a; Rhode-Parfet and Gonyou, 1991). Olfactory cues are located near

the nipple, rendering easier the nipple attachment (Morrow-Tesch and McGlone, 1990b) and the intake of colostrum, then milk. Mammary pheromones in milk, evoking attraction and oral grasping in newborns, have only been isolated in rabbits (Schaal *et al.*, 2003). Meanwhile, odours isolated from the nipple area of milking sow have been shown to reduce agonistic behaviours in piglets (Pageat and Tessier, 1998). The components of this putative maternal pheromone were identified as fatty acids in proportions that are species-specific (Pageat, 2001). The commercial analogue, Suilence®, has similar effects of reducing agonistic behaviours and its use increases the daily weight gain (McGlone and Anderson, 2002). The components of the putative maternal pheromone are bound by olfactory binding proteins (OBPs) in the nasal and vomeronasal mucosae of piglets (Guiraudie *et al.*, 2003), and may participate in their peripheral coding. These proteins belong to the lipocalin superfamily (Flower, 1996), members of which have already been identified in human amniotic fluid (Liberatori *et al.*, 1997) and colostrum (Murakami *et al.*, 1998). In addition, Piotte *et al.* (1998) identified three lipocalin-like proteins in the milk of marsupials. Two of them have similarities with OBPs, and the third with the major urinary proteins (MUPs) of rodents, known to be involved in the pheromone communication.

The objective of this study was to propose chemical and biochemical support to the transnatal olfactory continuity evidenced by behaviourists. In a first step, we investigated the presence of the putative maternal pheromone components in the maternal fluids, which are in contact with piglets around birth, and during fetal (amniotic fluid) and post-natal life (colostrum then milk). In previous work, we used a ligand-oriented approach to identify the proteins that bind the components of the putative maternal pheromone in the oral sphere of piglets (Guiraudie *et al.*, 2003). In the present work, we used the same strategy to functionally identify proteins that could act as carriers of these components in the maternal fluids.

Materials and methods

Animals and fluids

Three maternal fluids (amniotic fluid, colostrum and milk) were collected from sows (*Sus scrofa*; Large White breed) in the Nouzilly laboratory (INRA, France). The colostrum was collected during the 2 days following parturition, the milk was collected from the 3 weeks milking sows and the amniotic fluid was collected on their piglets just after birth. Each fluid was stored at −20°C until use.

Characterization of the maternal pheromone components in maternal fluids

The volatile composition of sow amniotic fluid, colostrum, and milk was determined by solid-phase microextraction (SPME), followed by gas chromatography–mass spectrometry (GC/MS) analysis. The SPME holder was equipped with a CW/DVB 65 μm fibre (Supelco®). The fibre was plunged into the solution for 1 min and then directly desorbed in the heated injector of the chromatograph. GC analyses were conducted on a Varian 3400 Cx gas chromatograph equipped with a 30 m, 0.32 mm i.d., 0.5 μm (df), Rtx-WAX column (Restek, Bellefonte, PA) using the following temperature program: maintained at 50°C for 1 min, heated to 160°C at 15°C/min, maintained at 160°C for 1 min and then heated from 160 to 245°C at 5°C/min, with helium as carrier gas at a pressure of 15 psi. Samples were injected in a splitless injector heated at 240°C. GC/MS analyses were conducted on a Nermag R30-10 quadrupole mass spectrometer coupled to a Delsi Di200 gas chromatograph. Mass spectra were recorded in electronic impact mode (70 eV) with a mass range of 40–340 amu. A 30 m, 0.32 mm i.d., 0.5 μm (deepth film (df)), RTX-5MS column (Restek) was used for analysis and the program temperature was raised from 50°C (maintained for 1 min) to 300°C at 10°C/min, with helium as carrier gas at a pressure of 12 psi. Samples were injected in a Splitless injector heated at 250°C. For identification, standards (Sigma-Aldrich) were co-injected with the samples and analysed in the same conditions as described above.

Analogues of pig appeasing compounds

Tritiated tetradecanoic acid ([9,10-^{3}H]myristic acid, [^{3}H]C14:0), hexadecanoic acid ([9,10-^{3}H]palmitic acid, [^{3}H]C16:0), 9-octadecenoic acid ([9,10-^{3}H]oleic acid, [^{3}H]C18:1) and 4-pregnene-3,20-dione ((1,2,6,7-^{3}H[N])progesterone, [^{3}H]Pro) were from Sigma-Aldrich. Radiolabelled analogues of decanoic acid ([1-^{14}C]capric acid, [^{14}C]C10:0), dodecanoic acid ([11,12-^{3}H]lauric acid, [^{3}H]C12:0) and 9,12-octadecadienoic acid ([9,10,12,13-^{3}H]linoleic acid, [^{3}H]C18:2) were purchased from American Radiolabeled Chemicals Inc. (Saint-Louis, MO). The tritiated 5-α-androst-16-en-3-α-ol ([^{3}H]androstenol, [^{3}H]And) was a gift from Dr Barbara Wasowska. The specific activity was 1.9 GBq/mmol for [^{14}C]C10:0, 2.2 TBq/mmol for [^{3}H]C12:0, 2 TBq/mmol for [^{3}H]C14:0, 1.8 TBq/mmol for [^{3}H]C16:0, 1.2 TBq/mmol for [^{3}H]C18:1, 1.4 TBq/mmol for [^{3}H]C18:2, 3.4 TBq/mmol for [^{3}H]Pro and 6.0 TBq/mmol for [^{3}H]And. For simplicity, the radiolabelled analogues are referred to as unlabelled molecules (e.g. C18:1 refers to [^{3}H]C18:1).

Preparation of protein samples

The proteins were extracted from 1 vol. of each of the three maternal fluids using 2 vols of chloroform/methanol (2:1) on ice. After centrifugation (15 000 *g* for 15 min at 4°C), the aqueous phase was collected, then evaporated in a speed-vac and stored at −20°C.

Electrophoresis, Western blot and binding assay

The crude protein extracts were solubilized in 20 μl electrophoresis buffer and subjected to non denaturing-polyacrylamide

gel electrophoresis (native-PAGE, 16.8% acrylamide) at 150 V, then the gels were stained with a colloidal Coomassie blue R solution (Nagnan-Le Meillour *et al.*, 1996). The protocol for immunodetection by Western blot was previously described (Guiraudie *et al.*, 2003). Rabbit antiserum against porcine odorant binding protein (OBP) (a gift from Pr Paolo Pelosi) was used as the primary antibody at a 1:10 000 dilution. Horseradish peroxidase-labelled anti-rabbit Ig (Amersham) were used as the secondary antibody at a 1:10 000 dilution. The signal was detected using the Enhanced ChemiLuminescence kit (ECL, Amersham), according to manufacturer's instructions. For the binding assay, 20 µl of crude extract of each maternal fluid was incubated with 1 µCi of each radiolabelled ligand (5 µl of ethanol solution) for 30 min on ice. Samples were immediately subjected to native-PAGE, then electroblotted 1 h onto ProBlott membranes (PVDF, Perkin-Elmer) at 4°C (400 mA constant current). Membranes were successively dipped 30 min in 7% formaldehyde and 1 h in 1 M salicylic acid for fluorography. 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-Aldrich, 0.2% in 1% acetic acid) in order to assign the radioactivity to protein bands.

In-gel digest, peptide extraction and MALDI-TOF MS analysis

Protein bands giving a radioactive signal on films were collected from gels run under the same conditions and stained with Coomassie blue. The gel bands were excised with a razor blade and put into Eppendorf tubes. Special attention was paid throughout the procedure to avoid contamination from dust and/or keratins. The excised bands were destained until clear by sequentially washing for 15–30 min with each of the two following solutions: (i) 25 mM ammonium bicarbonate and (ii) 50% acetonitrile. The gel pieces were then dehydrated by addition of acetonitrile. Excess solvent was removed, followed by drying for 20 min in a Speed Vac. Reduction was undertaken by covering gel pieces with 10 mM dithiothreitol for 45 min at 56°C. Excess solvent was removed and alkylation was performed with 55 mM iodoacetamide 30 min in the dark. After two rinses and drying, the gels were rehydrated by adding 15–30 µl of a solution containing 25 mM ammonium bicarbonate and 0.5 µg of trypsin (Promega) and incubating 10 min. Further ammonium bicarbonate (10 µl) was added to ensure complete hydration and digestion was carried out overnight at 37°C. The extracted peptides were loaded onto the target plate after mixing 0.5 µl of each solution with the same volume of matrix solution (4-hydroxy-α-cyanocinnamic acid, 10 mg/ml in 0.1% TFA/acetonitrile 2:1 v/v) and left to dry. Mass spectra were acquired on a Bruker Reflex III MALDI-TOF instrument equipped with a nitrogen laser with an emission wavelength of 337 nm. Spectra were obtained in the reflectron mode at an accelerating voltage of 19 kV. Deflection of the low-mass ions was used to enhance the target peptide signal. An external calibration using peptides covering the mass range 1000–3000 Da was performed for each measurement. Peptide mapping was compared with those of the Swiss-Prot database (http://www.expasy.org/tools/peptident.html).

Results

Identification of the maternal pheromone components in maternal fluids

The putative maternal pheromone is composed of six fatty acids in the following relative proportions (Pageat, 2001): hexadecanoic acid (C16:0, 35%), *cis*-9-octadecenoic acid (C18:1, 26%), (*cis*, *cis*)-9,12-octadecadienoic acid (C18:2, 22%), dodecanoic acid (C12:0, 8%), tetradecanoic acid (C14:0, 7%) and decanoic acid (C10:0, 2%). The GC traces obtained for the volatile fraction of sow maternal fluids indicated similar patterns for the three fluids (Figure 1). The comparison of mass spectra of peaks (a) to (f) with those of the NIH library in GC/MS and co-injections with standard compounds resulted in

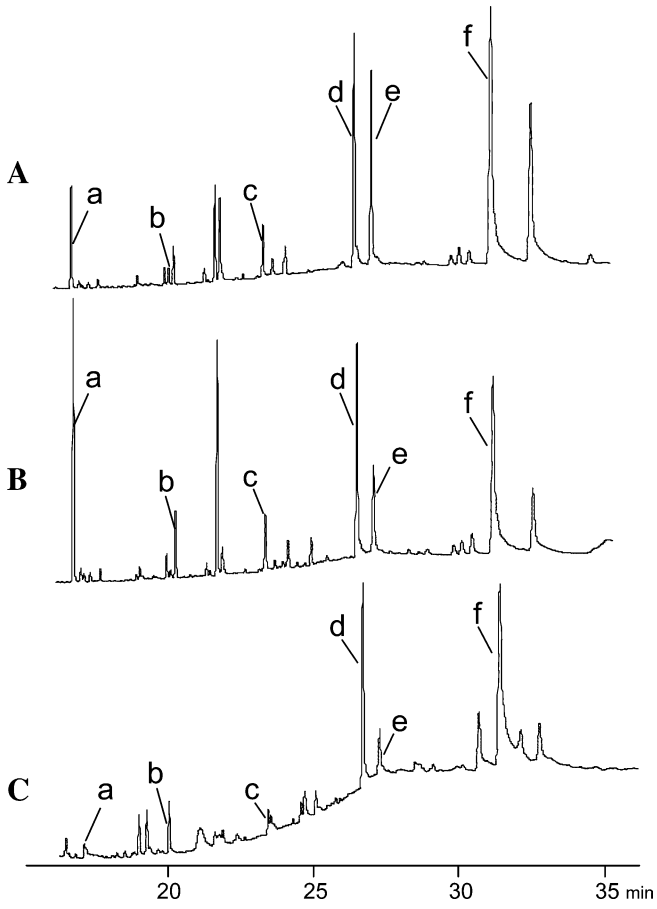

Figure 1 Gas chromatography traces of SPME analysis of sow maternal fluids. (A) Milk, (B) colostrum, (C) amniotic fluid; a, C10:0; b, C12:0; c, C14:0; d, C16:0; e, C18:1; f, C18:2.

identification of the six expected fatty acids in milk, colostrum and amniotic fluid in the proportions indicated in Table 1.

Functional identification of binding proteins in maternal fluids

The three maternal fluids issued from the same sow were compared to analyse the variability in their protein content. All experiments were performed on standardized extracts corresponding to the same quantity of each fluid. Extracts equivalent to 20 µl of each fluid were loaded on a gel and stained with Coomassie blue (Figure 2). An aliquot of piglet VNO extract was added for comparison. The protein profiles were quantitatively and qualitatively different even if some protein bands co-migrated. The number of proteins decreased from milk (M), to colostrum (C), to amniotic fluid (AF).

Radiolabelled analogues of pig appeasing compounds (fatty acids) were used as probes in binding experiments to identify binding proteins in the three maternal fluids. We additionally used two radiolabelled steroids: the progesterone, structurally related to the porcine sex steroid androstenone, and the androstenol, the other sex steroid produced by boars. Several bands differentially bound the radiolabelled compounds in the three fluids (Figure 3) and

Table 1 Relative proportions of the putative maternal pheromone components in sow maternal fluids

Fatty acids	Milk	Colostrum	Amniotic fluid
C10:0	6	18	6
C12:0	2	2	5
C14:0	3	5	2
C16:0	15	20	26
C18:1	44	39	49
C18:2	30	16	12

C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:1, oleic acid; C18:2, linoleic acid.

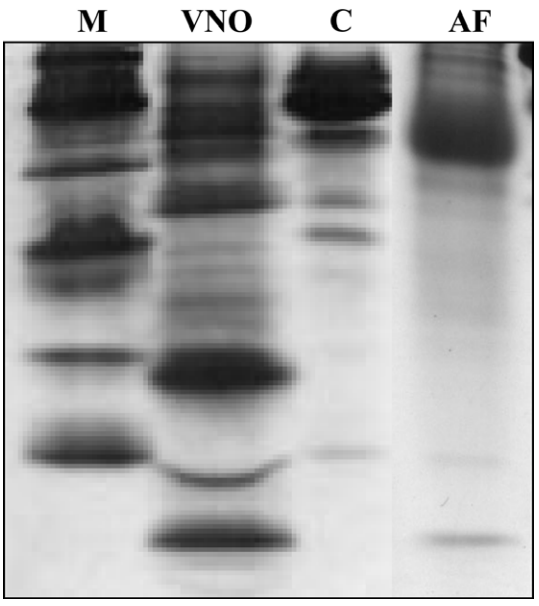

Figure 2 Protein content of sow maternal fluids extracts and piglet VNO extract. Each well contains 20 µl of maternal fluids extracts (M, milk; C, colostrum; AF, amniotic fluid) and piglet vomeronasal organ extract (VNO). Coomassie blue staining.

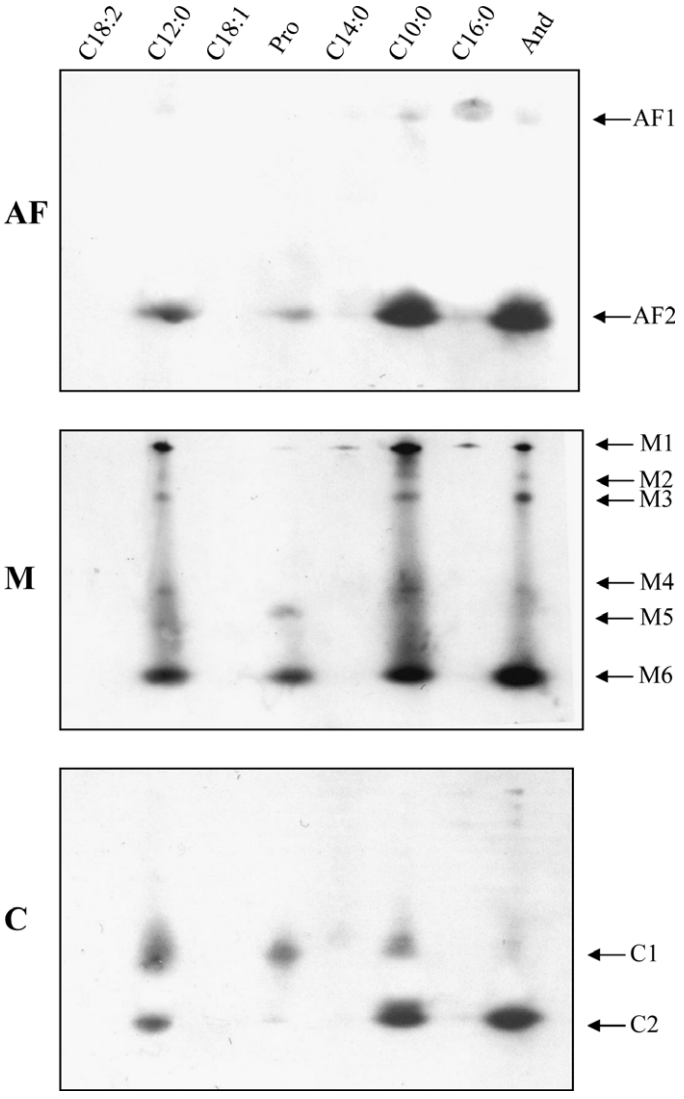

Figure 3 Binding properties of proteins extracted from sow maternal fluids. AF, amniotic fluid; M, milk; and C, colostrum. Fluorography: each well results on the incubation of 20 µl of each fluid sample (Coomassie blue staining in Figure 1) with 1 µCi of each radiolabelled compound (C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:1, oleic acid; C18:2, linoleic acid; Pro, progesterone; And, androstenol). Protein bands binding the radiolabelled analogues are numbered according to the fluids and increasing electrophoretic migration (AF1–AF2; M1–M6; C1–C2).

were analysed by mass spectrometry for characterization of their protein content.

In amniotic fluid (AF in Figure 3), protein bands numbered AF1 and AF2 were involved in binding the tested compounds. The band AF1 bound the saturated fatty acids and androstenol. Peptides map obtained after trypsin treatment matched at 78.2% with the map obtained for porcine alpha-1 acid glycoprotein (AGP; GenBank M35990, Table 2). Only small peptides of <800 Da (undetectable in our conditions) and the peptides bearing N-glycosylation sites on residues Asn5, Asn13, Asn36, Asn73 and Asn83 were not retrieved. The protein band AF2 bound the saturated fatty acids with an apparent decreasing affinity from C10:0 to C16:0, strongly bound the steroids but did not bind the unsaturated fatty acids. Peptide mapping resulted in 90.50% identity with those of porcine OBP (Swiss-Prot P81245, Table 3).

In milk, six bands (numbered M1–M6; M in Figure 3) were involved in the binding of fatty acids and steroids. Band M1 bound the saturated fatty acids C10:0 to C16:0 with an apparent decreasing affinity; the two steroids but did not bind the unsaturated fatty acids. The peptide mapping performed on M1 matched 75.7% of the sequence of porcine serum albumin precursor (SAP; Swiss-Prot P08835, Table 4). Faint binding was observed between band M2, C10:0, C12:0 and androstenol. Peptide mapping covered 44.3% of porcine whey acidic protein sequence (WAP; Swiss-Prot O46655, Table 5). This low recovery could be explained by the presence of a myristoylation (C14:0) site on lysine 39 that may disturb the digestion, generating a peptide too big to be detected. Band M3 weakly bound the small fatty acids C10:0 and C12:0, and androstenol. Peptide mapping covered 44.1% of alpha-2S casein sequence (CAS2; Swiss-Prot P39036, Table 6). Bands M4 and M5 were identified as porcine Beta-lactoglobulin I (BLG; Swiss-Prot P04119, Table 7) by peptide mapping, with 65.6 and 53.2% of sequence coverage, respectively. The proteins migrated to different positions in native-PAGE, indicating the presence of two isoforms of the BLG, together with different patterns after peptide mapping (Table 7). Indeed, the binding data showed distinct binding properties as M4 bound C10:0 and C12:0, while M5 bound the two steroids. The band M6 displayed the same binding profile as band AF2. Mass spectrometry analysis indicated 56% coverage of porcine OBP (Swiss-Prot P81245, Table 3).

In colostrum, two bands were involved in the binding with appeasing compounds (numbered C1 and C2; C in Figure 3). Peptide mapping of band C1, involved in binding with C10:0, C12:0 and the steroids (mainly progesterone), indicated a 56.3% coverage of porcine BLG (Swiss-Prot P04119, Table 7). The band C2 was identified as porcine OBP (100% identity, Swiss-Prot P81245, Table 3) and bound the appeasing compounds and steroids in the same pattern as bands AF2 and M6, but with a lower intensity.

Table 2 MALDI-TOF MS analysis of tryptic digests of reduced and carboxamidomethylated samples of band AF1 (amniotic fluid)

Peptide identification	Amniotic fluid AF1 measured mass	Theoretical mass	Modification
1–22	n.f.	2225.22	2 glycosylations
23–31	1162.53	1162.56	
32–40	n.f.	1078.49	glycosylation
41–57	1957.95	1957.96	
58–81	2934.32	2935.48	
82–88	n.f.	<800	glycosylation
89–103	1748.88	1748.91	
104–106	n.f.	<800	
107–117	1318.61	1318.68	
118–128	1227.60	1227.60	
129–137	1032.45	1032.50	
138–150	1556.67	1556.71	C_CAM :145
151–168	2125.97	2126.03	C_CAM :163
169–174	827.45	827.36	
175–176	n.f.	<800	
177–178	n.f.	<800	
179–180	n.f.	<800	
181–183	n.f.	<800	

Comparison with tryptic digests of porcine alpha-1 acid glycoprotein (AGP, GenBank No. M35990). C_CAM = carboxamidomethylated cysteine. Masses under 800 Da and over 3200 Da were not measured in our conditions. n.f., not found.

Immunodetection of OBP in maternal fluids

The presence of porcine OBP was searched in the three maternal fluids by Western blot with specific antibodies (Figure 4). Anti-OBP serum cross-reacted with the protein contained in band AF2 of amniotic fluid, band M6 of milk and band C2 of colostrum. It was necessary to increase the volume of milk and colostrum to obtain a detectable signal, suggesting that the OBP quantity in these fluids is much lower than those in amniotic fluid and VNO.

Discussion

Transnatal continuity for putative maternal pheromone components

The fatty acids described as the components of the pig putative maternal pheromone (Pageat, 2001) were identified in the three maternal fluids known to evoke positive

Table 3 MALDI-TOF MS analysis of tryptic digests of reduced and carboxamidomethylated samples of bands AF2 (amniotic fluid), M6 (milk) and C2 (colostrum)

Peptide identification	VNO OBP measured mass	Amniotic fluid AF2 measured mass	Milk M6 measured mass	Colostrum C2 measured mass	Theoretical mass	Modification
1–15	1711.77	1711.79	n.f.	1711.78	1711.79	PYRR :1
16–28	1498.69	1498.74	n.f.	1498.75	1498.74	
29–40	1408.64	1408.70	1408.70	1424.68	1408.70	MSO :39 on C2 (1424.70)
41–47		n.f.			853.40	
41–58			2196.64		2196.08	
41–50	1197.56			1197.58	1197.56	
48–58		1360.72			1361.70	
51–58	1017.46	1017.53			1017.54	
59–72	1596.76	1596.75	n.f.		1539.73	C_CAM :63
51–72				2538.24	2538.25	
73–87	1686.75	1686.74	n.f.	1686.73	1686.73	
88–120	3527.93	3527.86			3527.81	
121–133	1537.71	1537.68			1537.73	
134–152	2225.12				2224.15	
88–111			n.f.	2613.35	2613.29	
112–131			n.f.	2177.21	2177.10	
132–137		779.43	n.f.	779.43	779.44	
138–157		2240.13	2240.13	2240.13	2240.04	
153–158	648.28				648.26	

Comparison with tryptic digests of porcine odorant-binding protein (OBP, Swiss-Prot Q8WMH1 for VNO OBP and P81245 for maternal fluids OBPs). C_CAM = carboxamidomethylated cysteine; MSO = methionine sulfoxide. Masses under 600 Da and over 3600 Da were not measured in our conditions. n.f., not found.

orientation of mammalian newborns. The fluids contain other compounds that were not identified, our major goal being restricted to the search for fatty acids already described as components of the pig putative maternal pheromone.

The presence of free fatty acids in milk has extensively been demonstrated (e.g. Thompson and Brownhill, 1992; Gonzalez-Cordova and Vallejo-Cordoba, 2001), but only as nutritional elements (Aumaitre and Seve, 1978; Chilliard *et al.*, 2001) and never as potential olfactory cues. The young is successively in contact with amniotic fluid during fetal life, with colostrum in the hours following the birth, and with milk and putative maternal pheromone in the following days. During pre-natal life, the mammalian fetus develops in an environment with chemosensory information that imprints (Smotherman and Robinson, 1987; Lecanuet and Schaal, 1996) since newborns are able to discriminate the odours from amniotic fluid from the first hour after birth (Schaal and Orgeur, 1992). Lambs are even able to discriminate between amniotic fluid that bathed their chemoreceptors *in utero* and that from unfamiliar parturient ewes (Schaal *et al.*, 1995). Some authors have suggested that amniotic fluid and colostrum are similar in odour composition around birth (Marlier *et al.*, 1997). Our results suggest that the free fatty acids present in maternal fluids could constitute a chemical signature that contributes to the successful transition between the pre-natal and post-natal environments. The gradual transformation of colostrum to milk allows a progressive adaptation of the newborns to their new environment, increasing their survival (Aumaitre and Seve, 1978; Winberg and Porter, 1998; Burrin *et al.*, 1997). Behavioural tests of orientation towards these fatty acids individually or in a mixture are needed now to demonstrate that they play a role in the transnatal olfactory continuity in piglets.

Transnatal continuity for carrier proteins in maternal fluids

Binding assays, immunodetection and peptide mapping revealed that sow maternal fluids contained several proteins that could be potential carriers of putative maternal

Table 4 MALDI-TOF MS analysis of tryptic digests of reduced and carboxamidomethylated samples of band M1 (milk)

Peptide identification	Milk M1 measured mass	Theoretical mass	Modification
1–21	2496.32	2496.34	
22–32		<925	
33–42	1274.64	1274.64	
43–63	2496.28	2496.27	C_CAM:56
64–66		<925	
67–73		<925	
74–95	2497.08	2497.09	C_CAM :75.84
96–103	927.55	929.52	C_CAM :97
104–128	3193.37	3193.34	C_CAM:112.113.123
129–136		<925	
137–158	2621.28	2621.27	C_CAM :145
159–166	1083.53	1083.59	
167–183	n.f.	2164.11	
184–195	1429.59	1429.57	C_CAM :189.190
196–230		*	
231–243	1532.76	1533.86	
244–261		*	
262–283	2612.23	2612.16	C_CAM:266.267.274
284–295	1472.65	1471.67	C_CAM :286
296–307	1532.76	1532.78	C_CAM :299.300
308–315	927.55	929.45	
316–338	2768.35	2768.31	C_CAM :337
339–344		*	
345–357	1609.81	1609.78	
358–369	1455.82	1455.80	
370–384	1782.85	1784.85	C_CAM :381.382
385–397	1455.62	1455.64	
398–406	1072.56	1072.56	
407–410		<925	
411–418	1082.53	1081.49	
419–431	1479.80	1479.76	
432–435		<925	
436–450	1072.56	1071.56	
451–466		*	
467–480	1652.87	1652.83	C_CAM :469
481–487		<925	
488–505	2136.89	2136.09	
506–521	1909.99	1909.94	C_CAM :508
522–542	2485.14	2495.12	C_CAM :535
543–555	1512.67	1511.95	
556–578	2621.28	2621.39	
579–595	1918.82	1918.78	C_CAM :579.580.588
596–605		*	

Comparison with tryptic digests of porcine serumalbumin precursor (SAP, Swiss-Prot P08835). C_CAM = carboxamidomethylated cysteine; MSO = methionine sulfoxide. Masses under 725 Da and over 3200 Da were not measured in our conditions. n.f., not found. *, several peptides of mass <925 Da.

Table 5 MALDI-TOF MS analysis of tryptic digests of reduced and carboxamidomethylated samples of band M2 (milk)

Peptide identification	Milk M2 measured mass	Theoretical mass	Modification
1–39		>3200	
40–43		<800	
44–48		<800	
49–60	1309.71	1309.75	C_CAM:50
61–63		<800	
64–79	1879.95	1879.94	C_CAM :64.76
80–92	1526.57	1524.62	
93–102	1306.47	1306.41	C_CAM :94.95.99
103–113	n.f.	1201.54	

Comparison with tryptic digests of porcine whey acidic protein (WAP, Swiss-Prot O46655). C_CAM = carboxamidomethylated cysteine. Masses under 800 Da and over 3200 Da were not measured in our conditions. n.f., not found.

pheromone components and steroids, as they differentially bound these compounds *in vitro* (Table 8). They either belong to the lipocalin superfamily of carrier proteins (AGP, BLG and OBP) or have been described as carriers of different types of ligands in milk (SAP, WAP and CAS2).

Three proteins, SAP, WAP and CAS2, are milk-specific. SAP has been found before in sow milk (Klobasa *et al.*, 1987) and also that of humans (Murakami *et al.*, 1998). This protein is known to bind calcium, sodium and potassium ions, bilirubin, drugs, hormones and fatty acids (Perez *et al.*, 1993). Human SAP displays multiple binding sites that can be occupied by fatty acids and drugs (Rashid *et al.*, 1998; Colmenarejo, 2003). WAP is secreted by mammary glands, and its expression is under control of hormonal peptides, steroids and developmental signals during gestation

Table 6 MALDI-TOF MS analysis of tryptic digests of reduced and carboxamidomethylated samples of band M3 (milk)

Peptide identification	Milk M2 measured mass	Theoretical mass	Modification
2–25	2877.64	2876.33	MSO :4
26–33	908.53	908.49	
34–46	1539.81	1539.64	C_CAM :37.41
47–49		<900	
50–71	n.f.	2284.04	
72–85		*	
86–92	913.47	913.47	
93–114	n.f.	2682.33	
115–147		>3200	
148–159	n.f.	1446.61	
160–170	1248.57	1248.60	
171–176		*	
177–183	971.50	970.53	
184–191	1082.50	1082.56	
192–198		<900	
199–207	1124.55	1124.60	
208–218	1318.64	1318.71	
219–220		<900	

Comparison with tryptic digests of porcine alpha 2S-casein (CAS2, Swiss-Prot P39036). C_CAM = carboxamidomethylated cysteine, MSO = methionine sulfoxide. Masses under 900 Da and over 3200 Da were not measured in our conditions. n.f., not found. *, several peptides of mass<900 Da.

Table 7 MALDI-TOF MS analysis of tryptic digests of reduced and carboxamidomethylated samples of bands M4 and M5 (milk) and C1 (colostrum)

Peptide identification	Milk M4 measured mass	Milk M5 measured mass	Colostrum C1 measured mass	Theoretical mass	Modification
1–14	1603.79	1603.79	1603.79	1605.79	MSO:7
15–35	2171.11	2171.11	2171.11	2171.11	
36–40	n.f.	n.f.	n.f.	<800	
41–60	2211.11	2211.11		2213.20	
41–61			2369.33	2369.30	
62–74			1672.89	1673.86	C_CAM:66
61–74	1672.89	n.f.		1673.86	C_CAM:66
75–83	n.f.	n.f.	n.f.	1018.59	
84–124	n.f.	n.f.	n.f.	>3200	
125–133	1088.54		1088.54	1088.55	
125–135		1344.30		1345.69	
136–141		n.f.		<800	
142–160		2239.12		2240.18	C_CAM:158
134–148	1765.77		n.f.	1763.93	
149–159	1356.54		1355.82	1356.69	C_CAM:158

Comparison with tryptic digests of porcine beta-lactoglobulin (BLG, Swiss-Prot P04119). C_CAM = carboxamidomethylated cysteine; MSO = methionine sulfoxide. Masses under 800 Da and over 3200 Da were not measured in our conditions. n.f., not found.

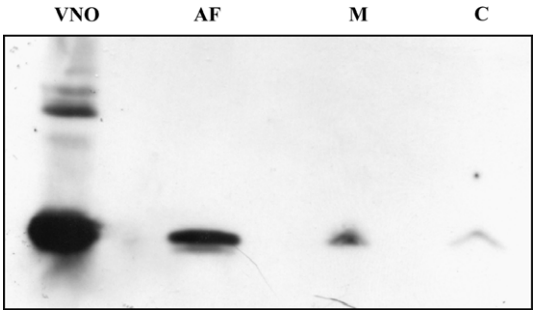

Figure 4 Immunodetection of OBP in sow maternal fluids by Western blot. AF, amniotic fluid (20 µl); M, milk (50 µl); C, colostrum (50 µl). A sample of piglet VNO (10 µl) was used as control. Anti-serum against porcine odorant binding protein (anti-OBP) was used at a 1:10 000 dilution. Enhanced chemiluminescence (ECL) detection (1 min exposure).

(Simpson *et al.*, 1998). WAP has been described as a protease inhibitor and may play an important role in the mammary gland development and tissue remodelling (Wall *et al.*, 1991). CAS2 is also a mammary gland protein secreted in milk (Alexander *et al.*, 1992).

Among the lipocalins, BLG was identified as common to colostrum and milk. This is a major protein of milk, present as two isoforms with either Gln or Thr at position 138 (Bell *et al.*, 1981). In addition, the protein can bear post-translational modifications, such as glycosylations, explaining why several predicted peptides were not retrieved after trypsin digestion (Table 7). Two isoforms could be observed in milk, differing in their migrating positions and binding properties (Figures 2 and 3). The M4 isoform bound C10:0 and C12:0, whilst M5 isoform bound the two steroids. A single form was observed in colostrum extracts, which bound the same fatty acids and the two steroids. Several binding kinetic studies between bovine BLG and hydrophobic ligands have previously been performed. Sawyer and Kontopidis (2000) showed that BLG binds fatty acids such as C12:0 and C16:0 with high affinity (K_ds of 7.0×10^{-7} and 1.0×10^{-7} M, respectively), and retinoic acid, retinol and cholesterol with similar K_d values. Bovine BLG also binds insect pheromones (Lamiot *et al.*, 1994) which are fatty acid derivatives: dodecanol ($K_d = 3.4 \times 10^{-7}$ M) and dodecyl acetate ($K_d = 2.6 \times 10^{-7}$ M). The binding affinity appears to be modulated by the double bonds position and the

Table 8 Comparison of the binding between the proteins identified in maternal fluids and the components of pig putative maternal pheromone and steroids (apparent affinities)

Compounds tested	Maternal fluids proteins									
	AGP AF1	SAP M1	WAP M2	CAS2 M3	BLG M4	BLG M5	BLG C1	OBP AF2	OBP M6	OBP C2
C10:0	+	++	++	++	+		+++	++++	+++	++++
C12:0	+	++	++	++	+		+++	+++	+++	++
C14:0	+	+	+				+++	++	+	
C16:0	+	+	+					+	+	
C18:1										
C18:2										
Pro						+		+++	++	
And	+		+	++	+	+	+	++++	+++	+++

C10:0, capric acid, C12:0; lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:1, oleic acid; C18:2, linoleic acid; AGP AF1, alpha-1 acid glycoprotein from band AF1 of amniotic fluid; SAP M1, serumalbumin precursor from band M1 of milk; WAP M2, whey acidic protein from band M2 of milk; CAS2 M3, alpha 2S-casein from band M3 of milk; BLG M4, beta-lactoglobulin I isoform from band M4 of milk; BLG M5, beta-lactoglobulin I isoform from band M5 of milk; BLG C1, beta-lactoglobulin I from band C1 of colostrum; OBP M6, odorant binding protein from band M6 of milk; OBP AF2, odorant binding protein from band AF2 of amniotic fluid and OBP C2, odorant binding protein from band C2 of colostrum.

aliphatic chain length. Porcine BLG behaves similarly as it binds the shorter and saturated fatty acids and not the *cis*-9 unsaturated acids (C18:1 and C18:2). In addition, bovine BLG bound to retinol remains able to bind insect pheromones, indicating that the protein possesses two different binding sites (Lamiot *et al.*, 1994). Nevertheless, the biological function of BLG is still speculative, although it is assumed to be a carrier of fatty acids and retinol (Sawyer and Kontopidis, 2000), a point supported by our results.

The protein content of amniotic fluid is particularly interesting: the two proteins, AGP and OBP, involved in the binding of selected ligands are known to bind these components in nasal and vomeronasal organ extracts of piglets (Guiraudie *et al.*, 2003). AGP specifically binds C12:0 in the oral sphere of piglets (Guiraudie *et al.*, 2003), and the other saturated fatty acids and androstenol in sow amniotic fluid (Table 8).

If the previous proteins were already found in the sow maternal fluids, the presence of OBP has never been reported outside the oral sphere. The protein was characterized both by mass spectrometry and using specific antibodies in the three sow fluids. When comparing the binding properties of OBP from piglet VNO (Guiraudie *et al.*, 2003) and OBP from amniotic fluid, they appear to differentially bind the selected ligands. OBP from the VNO binds the unsaturated fatty acids C18:1 and C18:2, binds the saturated fatty acids with an increasing apparent affinity according to the chain length, and does not bind progesterone (Guiraudie *et al.*, 2003). In this study, OBP from amniotic fluid did not bind the unsaturated fatty acids, bound the saturated fatty acids with a decreasing affinity according to their chain length and strongly bound the two steroids. The same observations were made for OBPs from milk and colostrum. Thus, OBP from piglets and OBP from sow maternal fluids differentially behave towards appeasing fatty acids and steroids. These binding properties could be explained by the results obtained by peptide mapping and mass spectrometry on the four OBP samples (Table 3). For OBPs contained in sow maternal fluids, a peptide of mass 2240,13 Da was obtained, corresponding to the C-terminal sequence ENGIPEENIVNIIERDDCPA (amino acids 138–157), typical of the OBP isolated from adult nasal mucus (Scaloni *et al.*, 2001; Swiss-Prot P81245). The digestion of OBP from piglet VNO gave a peptide of mass 648.28 Da, corresponding to the C-terminal sequence DDCPAK (amino acids 153–158), indicating the presence of an additional lysine residue, typical of the OBP sequence obtained by molecular cloning from piglet nasal and vomeronasal tissues (Guiraudie *et al.*, 2003; Swiss-Prot Q8WMH1). These data confirm the existence of a juvenile form of the porcine OBP with binding properties different from those of the adult form (Guiraudie *et al.*, 2003). Moreover, they suggest that OBP from amniotic fluid is of maternal origin, together with OBP(s) from milk and colostrum, which are secreted by the same mammary glands. If the six proteins identified in maternal fluids are able to act as carriers of putative maternal pheromone components and steroids and function as a relay between the maternal fluids, the OBP is the only protein common to the three fluids. The presence of proteins described in the oral sphere, in the amniotic fluid supports the hypothesis of their involvement in the detection of odours by the fetus. In the pig, the accessory bulb and vomeronasal organ are functional *in utero* (Salazar *et al.*, 2004), allowing detection of chemical and biochemical signals, to which the proteins described in this study could participate. It is noteworthy that similar proteins secreted in the oral sphere and maternal fluids, such as rodent MUPs in the nasal cavity and urine, are

involved in chemical communication. This addresses the question of a potential exchange of ligand between the maternal carriers proteins (fluids) and the juvenile carrier protein (nasal mucus) in the olfactory detection mechanism.

Despite the qualitative differences between amniotic fluid, colostrum and milk, several proteins can act as a relay of the proteins present in the previous biological fluid to transport the chemical signals emitted by the mother. This work represents the first investigation to highlight proteins involved in the transport of odorants from mother to young through the biological fluids, supporting the existence of a transnatal olfactory continuity (Coureaud *et al.*, 2000; Schaal *et al.*, 1995). Moreover, the ligand-oriented approach provides a powerful tool for functional identification of proteins involved in the binding of odour and pheromone signals in biological fluids.

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