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Pre-birth sense of smell in the wild boar: the ontogeny of the olfactory mucosa

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ABSTRACT

Animals recognize their surrounding environments through the sense of smell by detecting thousands of chemical odorants. Wild boars (*Sus scrofa*) completely depend on their ability to recognize chemical odorants: to detect food, during scavenging and searching partners, during breeding periods and to avoid potential predators. Wild piglets must be prepared for the chemical universe that they will enter after birth, and they show intense neuronal activity in the olfactory mucosa. With this in mind, we investigated the morpho-functional embryonic development of the olfactory mucosa in the wild boar (in five stages before birth). Using mRNA expression analysis of olfactory marker protein and neuropeptide Y, involved in the function of olfactory sensory neurons, we show early activation of the appropriate genes in the wild boar. We hypothesize olfactory pre-birth development in wild boar is highly adaptive.

1. Introduction

Olfaction plays a central role in wildlife adaptation, representing the most important and crucial sense for most mammals through a complex pathway starting in the olfactory epithelium (Nguyen et al., 2012). The relevance of olfaction during searching for food and intraspecific communication has been extensively investigated (Filsinger and Fabes, 1985; Rinaldi, 2007). Furthermore, even before birth, maternal amniotic odor can stimulate the fetus, particularly in kin recognition (Schaal and Orgeur, 1992), but the exact mechanism has not yet been elucidated. Young animals can start learning which food types are healthy and nutritious from the mother before weaning (Oostindjer et al., 2011) and even before birth.

In humans, at about 30 weeks amniotic fluid flows through the nasal and oral cavities to help the fetus "to smell" (Schaal et al., 1995); before that time tissues plug up the nasal cavities. It has also been observed that there is a highly selective neonatal response to familiar amniotic fluid odors, consistent with the hypothesis of detection and storage of the unique chemosensory information available to the fetus in the prenatal environment (Schaal et al., 1998). Interestingly, domestic piglets are most attracted to the odors associated with maternal feces and skin secretions at 12 h of age with a very acute ability to discriminate between mother and non-mother odors (Morrow-Tesch and McGlone, 1990). In several non-human mammals, the fetus is capable of olfactory learning and in some species neonates are attracted to the odor of amniotic fluid as a consequence of fetal exposure (i.e. prenatal olfactory learning) (Varendi et al., 1996). Studies on prenatal flavor learning have shown that the offspring of several species, such as humans and rats, show a preference to, or reduced aversion against, flavors to which they have been exposed before birth (Bilko et al., 1994; Hudson and Distel, 1999; Schaal et al., 2000; Mennella et al., 2001). A cross-species comparison among humans, rodents and sheep confirmed fetal olfactory learning (Schaal and Orgeur, 1992).

In the porcine genome a large olfactory receptor gene family has been found: over 1,301 olfactory receptor genes and 343 partial olfactory receptor genes (Groenen et al., 2012). This large number of genes could explain the ability to perceive a large spectrum of odors in *Sus*, implying a strong reliance on abilities such as scavenging for food or looking for a mate, for both the pig and the wild boar (Pearce and Hughes, 1987; Kristensen et al., 2001; Mendl et al., 2002; McLeman et al., 2005, 2008).

The wild boar is the most widespread ungulate in the world (Larson et al., 2005). Since the 1960s, wild boars have undergone a worldwide population expansion that has increased their overall geographic distribution as well as their population density in many areas within their range (Apollonio et al., 2010; Maselli et al., 2016). This spectacular demographic expansion was due to its adaptability (Rosell et al., 1998; Fulgione et al., 2016), its ability to invade different environments almost indiscriminately and human introduction (Abaigar et al., 1994; Baskin and Danell, 2003; Schley and Roper, 2003; Acevedo et al., 2006; Maselli et al., 2014b). It is impor-

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tant to point out that the two *Sus scrofa* forms dwell in very different habitats: wild boar inhabits forest and natural habitats, whereas pig lives in a man-made environment, depleted of biodiversity and offering fewer sensory stimuli (Kareiva et al., 2007). Probably domestication affects the olfactory abilities of some species, especially with respect to the detection and processing of environmental odorants in the female (Bisch-Knaden et al., 2014). In some mammals, there has been a strong reduction in the sense of smell and of brain size associated with domestication (Lega et al., 2015; Maselli et al., 2014b). Therefore, it seems that wild piglets, when compared with domestic pigs, must be ready for the chemical universe they will inhabit. Here we report for the first time on differences in the morpho-functional dynamics of the prenatal olfactory mucosa in the wild boar.

2. Materials and methods

2.1. Sample collection

Data on the litter size of wild boar are not easy to obtain because field observations can be inaccurate or incorrect, so hunted animals (especially pregnant) were used in our study (see also Gaillard et al., 1987; Fernández-Llario et al., 1999; Fernández-Llario and Mateos-Quesada, 2005; Maselli et al., 2016). In developing these studies, sows must be sacrificed without knowing whether or not they are pregnant and which developmental stage the fetuses have reached. This makes it very difficult to collect data according to a precise time sequence. In the present study, the age of fetuses was defined after shooting by their weight (±0.01 g). Given a suid gestation period of 120 days (Vericad, 1983), the age of the fetuses (T) in days was determined using the Vericad formula established in the wild boar by Huggett and Widdas (1951): $T = (Ps^{1/3} + 2.337)/0.097$, where Ps is the average fresh weight (g) of the fetus within the litter (Vericad, 1983). We analyzed 25 wild boar fetuses, all from different litters, 5 for each developmental stage.

Our activities were carried out in the Cilento, Vallo di Diano and Alburni National Park (CVD, South Italy, 181,000 ha) in accordance with Italian national laws (157/92 and 394/91 Laws). All field protocols were approved by the Ministry of Environment (ISPRA, prot. n 24581 20/07/2014). The animals studied originated from a culling plan carried out in the National Park to control wild boar abundance and were culled by specialized hunters.

2.2. Hybridization assay

In *Sus scrofa* hybridization between wild and domestic forms may locally reach high levels, as observed in some populations in Italy. In order to separate any hybrids in our samples, we used the melanocortin receptor 1 (MC1R) locus as genetic maker associated with introgression and hybridization (Fulgione et al., 2016).

From each wild boar sample, we extracted total genomic DNA by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. The entire coding region of the MC1R gene was amplified and sequenced by using primer combinations (Maselli et al., 2014a). All sequences were compared with the wild-type references (accession number KF780580; Fulgione et al., 2016) and all wild boar samples with one or more mutations were defined as hybrids and not considered in subsequent analyses.

2.3. RNA extraction

Olfactory mucosa sections were collected from fresh samples, isolating the whole olfactory epithelium using the RNAlater RNA Stabilization Reagent (QIAGEN) for immediate stabilization of RNA in tissues.

Table 1
Sequence of primers and size of amplicons used in the semi-quantitative and quantitative PCR. NPY, neuropeptide Y; OMP, olfactory marker protein; ActB, beta-actin; PCNA, proliferating cell nuclear antigen.

Primer	Sequence 5′–3′	Amplicon size
NPY – Forward	CTCGGCGTTGAGACATTACA	157 bp
NPY – Reverse	CACTTCCCATCACCACACAG	
OMP – Forward	ACCTCACCAACCTCATGACC	170 bp
OMP – Reverse	CCCGAAGGAGATGAGGAAAT	
ActB – Forward	AGAGCGCAAGTACTCCGTGT	210 bp
ActB – Reverse	AAAGCCATGCCAATCTCATC	
PCNA – Forward	AGTGAGAAGGCCTGGCAGTA	222 bp
PCNA – Reverse	CTTTGCAGCCAATCTCCTTC	

Total RNA was extracted from the olfactory epithelium using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and genomic DNA contamination was eliminated by using RNase-free DNase (QIAGEN) for 15 min. The RNA concentration and purity were quantified using NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA) and we selected pure total RNA with an A260/A280 ratio of 2 ± 0.2. The quality was determined by 1% agarose gel electrophoresis. The reverse transcription of 2 μg total RNA to cDNA was carried out using the Access RT-PCR System Kit (Promega, Fitchburg, WI, USA). Cycling parameters included a single-step cycle at 45 °C for 45 min followed by 95 °C for 2 min.

2.4. Gene expression

We assayed the variation in gene expression of the olfactory marker protein (OMP), neuropeptide Y (NPY), and the proliferating cell nuclear antigen (PCNA) by using the primers described in Table 1. OMP is a protein involved in signal transduction, found in mature olfactory receptor neurons of all vertebrates and is a modulator of the olfactory signal-transduction cascade. OMP is starting to become expressed by mature primary olfactory sensory neurons as early as during development in mice (Farbman et al., 1980; Monti-Graziadei et al., 1980; Lee et al., 2011). NPY is a 36 amino acid peptide that mediates its action via six identified G-protein-coupled (Y1–6) receptors. In the peripheral olfactory system NPY is expressed in sustentacular cells (Hansel et al., 2001), a subpopulation of the microvillar cells (Montani et al., 2006), in the olfactory ensheathing glia (Ubink and Hökfelt, 2000), and in the nervus terminalis where it appears to modulate the olfactory epithelial activity of hungry animals (Mousley et al., 2006). It participates in the regulation of hunger, and in the modulation of the vasoconstrictor response triggered by noradrenergic neurons (Di Bona, 2002). A significant reduction in the olfactory neuronal precursor proliferation occurs in NPY-deficient mice (Hansel et al., 2001) and in NPY Y1 receptor knockout mice (Doyle et al., 2008). Thus, NPY is a good candidate neurotrophic factor with which to test our hypothesis on the differential sense of smell in *Sus scrofa.*

PCNA plays an essential role in multiple cell cycle pathways, including DNA replication, DNA elongation and DNA excision repair (Kelman, 1997). PCNA is required throughout development and maternally encoded PCNA is essential for embryogenesis (Henderson et al., 1994). PCNA is highly conserved and has been identified in a range of eukaryotes (Kelman, 1997). PCNA expression provides a useful endogenous molecular marker to monitor cell proliferation in various types of tissues (Citterio et al., 1992; Dietrich, 1993; Iatropoulos and Williams, 1996; Chieffi et al., 2000).

We analyzed a fragment of 170 bp of the coding region of the OMP gene, 157 bp of the coding region of the NPY gene and 220 bp of the coding region of the PCNA gene. For each experiment, data were normalized to the expression of the Actin B (ActB) housekeeping gene. Each sample was tested and run in duplicate.

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