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The Prenatal Maturity of the Accessory Olfactory Bulb in Pigs

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Abstract

The morphological development of the accessory olfactory bulb of the fetal pig was studied by classical and histo-chemical methods, and the vomeronasal organ and nasal septum were studied histochemically. Specimens were obtained from an abattoir and their ages estimated from their crown-to-rump length. The accessory olfactory bulb was structurally mature in fetuses of crown-to-rump length 21–23 cm, by which time the lectin Lycopersicum esculentum agglutinin stained the same structures as in adults (in particular, the entire sensory epithelium of the vomeronasal organ, the vomeronasal nerves, and the nervous and glomerular layers of the accessory olfactory bulb). These results suggest that the vomeronasal system of the pig may, like that of vertebrates such as snakes, be functional at birth.

Key words: development, domestic mammals, vomeronasal system

Introduction

The vomeronasal system (VNS) is a secondary or accessory olfactory system composed of the vomeronasal organ (VNO), the accessory olfactory bulb (AOB), the vomeronasal amygdala (VNAg) and the nerves connecting them (Wysocki and Meredith, 1987). It is present in mammals, reptiles and amphibians, but there can be striking morphological and functional differences (Eisthen, 1997; Meisami and Bhatnagar, 1998) even between the vomeronasal systems of species as closely related as mouse and rat (Halpern *et al.*, 1998; Salazar and Sánchez Quinteiro, 1998; Brennan, 2001; Salazar *et al.*, 2001). Depending on species, the VNS has been attributed with a variety of functions, including the mediation of aggregation and agonistic behaviour (Halpern, 1987). However, the most intensively studied functions in mammals—mainly rodents, but also certain livestock species—relate to reproduction (Wysocki, 1979; Halpern, 1987; Keverne, 2002).

One of the most interesting histochemical techniques to have emerged in recent years is the use of lectins. Although the interactions of lectins with animal tissues are still not totally clarified, it is clear that in general they bind to the sugar moieties of glycoconjugates expressed on cell surfaces (Sharon and Lis, 1989). Since it is currently believed that such glycoconjugates may play important roles in intercellular communication, it is possible that lectin-binding studies may be able to contribute to the elucidation of intercellular communication, its development, and its role in the structural and functional development of tissues. It is an especially valuable property of lectins as a class that different lectins bind different sugars, albeit with varying degrees of specificity. This can not only throw light on the glycolconjugates expressed by the cells of particular anatomical structures, but can also reveal the existence of internal organization of a structure into substructures or cell subpopulations that are differentiated by their different glycoconjugates, as revealed by different lectin-binding patterns. Among the structures that lectin-binding studies have in this way helped to characterize is the nervous system (Jessell *et al.*, 1990); in particular, the utility of lectin-binding studies has been corroborated by Plendl and Sinowatz (1998) for the olfactory system, and by Sugai *et al.* (1997, 2000) for the AOB and other VNS components.

In adults of the pig, a macrosmatic species and one of the few mammals from which odorant receptor genes have been isolated (Mombaerts, 1999), the sensory epithelium of the VNO, the vomeronasal nerves (VNns) and the nervous and glomerular layers of the AOB are all stained by both the L-fucose-specific lectin UEA-I (from *Ulex europaeus*) and the (oligomeric *N*-acetyl-glucosamine)-specific LEA (from *Lycopersicum esculentum*), whereas the VNns and AOB of the adult sheep are stained by LEA but not by UEA-I (Salazar *et al.*, 2000); this difference may prove to reflect subtle differences between these relatively closely related species as regards glycoconjugate-mediated intercellular

communication in the VNS. Similarly, differences between the lectin-binding patterns exhibited by a given tissue in a given species at different stages of development, such as those observed in mouse (Salazar and Sánchez Quinteiro, 2003), may reflect changes in the roles played by glycoconjugates in the structural development and/or functions of that tissue.

We have previously reported that the cartilage and the sensory epithelium and other soft tissue of the VNO of the pig are well-developed some time before birth, with morphological characteristics similar to those of the adult (Salazar *et al.*, 1998, 2003b). In the work described here our main objective was to determine whether the AOB of the prenatal pig likewise attains a degree of morphological development suggesting the possibility of prenatal function. Secondly, we examined whether the possible morphological precocity of the VNO, VNns and AOB was accompanied by early development of adult lectin-binding patterns by these structures.

Material and Methods

Seventy-one pig fetuses of various developmental stages were obtained from the abattoir, and their ages were calculated from their crown-to-rump length (Marrable, 1971) (Table 1). The heads of three adults from the same source were also examined.

For light microscopy, smaller specimens were fixed directly over 24 h in 10% formalin or Bouin's fluid, while older fetuses were washed and perfused before their heads were removed and the whole brain dissected out and immersed in the fixative. Some of the largest fetal heads were decalcified before dissection by immersion for 1 h in gently stirred 10% EDTA at room temperature. After microdissection (when necessary), the olfactory bulb was embedded in paraffin wax and transverse, saggital and horizontal sections 8–10 μm thick were cut and stained with haematoxylineosin and/or Nissl stain and cresyl violet. The same procedure was followed with adult heads, except that in this case the whole olfactory bulbs were removed directly. For examination of the VNO, and nasal septum of fetuses, transverse sections 8 μm thick were cut and stained with haematoxylin–eosin.

Lectin Histochemistry

Biotin-conjugated Ulex europaeus agglutinin I (UEA-I) and Lycopersicum esculentum agglutinin (LEA; both from Sigma Chemical Co., St Louis, MO) were detected using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were dewaxed, transferred to phosphate buffer, and then (i) incubated albumin in 0.1 M Tris buffer (pH 7.2); (ii) incubated for 24 h at 4°C with lectin at various dilutions (15 and 30 μg/ml) in 0.1 M Tris buffer containing 2% bovine serum albumin; (iii) washed for 2 × 10 min in phosphate buffer; (iv) incubated for 90 min at room temperature with Vectastain ABC reagent (1:250 in phosphate buffer); (v) washed for 10 min in phosphate buffer; and (vi) washed for 5 min with Tris–HCl (pH 7.6). Peroxidase activity was visualized under a microscope following incubation in a solution containing 0.05% 3,3′-diaminobenzidine and 0.003% H_2O_2 in 0.2 M Tris–HCl buffer (pH 7.6) and reaction-quenching with 0.2 M Tris–HCl buffer. Controls were run without lectin and with preabsorption of lectin by an excess amount of the corresponding sugar. Some sections were lightly counterstained with cresyl violet.

Table 1 Estimation of age from crown-to-rump length according to Marrable (1971)

Crown-to-rump length (mm)	Age (days)
10	11
14	15
18	20
20	25
27	30
37	35
50	40
72	45
90	50
112	55
137	60
154	65
175	70
198	75
211	80
230	85
246	90
260	95
272	100
284	105
300	110

Results

In adult pigs, the main olfactory bulb (MOB) exhibits a typical laminar organization, comprising (from outside to inside) nervous, glomerular, external plexiform, mitral, internal plexiform and granular layers that are distinguished by their being composed of different cell types or combinations of cell types (Figure 1A). In the AOB the same layers are distinguishable (Figure 1B), although cell density is generally lower than in the MOB (in particular, glomeruli are less closely packed, and granular cells do not form such well-defined parallel bands as in the MOB). At the cell level, mitral cells are not distinguishable from tufted cells in the AOB, and the cells of the AOB mitral layer will accordingly

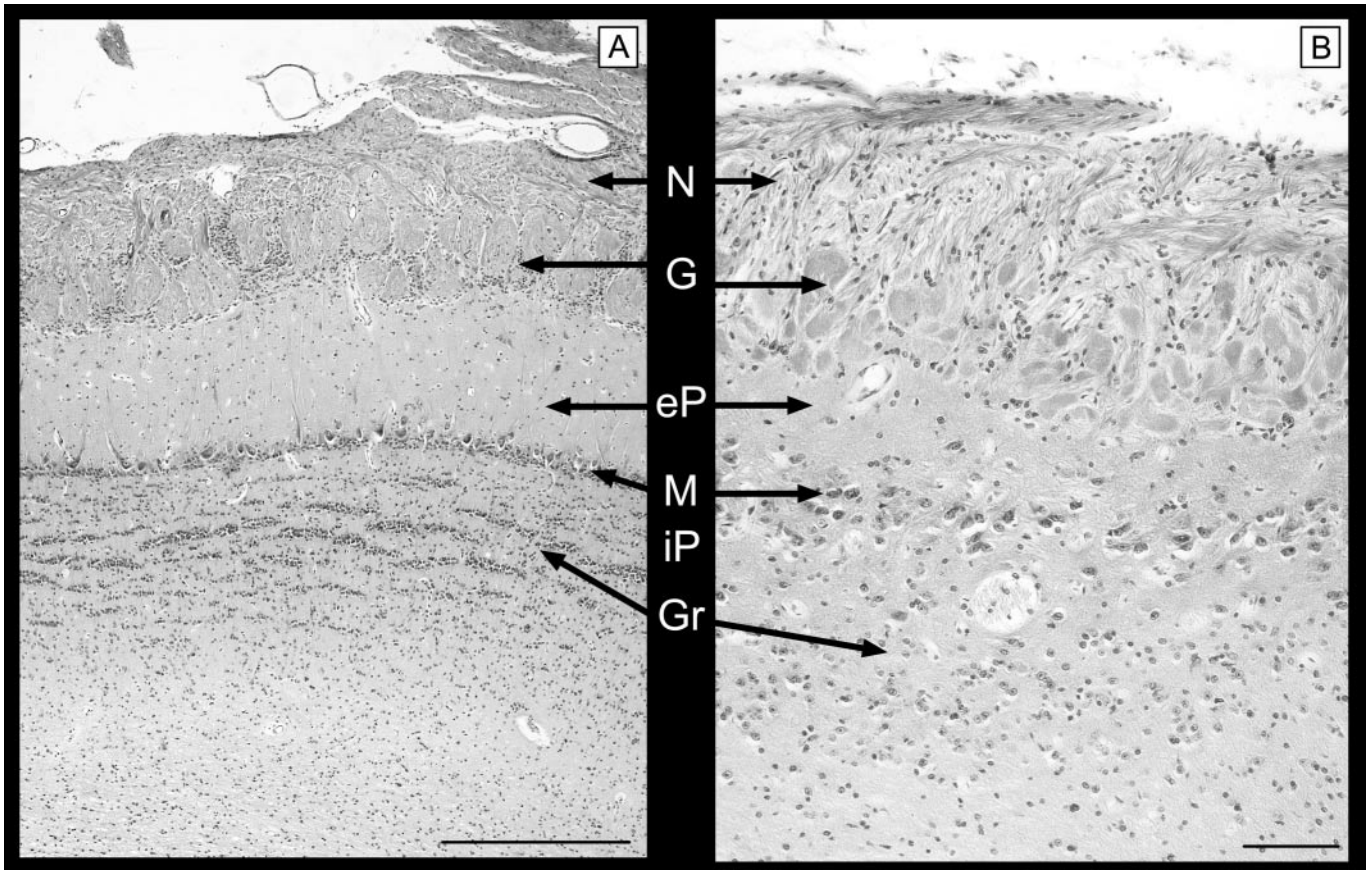

Figure 1 Nissl-stained sagittal sections of the main **(A)** and accessory **(B)** olfactory bulbs of adult pig, showing the correspondence between their nervous (N), glomerular (G), external plexiform (eP), mitral (M), internal plexiform (iP) and granular (Gr) layers. Bars = 500 μm (A), 100 μm (B). See Supplementary Material for colour version of this figure.

be referred to here as mitral/tufted (M/T) cells. Figure 2 shows sections of the structures involved in the transduction of chemical stimuli in the VNO and the transmission of the transduced signal to the AOB, together with a schematic diagram of the connections, in the glomerular layer of the AOB, between the VNns and the axons of the lateral olfactory tract (LOT), which carry the signal on to the VNAg.

Embryos and Fetuses

At about the 13 cm stage, the precursor of the AOB, composed of granular cells and variously developed M/T cells, is distinguishable as a protrusion on the dorsal surface of the MOB (Figure 3A). The granular and M/T cells persist and grow, and at the 14–17 cm stage it becomes possible to distinguish numerous small glomerulus precursors and, close by, a few isolated periglomerular cells (Figure 3B,C). By the 19 cm stage all six layers of the bulb are distinguishable (although the internal and external plexiform layers still have rather diffuse boundaries) and the M/T and granular cells are easily identified (Figure 3D). Stratification continues to progress, with clearer definition of the plexiform layers and the development of a very marked mitral layer, until about the 21–23 cm stage (Figure 3E–I); any apparent changes at subsequent ages seem to be due exclusively to changes in orientation and position of the AOB relative to the structures with respect to which the orientations and levels of sections were defined (Figure 3J–N). At these stages of development, the AOB is a complete, well-constituted tissue with a laminar organization that is very similar to that observed in adults.

Lectin Histochemistry

Of the two lectins used, only one, the *N*-acetylglucosamine-specific LEA, unequivocally stained certain structures in the tissues studied. From the 19 cm stage on, LEA consistently stained the entire sensory epithelium (SE) of the VNO and its mucomicrovillar surface (Figure 4A), the mucociliary surface of the respiratory epithelium of the VNO, the VNns in the lamina propia of the sensory mucosa (Figure 4A), and the nervous and glomerular layers of the AOB (Figure 4B) and MOB (Figure 4C). No other area of the tissues studied was labelled by LEA. The L-fucosespecific lectin UEA-I did not reproducibly stain any part of the VNS, although there sometimes occurred faint staining of the SE, the VNns or, in some sections of the larger fetuses, the nervous and glom-

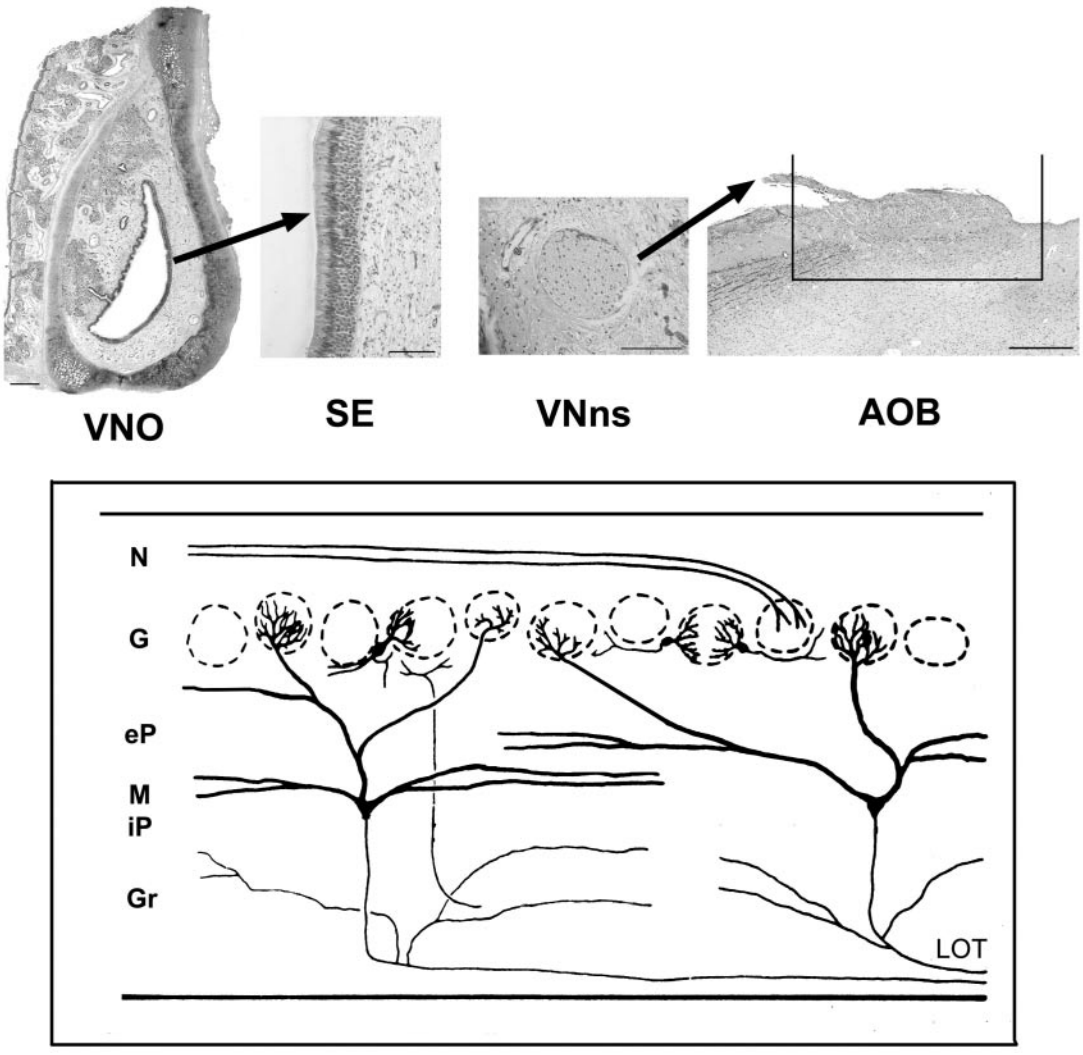

Figure 2 Top. Vomeronasal structures in adult pig: a transverse section of the vomeronasal organ (VNO), with higher magnifications of the sensory epithelium (SE) and a vomeronasal nerve (VNns); and a sagittal section of the accessory olfactory bulb (AOB). Stain, haematoxylin–eosin. Bars = 100 μm (SE, VNns), 500 μm (VNO, AOB). Bottom. Schematic representation of the layers of the AOB (abbreviations as in Figure 1).

erular layers of the AOB. All lectinless control sections were unstained.

Discussion

Studies of the olfactory systems of many adult mammals show general similarities of structure and function—the former especially—and also, in the case of species in which the adult possesses an accessory olfactory system, general morphological and functional similarities between this and the main system (Mori, 1987; Buck, 1996; Hildebrand and Shepherd, 1997; Mori *et al.*, 1999, 2000; Bozza *et al.*, 2002). Thus in spite of the between-species differences noted in the Introduction, the vomeronasal systems of the most extensively studied laboratory animals, the rodents mouse and rat, share a general pattern with those of the ungulates that have been most extensively studied in this respect, the sheep (Kendrick *et al.*, 1992, 1997; Booth and Katz, 2000; Salazar *et al.*, 2000, 2003a) and the pig (Signoret *et al.*, 1975; Leshin *et al.*, 1991; Dorries, 1992; Salazar *et al.*, 2000, 2003b); in particular, in all four cases the function of the highly developed adult VNS contributes to behaviour involved in reproduction and/or care of offspring (Estes, 1972; Wysocki, 1979).

Most newborn mammals, too, share a sense of smell founded on a well-developed main olfactory system, the morphological maturity of which is certified by the presence of an MOB with a well-formed glomerular layer (Hinds, 1968; Valverde *et al.*, 1992; Bailey *et al.*, 1999; Valverde, 1999; Puche and Shipley, 2001). Indeed, for altricial species such as the pig, which are deaf and blind at birth, neonatal olfaction is vital (Alberts, 1981; Hudson, 1993). However, relatively little is known of the status of the VNS at birth.

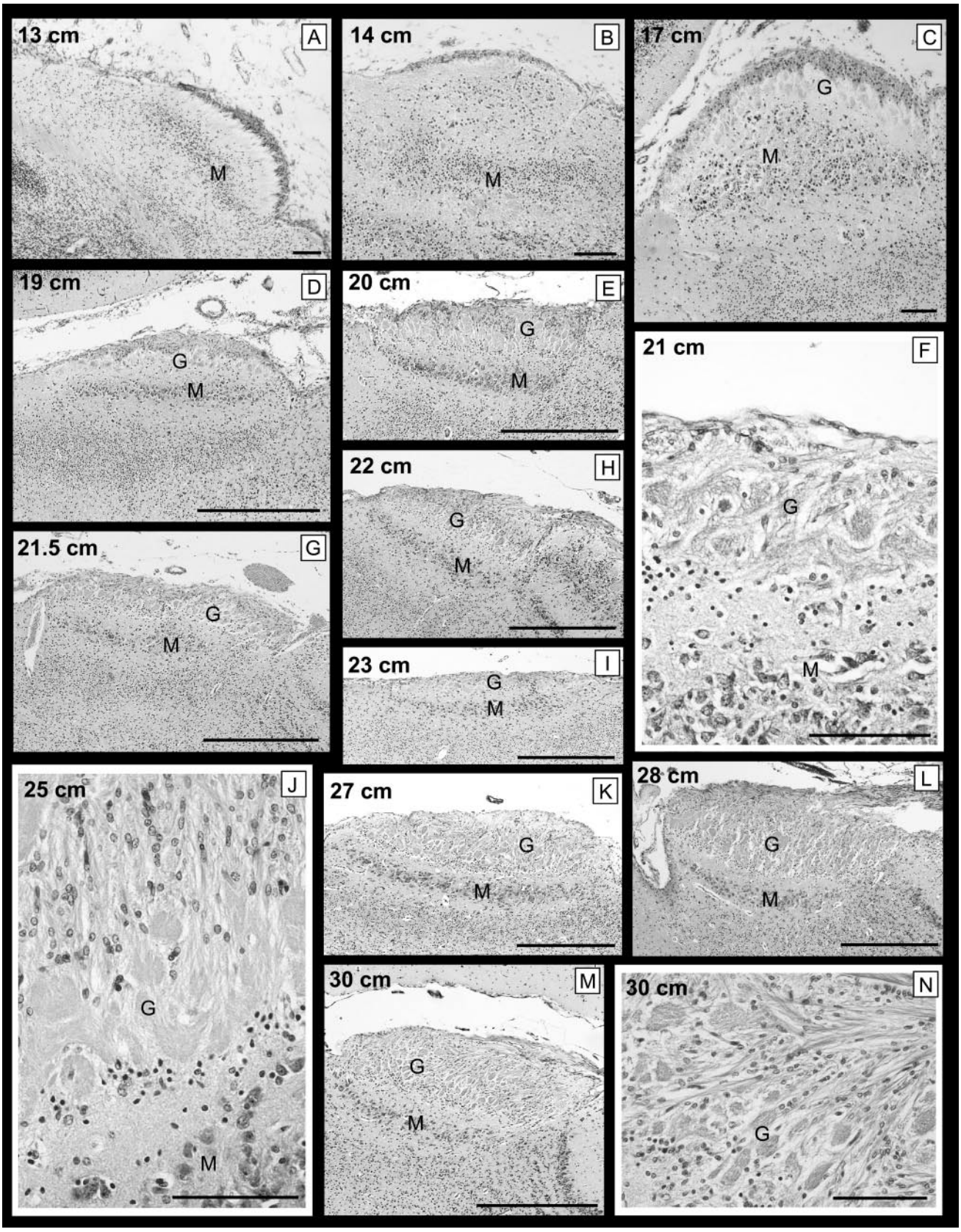

Figure 3 Nissl-stained transverse **(A–D)**, horizontal **(F, I, N)** and sagittal **(E, G, H, J–M)** sections of the AOB in fetal pigs at various developmental stages, showing the development of laminar structure and details of the glomerular layer (F, J, N). G, glomerular layer; M, mitral layer. Bars = 100 μm (A, B, C, F, J, N), 500 μm (others). See Supplementary Material for colour version of this figure.

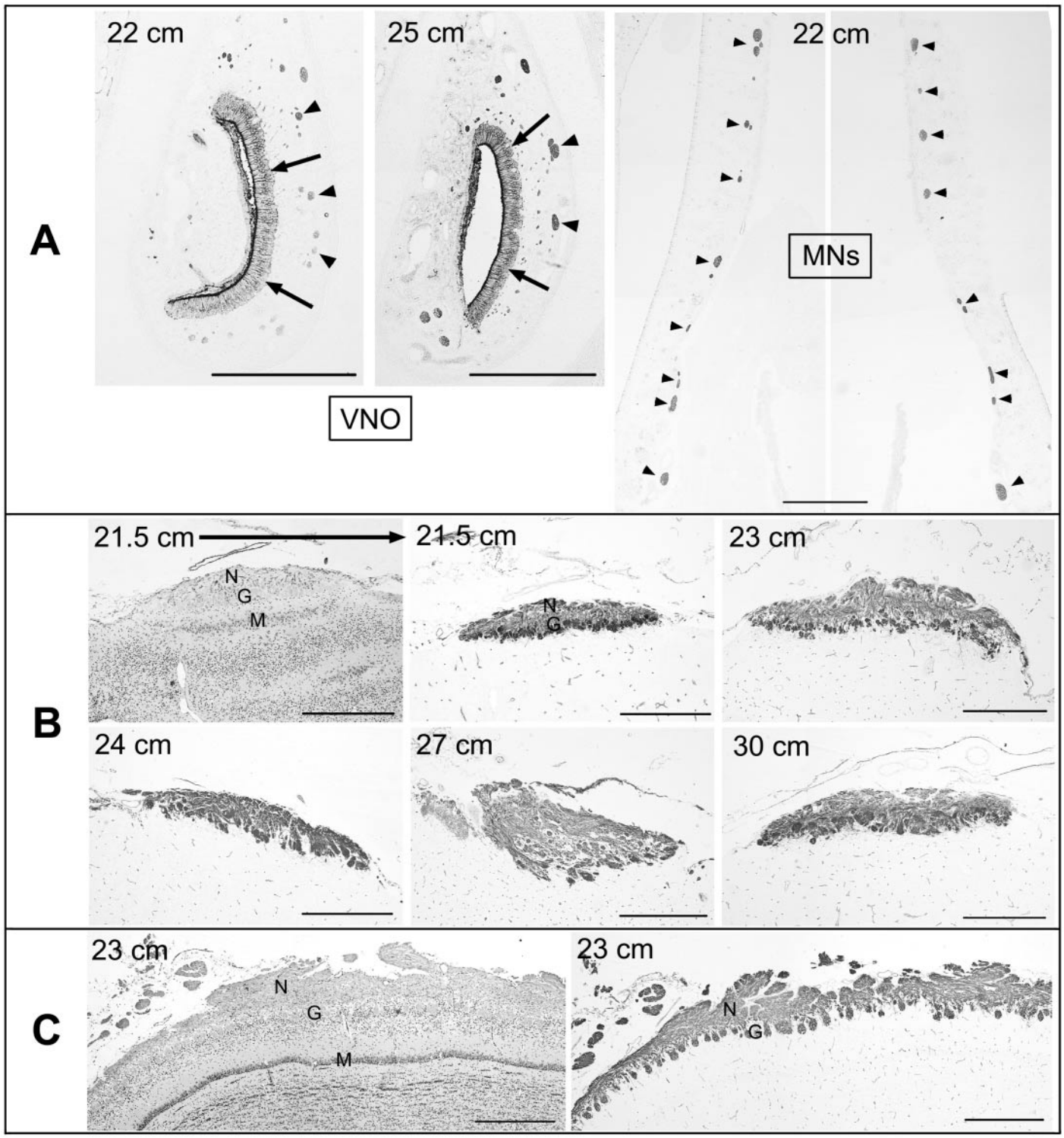

Figure 4 Olfactory structures in prenatal pigs at various developmental stages. **(A)** transverse sections of the vomeronasal organ (VNO) and of the mucosa of the nasal septum (MNs) stained with the lectin Lycopersicum esculentum agglutinin (LEA), which stained the whole of the sensory epithelium (arrows) and the vomeronasal nerves (arrowheads). **(B)** sagittal sections of the accessory olfactory bulb stained with LEA and haematoxylineosin (21.5 cm stage) or LEA alone (the others), showing in all cases LEA labelling of the nervous and glomerular layers. **(C)** sagittal sections of the main olfactory bulb stained with haematoxylin–eosin and LEA, showing labelling of the nervous and glomerular layers. G, glomerular layer; M, mitral layer; N, nervous layer. Bars = 500 μm. See Supplementary Material for colour version of this figure.

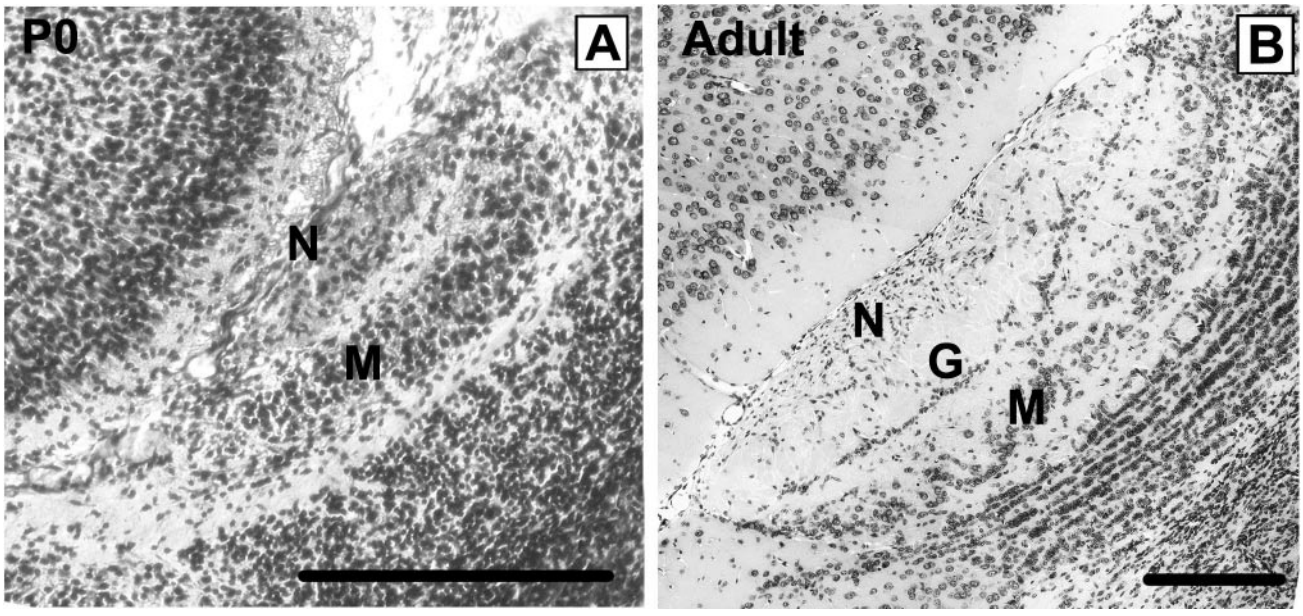

Figure 5 Nissl-stained sagittal sections of the mouse AOB. **(A)** Newborn (day 1). **(B)** Adult. G, glomerular layer; M, mitral layer; N, nervous layer. Bars = 250 μm. See Supplementary Material for colour version of this figure.

What is clear, is that neither the presence nor the absence of a fully developed VNS in the adult animal is necessarily paralleled at birth.

In mice, for example, the glomerular layer of the AOB—the last stratum to appear in vertebrates—only matures after birth (Figure 5), glomerulus formation being completed between days 3 and 5 (Salazar and Sánchez Quinteiro, 2003) [as in the case of the MOB (Bailey *et al.*, 1999; Valverde, 1999), it is necessary to distinguish between fully developed glomeruli and the 'protoglomeruli' of preceding stages]. In fact, as far as we know, a morphologically mature AOB has not been found before birth in any of the commonly used laboratory animals. On the contrary, in many animals in which a VNS develops during embryogenesis, it regresses before adulthood, either before birth or after; cases in point include crocodilians, some bats, and certain primates (Meisami and Bhatnagar, 1998); the human AOB, for example, is well documented in embryos and younger foetuses but has never been observed in adults (Humphrey, 1940; Bossy, 1980). In the case of sheep, however, the AOB, like the MOB, has a well-constituted glomerular layer well before birth, and the characteristics of this structure at this stage are retained by adults (Salazar *et al.*, 2000, 2003a).

The principal result of this study is that in pigs, as in sheep, a well-developed AOB is present not only in the adult but also at and before birth. Its presence at birth suggests that, like the main olfactory system, the VNS is functional at this stage, as in the case of certain reptiles in which the AOB also possesses all its cell layers immediately before birth, at Zehr stage 36 (Holtzman and Halpern, 1990). Certainly, a functional neonatal VNS would not lack stimuli to process, since by this stage olfactory stimuli can reach the pig VNO via its communication with the incisive duct (Wöhrmann-Repenning and Barth-Müller, 1994; Salazar *et al.*, 2003b) What the perinatal or immediately postnatal function might be will be more difficult to establish, but it is not impossible that it may be to detect pheromones triggering essential neonatal behaviour such as nipple-seeking; although the VNS is known not to be essential for this particular behaviour in rabbits (Hudson and Distel, 1986)—as could hardly be otherwise, the neonatal rabbit, like rodents, having an immature AOB—the wide between-species variations in the role of the VNS mean that its being required for nipple-seeking by the pig cannot be ruled out.

By the 21–23 cm stage, the lectin LEA behaved exactly as in the adult pig, staining all the components of the VNS up to the first relay stage (the SE, VNns and the nervous and glomerular layers of the AOB). It is therefore interesting that UEA-I, which also stains these structures in the adult, did not stain them prenatally in this study. Although this might suggest that the VNS is not fully mature at birth, it is also quite possible that what it reflects may be merely a difference between the functions of the pre- or neonatal VNS and those of the adult VNS, i.e. that the prenatal morphological maturity and expression of LEA-binding glycoconjugates allows the performance of functions that are required pre- or postnatally, while the performance of adult VNS functions requires the additional expression of UEA-I-binding glycoconjugates. In this regard, it would be interesting to determine whether the development of UEA-I reactivity between the neonatal and adult stages exhibits the same gradual

increase as has been observed in mice, or only begins at some near-adult stage (Salazar and Sánchez Quinteiro, 2003).

Supplementary Material

Supplementary material can be found at: http://www.chemse.oupjournals.org

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