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The olfactory system of the tammar wallaby is developed at birth and directs the neonate to its mother's pouch odours

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Abstract

In kangaroos and wallabies at birth the highly altricial newborn young climbs unassisted from the urogenital opening to the teat. Negative geotropism is important for the initial climb to the pouch opening, but nothing is known of the signals that then direct the neonate downwards to the teat. Here we show that the newborn tammar wallaby (*Macropus eugenii*) has the olfactory apparatus to detect smell. Both the main olfactory system and vomeronasal organ (VNO) are developed at the time of birth. Receptor cells of the main olfactory epithelium immunopositive for $G_{o\alpha}$-protein project to the three layered main olfactory bulb (MOB). The receptor epithelium of the VNO contains G-protein immunopositive cells and has olfactory knob-like structures. The VNO is connected to an area between the two MOBs. Next, using a functional test, we show that neonates can respond to odours from their mother's pouch. When neonatal young are presented with a choice of a pouch-odour-soaked swab or a saline swab, they choose the swab with their mother's pouch secretions significantly more often ($P < 0.05$) than the saline swab. We conclude that both olfactory systems are capable of receiving odour signals at birth, a function that must be a critical adaptation for the survival of an altricial marsupial neonate such as the tammar for its journey to the pouch.

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Introduction

Mammalian neonates need to be able to locate the teats to survive and grow and there is evidence that eutherian neonates rely on olfactory cues to locate them (Teicher & Blass 1977, Pedersen & Blass 1982, Stickrod *et al*. 1982, Hudson & Distel 1983). The main chemical cues involved in guiding the young can be detected by smell or taste and are apparently learned during fetal development through the presence of odour or taste in the amniotic fluid (Pedersen & Blass 1982, Stickrod *et al*. 1982). The major detecting site for these signals was thought to be the vomeronasal organ (VNO) of the accessory olfactory system (Graves & Duvall 1983). The VNO is present at some point in the development or life history of every extant tetrapod species examined so far and is regarded as a homologous feature throughout tetrapod evolution. However, the main olfactory system also senses pheromones. The mother–young aggregation pheromone in wild rabbits (Mykytowycz & Ward 1971) that guides the young rabbit to its mother's teat (Schaal *et al*. 2003) activates a specific area in the dorso-medial region of the main olfactory bulb (MOB; Teicher *et al*. 1980). Furthermore, rabbits with surgical ablation of the VNO can still locate the teats (Hudson & Distel 1986). Odour also guides newborn rats to their mothers' teats (Teicher & Blass 1977, Hudson & Distel 1983).

The detection of these pheromones depends on specific receptors in the olfactory epithelia (Kovach & Kling 1967, Tirindelli *et al*. 1998). There are two large families of G-protein-linked receptors that are expressed only in mature receptor cells of the VNO (Berghard & Buck 1996, Krieger *et al*. 1999). The V1R or V2R family receptors are unrelated to their counterparts in the main olfactory epithelium (MOE), suggesting that many active ligands are likely to act via the VNO receptors in addition to those in the main olfactory system (Halpern & Martinez-Marcos 2003). Thus, there is considerable overlap in function between the two systems.

The tammar wallaby, *Macropus eugenii*, delivers a highly altricial young after a 26.5 days pregnancy (Tyndale-Biscoe & Renfree 1987). When giving birth, kangaroos and wallabies usually sit with their tail passed forward between their legs (Tyndale-Biscoe & Renfree 1987, Renfree *et al*. 1989). The neonate crawls unassisted upwards on its mother's abdomen to reach the pouch entrance where it then switches direction and crawls downwards to the teat. There it will attach permanently for the next several weeks or months. Exactly how the newborn young manages this impressive feat of navigation is yet to be determined. The young placed on the fur while their mothers are inclined at different angles invariably climb upwards, suggesting that the young use gravity, at least initially (Cannon *et al*. 1976). Most

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marsupials have a functional vestibular system at birth complete with otoliths, cilia and microcilia (Hughes & Hall 1984, Gemmell & Nelson 1988, Gemmell & Rose 1989, McCluskey *et al*. 2008). However, since the neonates need to turn through ~180° to reach the teats within the pouch, there must be other signals that guide the young to the teats. There are olfactory sensory cells within the olfactory epithelium at birth so it is likely that olfaction plays a role in directing the young once it is near the pouch entrance (McCrady 1938, Hill & Hill 1955, Gemmell & Nelson 1988, Lin *et al*. 1988, Hughes *et al*. 1989, Renfree *et al*. 1989).

Once in the pouch, Merkel mechanoreceptors cells in the skin around the mouth in all marsupial neonates examined (Hughes & Hall 1984, Jones & Munger 1985, Gemmell *et al*. 1988, Gemmell & Rose 1989) may be important for the attachment to the teat and they may even function to stop the drive to climb upwards (Hughes *et al*. 1989). The tammar MOE primordium is visible as a thickened layer three days before birth and is connected via nerves to a pre-olfactory bulb condensation at birth (Hughes *et al*. 1989). There is also a VNO which has a tall columnar epithelium and is innervated by a branch of the nervus terminalis (Hughes *et al*. 1989). However, whether these organs are functional is not yet known, although young were not attracted to a cotton bud swab from the mother's pouch when smeared on a human forearm (Hughes *et al*. 1989). The neonatal tammar is developmentally at a stage equivalent to a 9 week human fetus or a 17-day-old rabbit fetus (Tyndale-Biscoe & Renfree 1987), and recent study of the neuronal connections of the olfactory epithelia in the tammar at birth suggested that the olfactory system is not sufficiently mature to be involved in guiding the neonate to the pouch and teat (Ashwell *et al*. 2008).

Despite its altriciality, the tammar is therefore equipped with at least rudimentary senses of balance, touch and smell, but direct evidence for the use of smell is lacking. Here we describe the structure of the olfactory systems and their function around birth in the neonatal tammar wallaby. We investigated the presence of $G_{o\alpha}$-protein and tested whether the neonatal wallaby uses smell to orientate itself towards and down into the pouch.

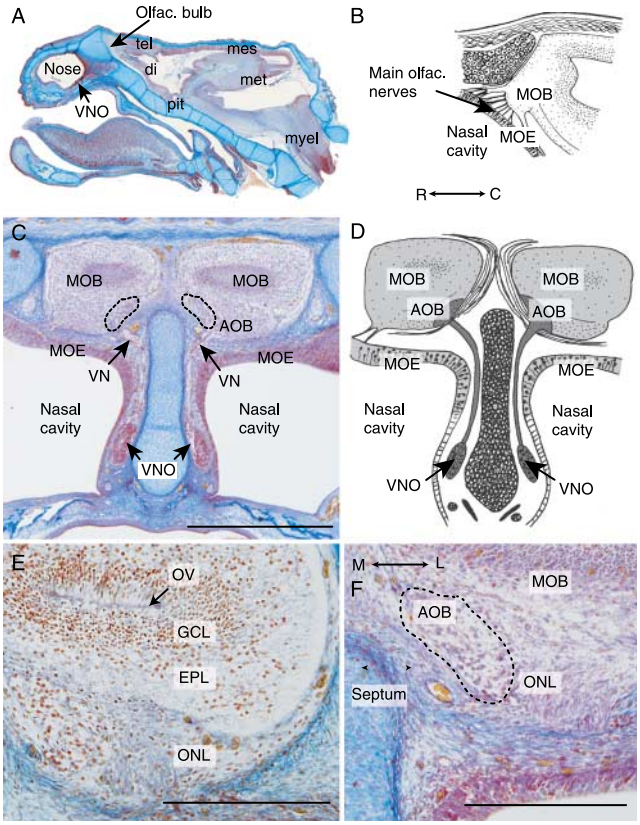

Figure 1 Olfactory bulbs and nasal cavity in the neonate tammar. (A) A sagittal section through the whole head of a neonate revealing the VNO at the base of the septum. di, diencephalon; mes, mesencephalon; met, metencephalon; myel, myelencephalon; tel, telencephalon; pit, pituitary. (B) Nerves are projecting from the main olfactory epithelium (MOE) to the olfactory bulb. R, rostral; C, caudal. (C) A coronal section of the head of a neonate tammar shows the vomeronasal organs (VNO) located medial to the nasal septum with the vomeronasal nerves (VN). Vomeronasal nerves lead along the nasal septum to the accessory olfactory bulb (AOB) dorsomedial to the main olfactory bulb (MOB; scale 500 μm). (D) A diagram of structures seen in C. (E) The neonate main olfactory bulb has an olfactory ventricle (OV, arrow) and three cell layers are visible: ONL, olfactory nerve layer; EPL, external plexiform layer; GCL, granular cell layer (scale 200 μm). (F) The AOB lies ventral from the MOB and shows only one cell layer (scale 200 μm).

Results

The main olfactory system

The overall structure of the main olfactory system, based on gross anatomy, and light and electron microscopic observations was similar in the fetus 24 h before birth and in the young in the neonatal pouch (data from neonates shown in Figs 1 and 2). The nostrils were open to the nasal cavities and the respiratory epithelium that lined the nasal cavity consisted of a single cell layer in which goblet cells were embedded. They were especially aggregated at the rostral end of the nasal cavity near the entrances of the naso-palatine ducts (NPDs) and the VNOs in the respiratory epithelium. Cilia covered the cell surfaces and goblet cells were less-electron dense than the surrounding epithelial cells and had mucous droplets in their cell bodies. The goblet cells were stained with periodic acid-Schiff's (PAS) and Alcian blue (AB) positive and the PAS staining was not altered through diastase treatment (data not shown).

The MOE lined the roof of the nasal cavity, starting rostral in the area of the entrance to the VNO. The epithelial height increased towards the caudal end of the nasal cavity reaching maximum height in the area where the VNO blindly ended. There were folds of the nasal cavity roof surface in the fetus and neonate (Fig. 3) that will form the ethmoturbinates in the adult.

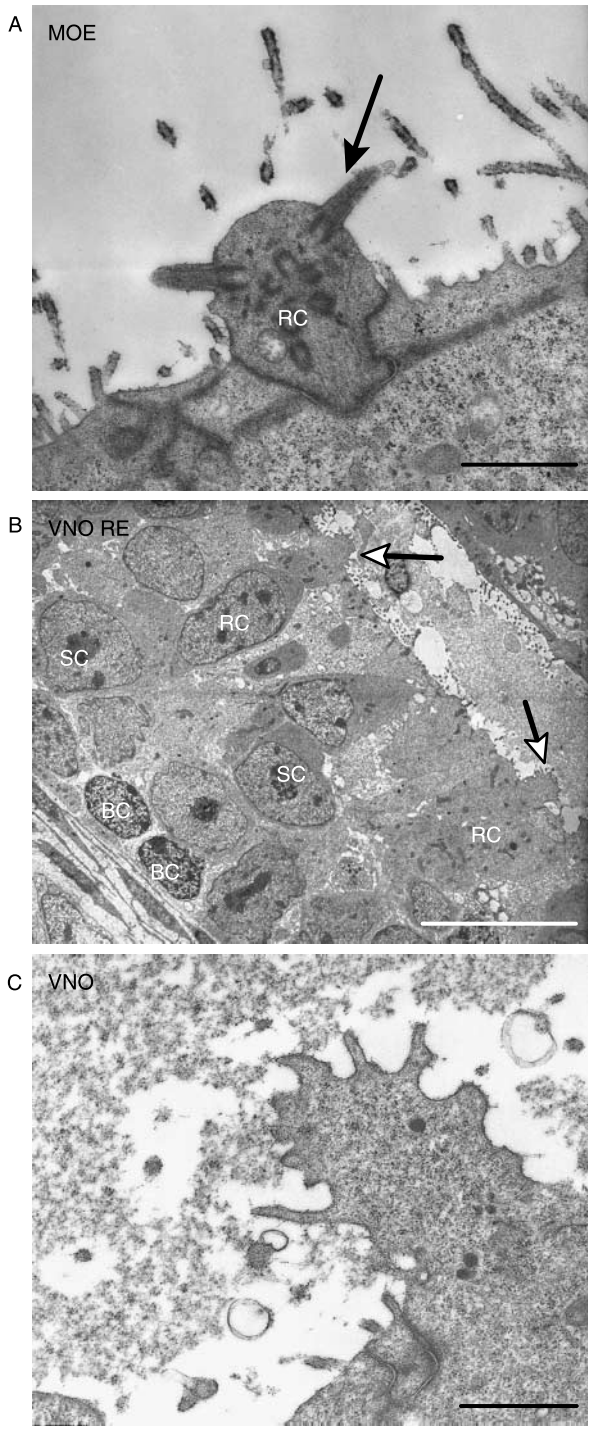

Figure 2 Electron micrographs of the main olfactory epithelium and sensory cells of the VNO of the neonate tammar. (A) The olfactory knobs on the main olfactory epithelium are covered by cilia (arrow). Microvilli are found on the supporting cells (scale 1 μm). (B) Three types of cells can be distinguished in the VNO receptor epithelium: basal cells (BC) with small electron dense nuclei located at the base of the septum, receptor cells (RC) with olfactory knob-like structures (arrows) on their surface which protrude into the VNO lumen and supporting cells (SC; scale 10 μm). (C) Olfactory knob-like structures found in the VNO receptor epithelium are covered with microvilli (scale 1 μm).

The MOE was a high pseudostratified columnar epithelium (Fig. 1C) readily distinguishable from the simple columnar respiratory epithelium. There was some positive PAS staining in the MOE and respiratory epithelium in the fetus. This disappeared or became less intense after diastase treatment. There was also some light AB positive staining of the receptor epithelial surface. In the neonate, there was some AB positive staining on the surface of the respiratory epithelium near the base of the nasal septum (not shown).

There were nerve bundles in the lamina propria underlining the MOE, of which some connected the MOE with the MOB (Fig. 1B). $G_{o\alpha}$-protein was detected in cells throughout the MOE (Fig. 3A) and in the MOB (Fig. 3B and C). Western blots for $G_{o\alpha}$-protein from mouse forebrain and tammar olfactory bulb and VNO gave a single band of the expected size at around 39 kDa (Fig. 4A). The cytoplasmic staining was found in the cell soma and in the dendrite projecting to the epithelial surface. The nerve bundles underlying the receptor epithelium were also darkly stained (Fig. 3A). There was no staining from IgG or negative controls (Fig. 4B).

The MOE was covered with microvilli and some cells also had cilia (Fig. 2A). Two types of cells were distinguishable in the MOE. One type of cell formed a bottleneck near the epithelial surface and ended with a knob-like structure that protruded into the nasal cavity (Fig. 2A). These protrusions looked like olfactory knobs and are presumably the receptor cells (Garrosa *et al.* 1998). Elongated mitochondria were very prominent in the receptor cells near the olfactory knob. The second type of cells had neither cilia on their surface nor any knob-like structure, but did have microvilli on their surface. These second type of cells were the supporting cells as previously described (Erhardt & Meinel 1979). Both cell types had large nucleoli or heterochromatin accumulations in their nuclei. There were up to four nucleoli visible in each nucleus. The epithelium was underlined by the basement membrane which was visible as a thin line under the epithelium. The lamina propria lay underneath the basement membrane which included blood vessels, nerve bundles and connective tissue.

The MOB and the lamina propria were separated by connective tissue. The MOB had three distinguishable layers in the fetus. The external layer was the olfactory nerve layer, with the external plexiform layer between it and the granular cell layer (Fig. 1E). The layers of the MOB were even more evident in the newborn. However, it was not clear if the stratum glomerulorum was present as no glomeruli were observed.

The accessory olfactory system

The VNO of the fetus was well developed and indistinguishable from the neonate (Hudson & Distel 1983). The VNO length was 0.625 ± 0.071 mm within a total head length of 7.2 ± 0.17 mm, similar to the ratio

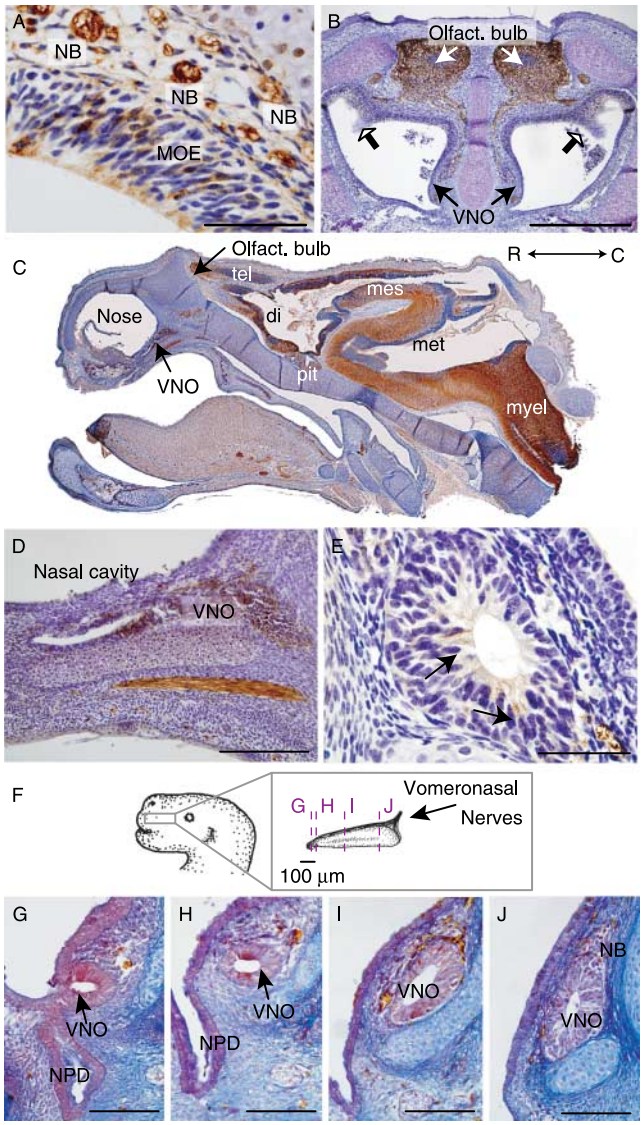

Figure 3 $G_{o\alpha}$ protein expression in the brain, MOB and VNO of the neonate tammar. (A) $G_{o\alpha}$ immunopositive staining is found in the MOE and nerve bundles (NB) underlining the epithelium (scale 50 µm). (B) A coronal section through the caudal region of the nasal cavities in the MOB show strong $G_{o\alpha}$-positive staining in the olfactory nerves and vomeronasal nerves (scale 500 µm). Ethmoturbinates (thick arrows) are visible. (C) A sagittal section through the head of a neonate that the developing brain, the VNO, trigeminal nerve and elements of the tongue were strongly $G_{o\alpha}$ immunopositive. di, diencephalon; mes, mesencephalon; met, metencephalon; myel, myelencephalon; tel, telencephalon; pit, pituitary. (D) The close-up of the sagittal section through the VNO shows many stained cells. (E) $G_{o\alpha}$ positive stained cells (arrows) are found in the receptor epithelium of the VNO (scale 50 µm). Sections A–E are counterstained with haematoxylin. (G–J) Coronal sections through the neonate's vomeronasal organ (VNO) from rostral to caudal as indicated in F. The nasopalatine duct (NPD) and VNO open into the nasal cavity (G). In J nerve bundles (NB) are visible leaving the VNO (Sections G–J stained with Mallory's Triple stain; scale for all 100 µm).

of the VNO length to head length of the newborn. The VNO opened to the nasal cavity more rostrally than the entrance of the NPD. The NPDs were already open and their entrances lay under the wing-like structures of the prominent papilla incisiva. In the most rostral region of the VNO the lumen was crescent shaped with the long axis in the horizontal plane. The long axis of the lumen rotated from the horizontal to the vertical plane towards its caudal end (Fig. 3F–J). The lumen of the tube-like organ increased in size towards the posterior end. The height of both receptor and non-receptor epithelium were similar. The receptor epithelium was pseudostratified columnar, while the non-receptor epithelium was simple columnar. No vomeronasal glands could be observed in the fetus or neonate and only small blood vessels accompanied the VNO. Some PAS and AB-positive staining was found in the VNO lumen in day 25 fetus. The PAS staining was not altered through diastase digestion. No PAS or AB positive staining could be observed in the VNO lumen of the pouch young at birth.

Structures similar to olfactory knob-like structures were found on the surface of the receptor epithelium of the VNO in two of the specimens (Fig. 2B and C). At the ultrastructural level, microvilli were found on both VNO epithelia while cilia were absent. Different types of cells were found in the receptor epithelium of the VNO. While basal cells could be identified by their position at the base of the epithelia, receptor or supporting cells could not be clearly distinguished from one another (Fig. 2B). No different cell types could be distinguished in the non-receptor epithelium which consisted of only one cell layer. Nucleoli or heterochromatin accumulations were found in the nuclei of all epithelial cells of the VNO. There was immunoreactivity to $G_{o\alpha}$ protein in a few cells in the receptor epithelium of the VNO of the day 25 fetuses (Fig. 3D and E).

The distinct layers of receptor and supporting cell nuclei found in the adult (Salazar & Sanchez Quinteiro 1998) were not observed in any of the pouch young. One big nerve bundle projected from the caudal end of each VNO along the septal cartilage to an area medial ventral between the two MOBs (Figs 1C, D and 3B). Only one cell type and no cell layers could be distinguished in this area (Fig. 1F).

Behavioural test

Neonates were placed on a tanned pelt where they had a choice to climb either towards a saline soaked swab or a swab with maternal pouch odour. All the neonates used in these studies were retrieved from their mother's pouch soon after birth, and all showed normal climbing behaviour when placed on the pelt (Fig. 5A and B). The neonates initially climbed uphill, but most veered away from the line straight up between the swabs (Fig. 5D). Eight pouch young turned away from the vertical path between the swabs, moving horizontally

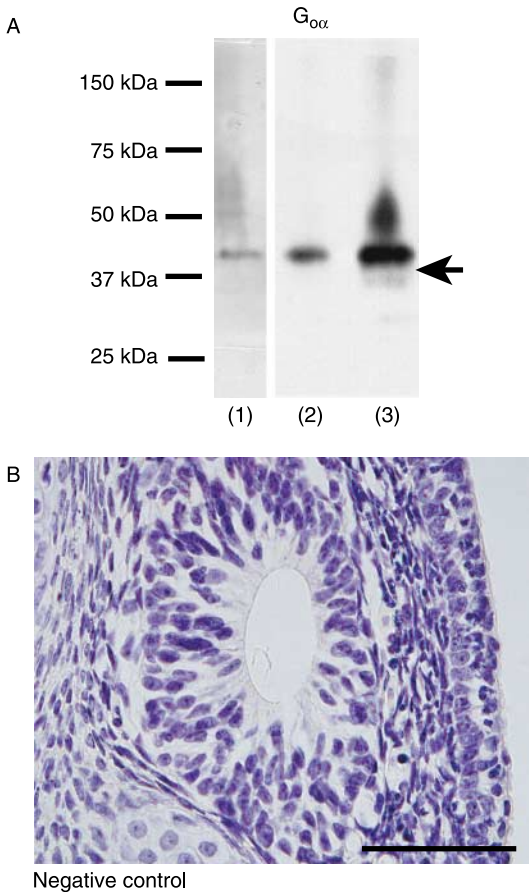

Figure 4 Controls for immunochemistry of $G_{o\alpha}$ protein. (A) There is a single band of $G_{o\alpha}$ at 39 kDa for mouse brain (2) as well as tammar olfactory bulb (3) and VNO (1) on the western blot. (B) Negative control: the first antibody was omitted, and there is no immunostaining visible.

towards one of the swabs. Once near the mother's odour swab, six neonates turned to face down the incline (Fig. 5C). Some pouch young then climbed downwards on the pelt for a short distance after turning, an action that may help the neonate enter the pouch, had there been a pouch present on the section of pelt used. Other pouch young did not show such strong directional changes, but six 'burrowed' their heads into the pelt near the swab with their mother's odour and ceased to move their head constantly side to side as they did during the initial climb. In total, 15 out of 20 young chose the swab with their mother's odour while five chose the saline swab ($P=0.022$). Three other neonates did not reach any of the swabs during the maximum 5 min of the trial because they became stuck in the pelt.

Discussion

Both the main olfactory and accessory olfactory systems of the tammar are well developed at birth and the receptors appear to be functional despite the fact that many other organ systems are poorly developed in the marsupial neonate.

The main olfactory system of the neonate appears to be functional at birth

Nearing birth, the nasal cavity has most of the structures that are present in the adult. The nasoturbinates have just started to form, and the respiratory epithelium consists of one cell layer. Even though mucous-secreting goblet cells are present before birth, there are no Bowman glands in any of the serial sections at birth, in contrast to the study by Hughes *et al.* (1989). In the adult, this structure increases the mucous-coated surface area of the nasal epithelia.

Although there are no ethmoturbinates on the day of birth, the MOE appears to be well developed enough to perceive an odour signal and has olfactory knobs on its surface as described in a number of marsupial newborns (Hill & Hill 1955, Hughes *et al.* 1989, Gemmell & Selwood 1994). The $G_{o\alpha}$-positive receptor cells are connected to the brain before birth, which would allow signal perception and transmission. The development of the MOE is similar to the E14 rat fetus (Farbman & Menco 1986). Since electro-olfactograms recorded between E14 and E19 in the rat are of a similar magnitude to those of adults (Gesteland *et al.* 1982), it is likely that the tammar olfactory receptor cells are also capable of a physiological response. In the full term fetus, we found that the olfactory bulb is visibly divided into the olfactory nerve layer, the external plexiform layer and the granular cell layer similar to

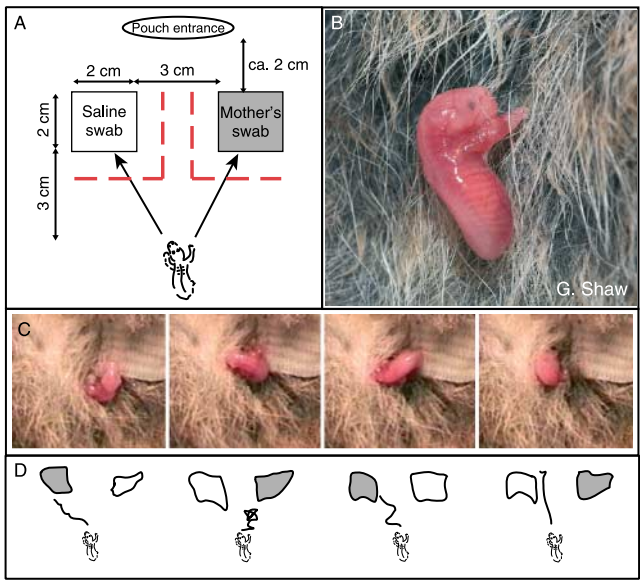

Figure 5 Behavioural test. (A) Y-maze set-up. (B) A neonate climbing towards the swabs. (C) Stills from video images of a neonate approaching a swab soaked with a wash from its mother's pouch (left panel), then turning and burrowing down into the pelt (top right panel). (D) Diagrams of paths taken by four different neonates traced on an acetate overlay of the video recordings. The dark figures indicate the swab from the mother's pouch; the white figures indicate the saline swab.

that described in neonatal tammars (Ashwell *et al*. 2008). $G_{o\alpha}$ protein is present in the olfactory bulb and the rest of the brain, as seen in the brain of the adult tammar (N Y Schneider, T P Fletcher, G Shaw & M B Renfree, unpublished data) and adult rat (Worley *et al*. 1986). This suggests that the brain even at this early stage of development is able to process incoming odour signals and that the main olfactory system in the tammar is functional at birth.

The neonate has a well-developed accessory olfactory system with anatomical correlates of functional VNO neurones

In contrast to newborn mice, hamsters and pigs, the VNO in the newborn tammar is open to the nasal cavity, so odours and pheromones could enter the lumen (Coppola *et al*. 1993, Wöhrmann-Repenning & Barth-Müller 1994, Taniguchi 2008). Since the mouth is permanently shut and filled with the teat after the neonate attaches to it, the direct opening of the VNO to the nasal cavity makes the perception of airborne chemical signals possible. The VNO lumen in the neonate rotates from a crescent shape in the horizontal plane adjacent to the entrance to the nasal cavity to a vertical plane at its caudal end. In the rat, the rotation of the VNO lumen is thought to be due to the development of the VNO from a groove in the septal wall (Estes 1972).

Mucous is produced to keep the epithelium lining the VNO lumen moist and to transport chemical cues along the length of the organ. The neonatal tammar has goblet cells around the entrance to the VNO lumen in the respiratory epithelium of the septum that could be a source of mucous for transport of pheromones into the VNO lumen. The blood vessels of the VNO are small, so it is unlikely that the newborn uses a pumping mechanism, as presumed in the adult tammar, to suck chemical cues into the lumen as suggested for rodents (Salazar & Sanchez Quinteiro 1998, Schneider *et al*. 2008), nor could the pouch young use the venturi effect of air-flow in the NPD (Matsuoka *et al*. 2001) as the VNO is not yet connected to the former. The most probable way for chemical cues to enter the VNO would be by diffusion in mucous produced by the goblet cells in the nasal cavity entering the VNO lumen through capillary action. While the pumping mechanism in rodents is conducted by one blood sinus found at the medial lateral side of each VNO lumen (Salazar & Sanchez Quinteiro 1998), contraction and expansion of the tammar VNO lumen could be achieved by the two big blood vessels on opposite sides of the VNO through pulsation of the blood or regulation of vascular tone (Schneider *et al*. 2008). The VNO of the neonate rat is similar that of the adult rat, but it lacks the bone capsule which surrounds the organ in the adult (Garrosa *et al*. 1992, 1998). A blood sinus is present in the neonate but whether it functions as part of a pumping mechanism at this age has not been investigated, but the lack of an enclosing bony capsule suggests that this pumping mechanism is not yet functional.

The receptor epithelium has olfactory knob-like structures before birth. These structures are part of vomeronasal receptor cells, as evidenced by the presence of G-protein that is coupled to V2R (Jia *et al*. 1997). Only mature receptor cells co-express this G-protein (Berghard & Buck 1996, Krieger *et al*. 1999). $G_{o\alpha}$-immunoreactive cells are also found in the neonatal grey short-tailed opossum (Garrosa *et al*. 1992). Thus, the day 25 tammar fetus has mature receptor cells in the VNO that are presumably functional. There is also $G_{o\alpha}$ protein in the vomeronasal nerves that project to an area in the medial MOB.

The VNO of the neonatal tammar is at a similar stage of development as that of the rat at E16 (Coppola *et al*. 1993). Although the development of the tammar VNO is not as mature as in the neonatal rat, there are features that suggest that the organ is functional at birth. The area of the brain in which the vomeronasal nerves of the tammar fetus terminate has no obvious cell layers, but is $G_{o\alpha}$ positive indicating functional VNO neurones are present.

Olfaction directs the young to the pouch

Our results show that the newborn tammar is able to detect odours. The newborn tammar wallaby shows a clear preference for a direction that leads them to the swab with the odour signals from the mother's pouch. The pouch young that approached swabs with maternal odour showed distinct behavioural changes. Six changed direction, and started crawling down the incline as if they were entering the pouch. Six others burrowed into the fur. These data suggest that neonates approaching the pouch respond to pouch odours with a pattern of movement that facilitates their entry into the pouch. Whether altered skin or fur structure around the pouch entrance would have caused a similar behavioural change could not be determined as the pouch opening was not near any of the swabs and because the tanning process hardened the thin pouch skin.

Around the time of birth the mother licks the fur between the pouch and the urogenital opening. This acts to keep the climbing neonate moist and prevent them sticking to dry fur, as happened to three of the neonates in this experiment that became stuck on the fur and never reached either of the swabs. This saliva might potentially provide a taste-trail that could also assist the neonate's navigation. This could not be determined in this experiment because we used a tanned pelt so no maternal saliva was present. However, since the mother licks generally over the fur of the lower abdomen (Renfree *et al*. 1989), rather than licking a specific track towards the pouch, this seems unlikely to provide much directional assistance to the young.

Ashwell *et al.* (2008) identified neural connections from the olfactory epithelium to the olfactory tubercle and basal forebrain but saw few connections past the olfactory bulb. They concluded that the olfactory system of newborn tammar is not sufficiently developed to play a role in guiding the young to the teat. However, this sensory system is required only for a simple switch in direction so minimal development may be sufficient. Our behavioural observations disagree with the conclusions of Ashwell *et al.* (2008) and show for the first time that the pouch young of the tammar wallaby is attracted to the odours from the mother's pouch (although our experiments did not assess the specificity of the behavioural response to odour). We conclude that both olfactory systems are capable of receiving odour signals at birth, a function that must be a critical adaptation for the survival of an altricial marsupial neonate such as the tammar for its journey to the pouch.

Materials and Methods

Tammar wallabies, *M. eugenii*, were maintained in open grassy yards in our breeding colony using standard husbandry conditions (Renfree & Tyndale-Biscoe 1978). All experimental procedures and collection of tissues were approved by The University of Melbourne Animal Ethics Committees and conformed to Australian National Health and Medical Research Council guidelines (National Health and Medical Research Council 2004).

Tissue preparation

Heads of near term fetuses (day 25 of the 26.5 day gestation, $n=3$), and neonates ($n=6$) were collected for light microscopy and immunohistochemistry. Heads were fixed in 4% paraformaldehyde for one day, washed twice with PBS and stored in 70% ethanol. All samples were embedded in paraffin for serial section (7 μm). Samples from the VNO and MOE ($n=3$) of near term fetuses and neonates ($n=3$) were collected for electron microscopy.

Light microscopy

Coronal sections were cut through the VNO of fetuses (day 25) and neonates ($n=3$ at each stage). Every third slide was stained with Mallory's trichrome stain. Alternate sections were used for immunohistochemistry and the four sections of the caudal end of each VNO were stained with PAS, PAS diastase digestion technique and AB. The length of the VNO was determined by counting the coronal sections on which the VNO was visible (including sections that were not stained) and multiplying by the section thickness. An additional three heads of newborn were cut in a sagittal plane.

Transmission electron microscopy

Samples were fixed in superfix at room temperature for 4 h or overnight at 4 °C. The samples were washed three times for 10 min with 0.1 mol/l cacodylate buffer at room temperature, placed in osmium tetroxide for 1–2 h (1% OsO_4 in 0.2 mol/l cacodylate buffer), washed with 0.1 mol/l cacodylate buffer, dehydrated, embedded in resin (epon araldite) and cut with the ultramicrotome. Thick sections (1 μm) were stained with 1% toluidene blue. Ultrathin sections (90 nm) were stained with uranyl acetate and Reynold's lead citrate and examined under the transmission electron microscope.

Western blot

Protein for western blot was extracted from snap frozen mouse forebrain, tammar olfactory bulb and tammar VNO using protease inhibitor cocktail (Calbiochem, Kilsyth, Australia; # 535141) in RIPA buffer. Protein concentrations were measured with a spectrophotometer (NanoDrop ND-1000; NanoDrop, Wilmington, DE, USA). Samples were run on a 10% separating gel (SDS-PAGE) and blotted to a Hybond-p membrane (Amersham Biosciences). The membrane was blocked with 5% skim milk in TTBS. The membrane pieces were incubated with a polyclonal rabbit antibody (Upstate, New York, NY, USA; # 07-634) raised against purified native bovine $G_{o\alpha}$-protein in 5% skim milk/TTBS for 1 h in the dark on a roller. The membranes were washed three times for 5 min in TTBS and incubated with HRP-conjugated goat anti rabbit IgG (Santa Cruz, Santa Cruz, CA, USA; # SC-2004) at 1/20 000 dilution for 30 min. The membranes were washed ×3 for 5 min in TTBS, exposed for 1 min to ECL (Santa Cruz) and exposed to a Hyperfilm (Amersham Biosciences) for 7 min.

$G_{o\alpha}$ immunohistochemistry

Coronal sections from the middle of the VNO of the d25 fetuses and neonates were dehydrated and transferred into PBS. They were treated with 5% hydrogen peroxide for 5 min, washed with PBS (3×5 min), blocked with 10% goat serum and incubated overnight at 4 °C with $G_{o\alpha}$-antibody (Upstate, # 07-634) diluted 1/200 plus 2% goat serum in 0.1% BSA in PBS. Control sections from each animal were incubated with rabbit IgG (Dako Australia, Kingsgrove, Australia; # X0903), 1/200 dilution in 2% goat serum in 0.1% BSA or with 0.1% BSA in PBS, omitting the first antibody (negative control) at 4 °C overnight. The slides were washed ×3 for 5 min with PBS and incubated with biotinylated goat anti-rabbit (Dako, # E0432); diluted 1/500 for 30 min. After washing ×3 for 5 min with PBS, the slides were incubated with Strept ABComplex/HRP kit (Dako, # K0377) for 30 min. A final washing step (×3 for each 5 min with PBS), preceded addition of DAB Chromogen (Dako, # S3000) with 1 μl H_2O_2 per 1 ml for 2–3 min. Haematoxylin was used as counterstain.

Behavioural test

In total, 23 neonates were used for behavioural observations. Ten neonates were recovered within 2 min *post partum* from females that were held in small pens and watched continuously for births (Renfree *et al.* 1989). The remaining neonates were recovered from females that were held in small grassy enclosures and checked every morning from day 25 of gestation. No behavioural differences were noted between

these two sets of neonates. All pouch young used were able to navigate, since all had entered the pouch before recovery for use in this study. Pouch young were gently removed from the pouch and kept moist and warm. A 2 ml 0.9% saline wash was collected from each mother's pouch by pipette after gently massaging it in the pouch. During this process the inside of the pouch was not touched. This mix of odour from the pouch and saline only contained water soluble components. This saline wash was placed in a sterile vial containing a 2×2 cm cotton swab. A second cotton swab of the same size also in a sterile vial was soaked in 2 ml of 0.9% saline. The samples in the vials including the swabs were warmed to hand temperature immediately before use by holding the vials in the palm of the gloved hand. The swabs were placed with clean forceps on the pelt using a template to ensure consistency in the set up. Each neonate was exposed to the swab from its own mother's pouch. The young were handled with bare hands as they are delicate and the rubber of gloves snags their skin. All pouch young handling and collection of odour samples were performed by the same person who had also tanned the pelt (see below) so that all young were equally exposed to this person's smell.

A piece of tanned tammar belly pelt, including the pouch opening, obtained from animals killed for other ethically approved experiments was placed inside an incubator (34 °C) at an angle of 42.5–60.5°. The pelt was moistened with a spray of 0.9% saline. The two swabs, one with the mother's pouch odour and one with saline were placed on a horizontal line 3 cm apart (from edge of one swab to the edge of the other swab) on the pelt around 2 cm below the pouch entrance (Fig. 5A). Each pouch young was placed at the bottom of the 'Y' with its head oriented in the direction of the pouch about 3 cm from the swabs (neonates normally have to climb about 5–8 cm from the urogenital opening to the pouch) and allowed to crawl for up to 5 min in up to three trials. Neonates that had not reached either swab within 5 min were were replaced at the base of the Y and given repeated trials. The set up of the swabs were changed at random at each different trial of a young. Two pelts were used, one for placement of the swab soaked in clean saline on the left arm of the 'Y' and one for placement of the clean saline swab on the right arm of the Y, as cleaning all odours from the pelts was not practical in the time frame of the experiments. It is unlikely that neonates left a significant odour trail behind them as they climbed since there was no evidence from traces derived from the video recordings of successive young following the path of the young before them. The movements of the young were watched continuously and recorded with a video camera. Young were kept moist using a fine saline spray. At the end of the experiment the pouch young were placed back in their mother's pouch.

The path that the newborn took was drawn by placing a transparent sheet over a screen projecting the video. A dot was made for the position of the newborn (between the young's scapulae) every 10 s and the sequential dots connected by a line. An area of 1 cm around each swab was marked on the transparent sheet and a swab was considered 'chosen' when a newborn spent 30 s with its nose within 1 cm of that swab. The result of the first trial in which a pouch young made a choice was counted for the results. Three of the 23 pouch young became stuck in the dry pelt at the starting point and so were not included in the data analysis. Data were analysed using a one-tailed binomial test (R, version 2.5.1; http://www.R-project.org/).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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