Early differentiation and migration of cranial neural crest in the opossum, *Monodelphis domestica*

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SUMMARY Marsupial mammals are born at a highly altricial state. Nonetheless, the neonate must be capable of considerable functional independence. Comparative studies have shown that in marsupials the morphogenesis of many structures critical to independent function are advanced relative to overall development. Many skeletal and muscular elements in the facial region show particular heterochrony. Because neural crest cells are crucial to forming and patterning much of the face, this study investigates whether the timing of cranial neural crest differentiation is also advanced. Histology and scanning electron microscopy of *Monodelphis domestica* embryos show that many aspects of cranial neural crest differentiation and migration are conserved in marsupials. For example, as in other vertebrates, cranial neural crest differentiates at the neural ectoderm/epidermal boundary and migrates as three major streams. However, when compared with other vertebrates, a number of timing differences exist. The onset of cranial neural crest migration is early relative to both neural tube development and somite formation in *Monodelphis*. First arch neural crest cell migration is particularly advanced and begins before any somites appear or regional differentiation exists in the neural tube. Our study provides the first published description of cranial neural crest differentiation and migration in marsupials and offers insight into how shifts in early developmental processes can lead to morphological change.

INTRODUCTION

One of the defining features of living marsupials is that they are highly altricial at birth. The overall state of development of a marsupial neonate resembles an 11- or 12-day-old embryonic mouse or an approximately 10- to 12-week-old human embryo. Yet the almost embryonic newborn marsupial must travel to, identify, and attach to the teat and suckle. Therefore, a number of elements must be functional at birth despite the overall level of development. For instance, the marsupial oral–pharyngeal apparatus must be sufficiently developed to suckle, and the major physiological systems (circulatory, respiratory, digestive, and excretory) must be capable of basic function. There is an overall rostral–caudal gradient in development of the axial and limb skeleton, so that the cervical region is the most robust portion of the vertebral column and the forelimb is significantly more developed at birth than the hindlimb. In the head the chondrocranium is massive, especially in the nasal region, and the bones around the oral apparatus develop precociously (Clark and Smith 1993). Most craniofacial muscles are well developed at birth, and the tongue is particularly large (Smith 1994). In contrast, the brain, in particular the forebrain, is at an embryonic state of development, and the bones of the calvarium have not yet developed (summarized in Smith 1996, 1997, 2001a; Nunn and Smith 1998 and references therein).

Thus far, such heterochronies have been examined primarily in organogenic stages, and the developmental processes underlying these morphological transformations are unknown. Here we examine the hypothesis that the developmental processes modified to produce the advancement of the skeletal and muscular elements in the orofacial region include changes in the timing and/or pattern of neural crest cell migration. The neural crest is of particular interest because neural crest cells give rise to much of the bone, cartilage, and connective tissues of the face and appear to be critical in patterning muscular organization (e.g. Noden 1983, 1984; Hall 1999; Le Douarin and Kalcheim 1999). Details of cranial neural crest cell differentiation, patterning, and migration in marsupials have not been published but were studied in a variety of species in the early twentieth century (Hill and Watson 1958).

This study grows out of preliminary data presented by Smith (2001a) on early development of the neural plate, neural crest, and facial region in the marsupial *Monodelphis domestica*. Here we present for the first time data derived from scanning electron microscopy (SEM) studies. We focus on the initial differentiation and migration of cranial neural
crested cell populations and the early differentiation and morphogenesis of the brain. We describe spatial and temporal aspects of cranial neural crest cell differentiation, patterning, and migration in *Monodelphis* relative to axial, neural tube, and sensory organ development. The patterns observed in *Monodelphis* are compared with published observations for eutherian mammals and a variety of other vertebrates.

**MATERIALS AND METHODS**

**Embryos**

Embryos of specific stages were obtained from a breeding colony of *M. domestica* maintained at Duke University. This colony consists of animals obtained from the Southwest Foundation for Biomedical Research (San Antonio, TX, USA) and their descendants. A number of published articles discuss care, maintenance, and breeding of *Monodelphis* (Fadem et al. 1982; Trupin and Fadem 1982; Cuthran et al. 1985; Fadem and Rayve 1985). To obtain timed embryos, breeding pairs were filmed nightly and the specific date and time of mating recorded (see also Mate et al. 1994). Females were killed at the appropriate time (as determined by an age-stage table developed in our laboratory) by an intraperitoneal injection of 0.5 ml Nembutal. The uteri were removed and placed in phosphate-buffered saline, and embryos were removed, photographed, and fixed as appropriate (see below). Embryos were staged according to criteria originally developed by McCrady (1938) for *Didelphis* and modified by Mate et al. (1994) and our laboratory for *M. domestica*.

**Scanning electron microscopy**

Head and neural tube development and neural crest cell migration were observed using SEM (model 501, Philips Electron Optics, Eindhoven, The Netherlands). Embryos were separated from the amnion and fixed in modified Karnovsky’s fixative (50% cacodylate buffer, 10% paraformaldehyde [from 15% stock], and 6% glutaraldehyde [from 25% stock]) (Sire 1987). After a fixation time of 10–25 min, depending on the age and size, embryos were placed in cold phosphate-buffered saline. The epidermis was removed using finely pulled glass micropipettes, after which embryos were immediately placed back into modified Karnovsky’s fixative. After overnight fixation samples were rinsed in 0.2 m cacodylate buffer at pH 7.0 with 10% sucrose. They were then postfixed in OsO₄, rinsed a second time in cacodylate, dehydrated, and critical point dried using hexamethyldisilazane (Ted Pella, Inc., Redding, CA). Specimens were sputter coated before viewing. Several specimens from each stage (and often from several litters) were prepared to account for variation in staging and preparation (Table 1).

**Histology**

Embryos were fixed in Carnoy’s solution (Humason 1972), rapidly dehydrated through a graded ethanol series, cleared, embedded in paraffin, and sectioned at 7 µm. The sections were then deparaffinized and stained using Weigert’s hematoxylin and picronorceau (Table 1).

**Three-dimensional reconstructions**

Morphological reconstructions were generated from digital images of histological sections using Surf Driver software version 3.5 (Kailua, Hawaii). The images of sections were aligned manually for overall visual best fit using the “adjust object” module in the program. In the cranial region, every second section was used in the reconstructions (thus slices were 14 µm apart); every fourth section was reconstructed in more posterior parts of the embryo.

### Table 1. Age¹ stage, and number of *Monodelphis* specimens examined using histology and SEM

<table>
<thead>
<tr>
<th>Litter</th>
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<th>Stage</th>
<th>Preparation</th>
<th>No. of embryos</th>
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<td>23</td>
<td>Histology</td>
<td>3</td>
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¹Values are days and hours after observed copulation.

**RESULTS**

**Neural crest cell identification**

This study used SEM and histology to observe the differentiation and migration of neural crest cells. No absolute markers differentiate neural crest cells from other undifferentiated mesenchymal cells (we have found that HNK-1, which is useful in some species, does not label neural crest cells in *Monodelphis*). Therefore, our identification of neural crest cells relies on cell shape and positional information, as in many other studies using SEM (e.g., Tan and Morriss-Kay 1985, 1986; Horigome et al. 1999; Falck et al. 2000). Our primary criterion was whether the mesenchymal cells were in clear physical continuity with the neuroepithelium or were part of a mass that was in continuity with the neuroepithelium. Such cells were identified as neural crest cells. This inference was bolstered by the fact that these cells were in all cases adjacent to the regions of the neural plate that have been shown to produce neural crest cells in other vertebrates. Identification of neural crest cells in marsupials is aided by the fact that at the earliest stages, the embryo is completely flat and virtually transparent. Condensations of mesenchymal cells can be easily identified as they spread laterally between the ectoderm and endoderm of the flat embryo.
We identified a second distinct population of mesenchymal cells as the paraxial mesoderm. In early stages the paraxial mesoderm consists of a thin layer of cells underlying the neural plate. This layer is continuous along the length of the anterior part of the embryo (i.e., it is present in regions of the head that show no evidence of neural crest) and is in continuity with the developing somites posteriorly. In older embryos (e.g., stages 25–27), neural crest merges with this mesoderm and the two populations of mesenchymal cells cannot be distinguished on the basis of position or morphology, except for those neural crest cells still in continuity with the neuroepithelium.

Stage 22: first somite (approximately 10 days)
Stage 22 occurs approximately 10 days after mating and within a few hours of primitive streak formation (stage 18) (Mate et al. 1994). The broad flat neural plate occupies much of the surface of the embryo. Preotic (future rhombomere 2/3 boundary) and otic (rhombomere 5) sulci are present as small nicks on the lateral margin of the neural plate. There is no morphological evidence for subdivision of the neural tube anterior to the preotic sulcus, and no somites are present. In the region just anterior to the preotic sulcus, a population of mesenchymal cells leaves the lateral part of the neural plate. These cells accumulate between the ectoderm overlying the lateral margins of the embryo and the endoderm overlying the yolk sac. We identify these cells as first arch neural crest cells and consider this the onset of neural crest migration (Table 2).

Stage 23: four somites (approximately 10.25 days)
At stage 23 head and trunk regions can be identified as subcphallic folding lifts the anterior end of the relatively flat embryo off of the extraembryonic mesoderm. Approximately four somites are present in stage 23 embryos. The neural tube is flat and open along the entire axis. The preotic and otic sulci are distinct, but it is not yet possible to distinguish boundaries between forebrain, midbrain, and hindbrain (Fig. 1. A and B). No sensory anlagen are present at this stage except for a thickening in the region of the future optic pits. At this stage a substantial population of mesenchymal cells has accumulated beneath the neural plate and ectoderm anterior to the preotic sulcus (see Fig. 3A). Many cells in this condensation are in direct continuity with cells in the neuroepithelium. The approximate site of their migration is marked by two lateral grooves on the dorsal surface of the neural plate (Fig. 1B). These cells condense in the space between the flat neural plate and ectoderm and the underlying paraxial mesoderm (Fig. 2). There is no evidence of distinct populations of neural crest cells within this mass that might have arisen from the future hindbrain, midbrain, or forebrain regions. Although we refer to this population as first arch neural crest, these cells underlie the entire anterior portion of the neural plate and will populate the frontonasal process as well as the first arch region.

Stage 24: six to eight somites (approximately 10.35 days after mating)
Between six and eight somites are present in stage 24 embryos, and for the first time heart tubes and vessels appear. The neural tube has begun to close in the cervical region, although there is not yet contact of the neural folds (Fig. 1. C and D). The forebrain extends as a thin flap projecting from the anterior end of the embryo (see Fig. 4, A and B). The slit-like optic pits have begun to invaginate ventrally (Figs. 3 and 4). Posterior to the optic pits, shallow furrows are forming perpendicular to the embryonic axis and mark the rhombomeric divisions of the hindbrain (Fig. 1D). More posteriorly (level of rhombomere 5), the otic vesicles begin to invaginate (Fig. 3E). There is still no flexion or folding of the neural plate, and no morphological boundary is apparent between the midbrain and either the hindbrain or forebrain.

At this stage a substantial mass of neural crest cells has accumulated in the anterior region of the embryo. The flattened opaque condensations of neural crest cells begin to differentiate into the broad raised swellings of the mandibular or first arch (Figs. 3C, 4A, and 5, A–C) and a thinner band of cells in the frontonasal region, underlying the developing forebrain (Figs. 3A and 4B). Stage 24 marks the first migration of second or hyoid arch neural crest cells, which accumulate around the level of rhombomere 4, posterior to the preotic sulcus and anterior to the otic vesicles (Figs. 3D, 4C, and 5, A–C).

The relations of the neural crest and paraxial mesoderm may be observed in serial sections through an early stage 24 embryo (Fig. 3). The mesodermal cells lie in a thin sheet between the midline (notochordal region) of the embryo and extend laterally to the developing heart vessels, thereby including both paraxial and lateral mesoderm. This sheet of mesoderm is distinct in areas without neural crest (e.g., Fig. 3, D and E), but in the region of the first arch the neural crest and paraxial mesoderm mix (Fig. 3, B and C). In posterior regions (Fig. 3F) the mesoderm differentiates into somites. There is no evidence of localized condensations of mesoderm in the cranial region (somitomeses); however, such structures are not generally visible in histological sections, and SEM specimens were not prepared in a manner that would reveal such condensations (Trainor and Tam 1995). Figure 5, A–C, illustrates the relations of the neural tube, neural crest, and paraxial mesoderm, respectively, at this early stage.

Stage 25: 12 to 13 somites (approximately 10.5 days)
Stage 25 embryos have 12–13 somites. In this stage neural folds first make contact in the postotic/cervical region (Fig.
Fig. 1. SEM images of *Monodelphis* embryos. (A and B) Stage 23 embryos. The head is to the right. Indentations along the lateral head mark the preotic sulci (POS) and otic sulci (OS). The accumulation of first arch neural crest cells under the epithelium and neural plate is responsible for the blurring of the margin of the embryo anterior to the preotic sulcus. Arrowheads indicate lateral grooves that span the midbrain and anterior hindbrain and may represent regions where neural crest cells have migrated from the neural tube. (C and D) Stage 24 embryos. The neural tube remains open, although the folds are drawing toward the midline in the cervical region. Rhombomeres (R1–R5) appear and the optic pits invaginate as mesenchyme accumulates in the mandibular (MA) and hyoid (HA) arches. (E and F) Late stage 25 embryos. The neural folds have met between the levels of somite 6 and just posterior to the otic vesicle (OTV). The mandibular and hyoid arches are present as distinct swellings. Cephalic flexure has begun to shift the orientation of the fore- and midbrain to a more dorsoventrally directed position. Scale bars: A–C and E, 200 μm; D and F, 100 μm.
1, E and F). The anterior parts of the hindbrain, midbrain, and forebrain remain open. The hindbrain and the forebrain/midbrain regions may be distinguished as a slight cephalic flexure appears at this stage. In addition, the hindbrain is more clearly delineated by rhombomeres (Fig. 6). The optic vesicles continue to invaginate and project ventrally as the floor of the diencephalon has not yet expanded (Fig. 5E). The otic vesicles have become well-formed cups (Fig. 5, D and E). There is no evidence of nasal pits. The end of stage 25 is marked by more complete cephalic flexure, a deepening of the rhombomeric furrows, and by a merging of neural folds just posterior to the invaginated otic vesicles. The pulling together of neural folds creates a "flaring" effect of the lateral hindbrain.

The first two arches are easily identified, especially the robust mandibular arch that spans much of the area ventral to the midbrain and first two rhombomeres (Figs. 5D and 6). Neural crest cells continue to migrate in the mandibular arch region. These cells appear to originate from the lateral third of the ventral surface of the neural plate (Fig. 7). The neural crest cells of the hyoid arch also continue to migrate as a broad diffuse sheet (Fig. 8, C and D). Although some neural crest cells are deposited at the first branchial arch, other cells continue to migrate posteriorly, and yet others migrate anteriorly from above the otic capsule. Histological sections reveal that neural crest cells continue to migrate from the closing edges of the neural folds in the hindbrain (Fig. 9). A dense population of mesenchymal cells has accumulated in the frontonasal region (Fig. 9A), and the olfactory placodes appear as thickenings in the epithelium of this region. Some neural crest cells continue to migrate from the mid- and hindbrain into the regions of the mandibular and hyoid arches (Fig. 5, F and G). The neural crest cells that contribute to the branchial arches proliferate from the edges of the neural plate at the level of rhombomeres 6–7 and migrate as a broad diffuse sheet (Fig. 8, C and D). Although some neural crest cells are deposited at the first branchial arch, other cells continue to migrate posteriorly, and yet others migrate anteriorly from above the otic capsule. Histological sections reveal that neural crest cells continue to migrate from the closing edges of the neural folds in the hindbrain (Fig. 9, D and E). More posteriorly, neural crest cells are observed to migrate along a medial path at the level of somites 1–4 (Fig. 8C).
Fig. 3. Neural crest (NC) cell migration in early *Monodelphis* embryos. (A) Parasagittal section of a stage 23 embryo; anterior is to the right. Neural crest cells are migrating anteriorly and ventrally from the midbrain and anterior hindbrain. Inset: Schematic of a stage 24/25 embryo. The horizontal line indicates the approximate longitudinal plane for image A; the vertical lines indicate the planes of sectioning in images B–F. POS, preotic sulcus; OS, otic sulcus. (B–F) Early stage 24. (B) Neural crest is migrating at the diencephalic region (arrowheads) adjacent to the invaginating optic pits (OPP). Paraxial mesoderm (PAM) is visible as a thin layer of cells sandwiched between the overlying neural plate and underlying endoderm. (C and D) Neural crest cells are migrating from the border of epidermal and neural ectoderm (arrowheads) at the level of the mandibular arch (C) and at the level of the hyoid arch (D). (E) No neural crest is migrating at the level of the otic vesicle (OTV); however, a small stream of neural crest cells is migrating from the anterior trunk (arrowheads, F). Scale bar, 50 μm.
during stage 27. At early stage 27 the neural tube remains open anterior to the otic capsule, but by late stage 27 the neural tube has begun to fuse over the midbrain. The maxillary process is a distinct mass, and the frontonasal processes continue to expand. The hyoid arch is more robust, and the first branchial arch is separated from the posterior arches as the third pharyngeal pouch appears. Olfactory pits are present, and the eye begins to appear on the lateral surface of the embryo. The otocyst remains open. We observed few cranial neural crest cells leaving the neural tube after this stage.

**Subsequent development**

In subsequent stages the cranial region reaches full matura-
tion. By stage 28 (11.4 days) the otocyst and anterior neuropore are closed. Deep olfactory pits are present, and the lens placode begins differentiation. The maxillary processes begin to fuse with the frontonasal region. By stage 30 (11.75 days) the posterior neuropore closes and many structures of the face are recognizable. All cranial nerves (with the exception of the motor nerves to the eye) are present. In stages 31–32 (days 11.5–12.5) the telencephalon begins evagination and cartilage first appears in the head. *Monodelphis* is born just after stage 33, approximately 14.5 days after mating.

**DISCUSSION**

**Comparative patterns of neural crest cell migration**

Despite slight differences in the timing and/or sequence of cranial neural crest cell migration, the pattern of cranial neural crest migration is remarkably conserved among amniotes, and indeed among the vertebrates, thus far described. In general, neural crest cells differentiate from the neural ectoderm/epidermal ectoderm boundary just before, during, or soon after the neural folds meet. There are conserved patterns in the specific regions of the brain from which populations of crest cells arise, and neural crest appears to migrate in three major streams of cells. Finally, relatively few cells appear to migrate from rhombomeres 3 and 5 (Hall and Hörtadius 1988; Hall 1999; Le Douarin and Kalcheim 1999). Below we discuss the condition commonly found in amniotes for each of these features and compare it with our observations of *M. domestica*.

In all vertebrates described, cranial neural crest cells typically arise as three distinct populations of cells. The most

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![Fig. 4. Head and pharyngeal arch development in late stage 24 *Monodelphis*. Anterior is to the right. A portion of the neuroepithelium in the fore- and midbrain has been removed, and surface ectoderm (e) has been removed lateral to the head. (A) The optic vesicles (OPV) mark the forebrain. At this stage the mandibular arch (MA) is robust, and the hyoid arch (HA) is developing as a narrow column of cells. White boxes are enlarged in B and C. (B) Mesenchymal cells thought to be of neural crest origin have accumulated beneath the neuroepithelium in the frontonasal region (Frn). (C) Enlarged view of the hyoid arch. Scale bars, 200 μm.](image-url)
rostral and largest population spans the anterior part of the neural tube rostral to the rhombomere 2/3 boundary and is often referred to as mandibular, first arch, or trigeminal crest. This population not only provides neural crest cells to the first arch, but also the frontonasal region. Cells migrate from the first two rhombomeres, the midbrain, and, in some vertebrate species, the caudal forebrain. The second population is referred to as second arch, or hyoid crest, and arises from the level of rhombomere 4. The third and most caudal cranial population, sometimes referred to as the circumpharyngeal crest (Shigetani et al. 1995), or postotic crest, migrates from the postotic rhombomeres as a sheet of cells, which then populates the branchial arches (Epperlein et al. 2000; Kuratani and Horigome 2000). In most vertebrates the three major populations of cranial neural crest migrate in a clear rostral–caudal order. Several authors have reported that in eutherian mammals (at least mouse and rat; Tan and Morriss-Kay 1985, 1986; Osumi-Yamashita et al. 1996) postotic crest cells migrate before second arch crest. In *Monodelphis* we observe three distinct populations of crest cells that arise from the same regions of the brain documented in other taxa and populate the same series of pharyngeal arches. Crest cells clearly
arise from the margins of the optic vesicles and therefore from posterior forebrain regions. Like most other vertebrates, and unlike eutherians, the three streams commence migration in a rostral–caudal sequence.

Although neural crest cells are produced throughout most of the neural tube, gaps in cranial neural crest migration from rhombomeres 3 and 5 have been reported (e.g., Serbedzija et al. 1992; Farlie et al. 1999; Hall 1999; Horigome et al. 1999). The paucity of migrating neural crest cells from rhombomeres 3 and 5 has been attributed to a number of factors, including cell death, deflection into other streams, or mesodermal inhibition (e.g., Graham et al. 1993; Birgbauer et al. 1995; Eickholt et al. 1999; Farlie et al. 1999; Golding et al. 2000). In early stage *Monodelphis*, the sites of rhombomeres 3 and 5 also appear to be crest free. In particular, the preotic sulcus, which lies at the eventual level of rhombomere 3, appears to provide a sharp posterior boundary to migrating first arch cells in stage 22–24 embryos. However, at slightly later stages neural crest cells appear to arise from the dorsal edges of the neural tube overlying the otic capsule (level of rhombomere 5) and appear to migrate anteriorly and posteriorly to connect with preexisting streams. Thus, in many aspects the basic pattern of differentiation and migration of neural crest cells in *M. domestica* exhibits the primitive, and highly conserved, condition.

**Timing of neural crest migration**

The most notable difference in the pattern of cranial neural crest migration in *Monodelphis* concerns the timing of differentiation and migration relative to neural tube maturation. In all vertebrates thus far examined, neural crest commences migration after the neural tube exhibits significant regional differentiation and after it begins closure. In birds and many reptiles (Hall 1999; Le Douarin and Kalcheim 1999) and many fish (Falck et al. 2000; Horigome et al. 1999), neural crest leaves the closed neural tube. In eutherian mammals (Tan and Morriss-Kay 1985, 1986) and some amphibians (Olsson and Hanken 1996), migration occurs before the neural folds in the head meet but after closure has begun. In no animal thus far reported does neural crest migrate from a flat undifferentiated neural plate. In the discussion below we explore this heterochrony with detailed comparisons with eutherians. We focus on eutherians (a) because among vertebrates neural crest migration in eutherians is relatively early compared with neural tube closure and (b) because the Eutheria is the sister taxon to marsupials, it is the most relevant comparison phylogenetically. Although most available data are derived from mice and rats, it appears to be consistent with observations of other eutherians (Peterson et al. 1996).

To make this comparison we document the relative timing of a number of events in neural tube and cranial sense organ maturation (Table 2). We also compare the timing of these events relative to somite differentiation. These data allow us to assess the relative timing of events such as neural tube closure or neural crest migration in the context of a number of different kinds of morphological features. Finally, these events are all expressed in relative terms. Thus, when we conclude that an event in *Monodelphis* is late relative to mice, it is the same thing as saying it is early in mice relative to *Monodelphis*. At least four major differences are apparent when *Monodelphis* is compared with mice and rats.

First, in *Monodelphis* neural tube closure (both first contact of neural folds and closure of the anterior neuropore) is late relative to somite and sense organ differentiation. In *Monodelphis* approximately 12–13 somites are present when the neural folds first make contact; in mice first contact appears at 6–8 somites. In addition, in *Monodelphis* neural...
folds first make contact after the optic pits have evaginated, the hyoid and postotic crest has begun migration, and the otic placode appears. In mice these latter events occur at the same stage or after first neural fold contact. It is noteworthy that in *Monodelphis* neural tube closure appears to pause once it has reached the otic vesicle. The neural folds fuse just posterior to the otic vesicle at stage 25 and do not fuse further anteriorly until well into stage 27. During this time there is considerable growth, expansion, and differentiation of the neural tube and cranial region. This pause is in part reflected in the timing of anterior neuropore closure. In mice the anterior neuropore closes at somite stage 20, before the otocyst closes or the olfactory placode appears. In *Monodelphis* these latter events precede anterior neuropore closure, which occurs at 25–29 somites. We infer from these data that the delay in brain development in marsupials noted by Smith (1996, 1997) for later stages of organogenesis exists at this early stage.

Second, neural crest migration in *Monodelphis* is early relative to other events in embryonic differentiation, including both neural tube closure and differentiation of somites. In mice and rats, first arch neural crest cells begin to differentiate at somite stages 2–3 and begin to migrate at approximately somite stage 4–5, just before the neural folds first make con-
tact (Nichols 1981, 1986; Tan and Morriss-Kay 1985; Morriss-Kay et al. 1993). In *Monodelphis* large populations of crest have accumulated at the first arch, and significant crest leaves the second arch while the neural plate is still completely flat and before more than a few somites appear.

Third, the sensory organs differentiate early in *Monodelphis* relative to aspects of neural tube or somite differentiation. It is possible that early development of the otic and olfactory organs is important in orienting the neonate during its migration to the teat, and the olfactory apparatus appears to be particularly advanced in marsupial development. However, as the eyes do not open until postnatal day 35, the advancement of the optic vesicle is not likely to be explained on functional grounds.

Fourth, as mentioned earlier, in *Monodelphis* there is a clear rostral–caudal gradient in the timing of migration of the three major streams of neural crest. This resembles the primitive condition reported for chick and other vertebrates but differs from that reported in eutherians (Nichols 1981, 1986, 1987; Tan and Morriss-Kay 1985, 1986; Serbedzija et al. 1992; Morriss-Kay et al. 1993; Osumi-Yamashita et al. 1996). We conclude that in marsupials, relative to eutherians, the timing of the differentiation and migration of neural crest is significantly advanced relative to either the neural tube or the paraxial mesoderm (somites). The neural crest populations that will inhabit the mandibular arch and frontonasal regions are particularly advanced relative to surrounding tissues. Because eutherians in most aspects resemble other vertebrates, we conclude that the pattern in marsupials is derived. There is, however, a complex pattern of heterochrony among the structures of the neural tube, sense organs, and neural crest, so that we cannot simply say a rostral–caudal

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**Fig. 9.** Histological sections of head development and neural crest cell migration in stage 26 *Monodelphis*. (A) Anterior is to the right. Parasagittal section showing optic vesicle (OPV) and condensations of mesenchymal cells in the mandibular, hyoid, and first branchial arches (MA, HA, BA1). The box inset is a schematic of a stage 26 embryo. The lines indicate the plane of sectioning in images B–E. MB, midbrain; FB, forebrain; ftn, frontonasal process; mn, mandibular process; mx, maxillary process. (B) The lateral orientation of the developing optic vesicles can be seen in this section through the forebrain and midbrain area. (C–E) Cross-sections through the mandibular, hyoid, and first branchial arches. Neural crest cells are migrating from the closing or closed neural folds at the level of the second and third arches (arrowheads in D and E). Scale bars: A, 100 μm; B–E, 50 μm.
Thus, in the vertebrates thus far studied, gene expression, expressed well before the onset of neural crest migration. To either signal or pattern regional specificity in the brain are when crest migrates. Further, many of the genes that appear in mice and chick morphological differentiation has begun. In both marsupials and other amniotes. Of particular interest is the fact that the neural tube in marsupials exhibits little or no regional differentiation at the time of first arch neural crest migration. In both mice and chick, rapid forebrain growth during neurulation is a characteristic mammalian feature. However, in marsupials although forebrain development is delayed, the preotic sulcus is a prominent early feature. We suggest that the preotic sulcus in marsupials may relate to neural crest differentiation. Specifically, we suggest that the preotic sulcus is found uniquely in mammals because rapid forebrain growth during neurulation is a characteristic mammalian feature. However, in marsupials although forebrain development is delayed, the preotic sulcus is a prominent early feature. We suggest that the preotic sulcus in marsupials may relate to neural crest differentiation. Specifically, we suggest that the preotic sulcus serves as a mechanism to segregate the regions of the neural plate that give rise to first arch crest (anterior to rhombomere 2) from more posterior regions. This hypothesis is consistent with the appearance of the preotic sulcus at, or before, the onset of migration of first arch neural crest cells and its location at the boundary between rhombomeres 2 and 3.

### Table 2. Relative timing of early developmental events in a placental and marsupial mammal

<table>
<thead>
<tr>
<th>Mouse, rat</th>
<th>Number of somites</th>
<th>Monodelphis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preotic sulcus</td>
<td>0–1</td>
<td>Preotic sulcus</td>
</tr>
<tr>
<td>Otic sulcus</td>
<td>2–3</td>
<td>Otic sulcus First arch neural crest</td>
</tr>
<tr>
<td>First arch neural crest</td>
<td>4–5</td>
<td>Optic pits Second arch neural crest</td>
</tr>
<tr>
<td>First contact of neural folds</td>
<td>6–8</td>
<td></td>
</tr>
<tr>
<td>Optic pits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postotic neural crest</td>
<td>8–9</td>
<td>Postotic neural crest</td>
</tr>
<tr>
<td>Second arch neural crest</td>
<td>12–13</td>
<td>Otic placode First contact of neural folds</td>
</tr>
<tr>
<td>Otic placodes</td>
<td>15–18</td>
<td>Olfactory placode</td>
</tr>
<tr>
<td>Anterior neuropore closes</td>
<td>25–29</td>
<td>Olfactory placode</td>
</tr>
<tr>
<td>Olfactory placode</td>
<td>30–34</td>
<td></td>
</tr>
</tbody>
</table>

1Ruberte et al. (1997).
3Tan and Morriss-Kay (1986).
Conclusions
Smith (1997, 2001b) observed that in marsupials, relative to placentalts, the differentiation of the central nervous system is delayed during organogenetic stages and the skeletal-muscular system of the face is advanced. In this study we demonstrate that this heterochrony is also present at early events in cellular differentiation. This observation suggests that the changes in developmental timing that characterize marsupials are not simply late shifts in relative rates of growth but instead extend back to some of the earliest events in embryonic differentiation. It is of note that the shifts we observe occur during the period normally considered to constitute a highly conserved phylotypic stage in vertebrates (Duboule 1994). The observation of such significant changes in the timing of early events confirms recent studies demonstrating that this phylotypic stage may not be highly conserved (Richardson 1995).

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REFERENCES


