Craniofacial Development in Marsupial Mammals: Developmental Origins of Evolutionary Change

Kathleen K. Smith*

Biologists have long studied the evolutionary consequences of the differences in reproductive and life history strategies of marsupial and eutherian mammals. Over the past few decades, the impact of these strategies on the development of the marsupial embryo and neonate has received attention. In this review, the differences in development in the craniofacial region in marsupial and eutherian mammals will be discussed. The review will highlight differences at the organogenic and cellular levels, and discuss hypotheses for shifts in the expression of important regulatory genes. The major difference in the organogenic period is a whole-scale shift in the relative timing of central nervous system structures, in particular those of the forebrain, which are delayed in marsupials, relative to the structures of the oral–facial apparatus. Correlated with the delay in development of nervous system structures, the ossification of the bones of the neurocranium are delayed, while those of the face are accelerated. This study will also review work showing that the neural crest, which provides much of the cellular material to the facial skeleton and may also carry important patterning information, is notably accelerated in its development in marsupials. Potential consequences of these observations for hypotheses on constraint, evolutionary integration, and the existence of developmental modules is discussed. Finally, the implications of these results for hypotheses on the genetic modulation of craniofacial patterning are presented. ***Developmental Dynamics 235:1181–1193, 2006.*** **© 2006 Wiley-Liss, Inc.**

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INTRODUCTION

For the past two centuries, biologists have recognized that marsupial and eutherian mammals are characterized by major differences in life history, reproduction, and development. The evolutionary origins of these differences, as well as the potential evolutionary constraints imposed by one or the other strategy have long been topics of broad interest and controversy (e.g., Tyndale-Biscoe, 1973; Lillegraven, 1975; Kirsch, 1977a,b; Lee and Cockburn, 1985; Lillegraven et al., 1987; Tyndale-Biscoe and Renfree, 1987; Hughes and Hall, 1988; Cockburn, 1989; Maier, 1993, 1999; Sears, 2004; and references therein).

Marsupials are characterized by a very short period of intrauterine gestation. The young are born at a highly immature or altricial state, and at birth must travel to the teat area or pouch, recognize the nipple, attach, and suckle with no assistance from the mother. They remain firmly attached to the teat for 12–14 days in some species, to over 100 days in others. They then periodically detach from the teat, but continue their development while being nourished through lactation for periods ranging from 60 days to well over a year. Several evolutionary scenarios have been presented to account for the differences between marsupials and eutherians. Some scenarios emphasize constraint, claiming that marsupials have been unable to develop suffi-

Department of Biology, Duke University, Durham, North Carolina
Grant sponsor: NSF; Grant number: IBN 0316353.
***Correspondence to: Kathleen K. Smith, Box 90338, Duke University, Durham NC 27708. E-mail: kksmith@duke.edu**

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TABLE 1. Ranges in the Time from Primitive Streak to Birth in Marsupials[a]

Species	Maternal weight (grams)	Primitive streak to birth (days)	Gestation length (days)	Neonatal weight (mg)	Litter size (number)	Time to weaning (days)
Monodelphis domestica Gray short-tailed opossum	80–100	~4.5	14.5	100	10–13	60
Sminthopsis crassicaudata Fat-tailed dunnart	12–18	~2.5	13	10	7–8	65–68
Dasyurus viverrinus Eastern Quoll	1350	~2.5	19	12.5	5–6	135–140
Trichosurus vulpecula Brush-tailed opossum	1500–3500	~6.5	17.5	200	1	275
Macropus eugenii Tammar wallaby	5000	~10	26.5	370	1	270

[a]Data from Tyndale-Biscoe and Renfree (1987). *Monodelphis* data collected from K.K. Smith Lab colony.

ciently effective placentation methods or immunological protection of the fetus (e.g., Lillegraven, 1975; Lillegraven et al., 1987), while others argue that the marsupial mode of reproduction has evolved in response to distinct selective pressures and has significant advantages in some circumstances over the mode of reproduction seen in eutherians (e.g., Kirsch, 1977a,b; Parker, 1977; Hayssen et al., 1985). Others have claimed that marsupials and placentals represent ends of a continuum and that the reproductive strategies should not be seen as distinct alternatives, but merely differences in emphasis, perhaps the consequences of initial minor differences that have become magnified over time (e.g., Renfree, 1983, 1995; Tyndale-Biscoe and Renfree, 1987).

Whatever the origins and evolutionary consequences of these reproductive strategies, the morphology of the newborn marsupial is quite different from even the most altricial eutherian mammal. The craniofacial region in particular is characterized by several unusual features and is of interest for several reasons. First, it has long been recognized to be one of the most complex regions of the body structurally, functionally, and developmentally. The craniofacial region contains structures involved in several different systems, many of which must be functional at birth. These include the feeding and respiratory systems and the brain and sense organs. Second, these different systems are highly integrated during development with one or another element known to have major effects on the development of other elements. These effects include inductive interactions as well as physical and mechanical relations that impact later growth and form. Because many of the systems of the craniofacial region must be functional in this highly altricial neonate, the newborn marsupial presents a mosaic of elements, with some accelerated in development when compared with eutherians or other amniotes, and others significantly delayed. It is, therefore, an excellent model for the study of heterochrony. A study of the developmental details of this region, furthermore, provides a model system with which to study the degree to which individual elements or suites of elements are independent evolutionarily and developmentally.

In this review, I briefly examine craniofacial development in marsupial mammals, summarizing work appearing during the past two decades. My goal is to present an overview of the differences between marsupials and eutherians and other amniotes, and to highlight some of the ways that the marsupial model may illuminate basic patterns and processes in cranial development in amniotes.

AN OVERVIEW OF MARSUPIAL DEVELOPMENT

The marsupial neonate is often likened to a 10–12 day mouse embryo, or a 10-week human embryo. This comparison provides a rough estimate of overall development (in particular neural development), but hides the fact that the newborn marsupial presents a remarkable mosaic of advanced and delayed features.

Marsupial mammals are all characterized by a very short intrauterine gestation period. Gestation time generally ranges from around 35 days in the largest macropodids (kangaroos and wallabies) to less than 14 days in some of the smaller dasyurids or didelphids (Tyndale-Biscoe and Renfree, 1987). Even more remarkable is the period of organogenesis. In marsupials, the time from primitive streak to birth ranges from 64 hr to approximately 10 days (Table 1). In comparison, this period is 11 days in mice, 50 days in the domestic cat (Noden and de Lahunta, 1985), and over 250 days in humans (Larsen, 2001). Unlike the majority of eutherians, there is little relation between maternal size and gestation length, embryo size, or total litter weight (Eisenberg, 1981; Tyndale-Biscoe and Renfree, 1987). Nor does one see the wide range of degree of neonatal maturity seen in eutherians, from the helpless altricial newborn in species such as mice, cats, or humans, for example, to highly precocial and nearly independent neonatal elephant shrews, dolphins, or horses. All marsupials are extremely altricial at birth and far less developed than any placental mammal.

Nonetheless, there is variation in the relative degree of development of the marsupial neonate (Hall and

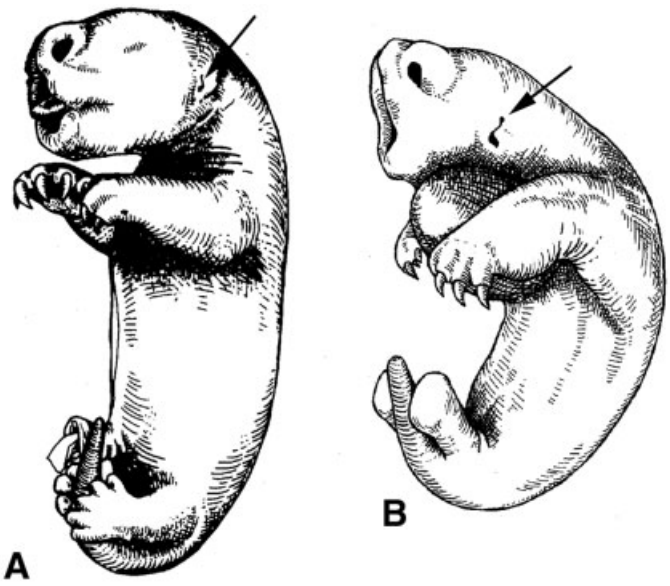

Fig. 1. Drawing of newborn marsupials. **A:** *Trichosurus vulpecula*. **B:** *Dasyurus viverrinus*. *Trichosurus* is redrawn from Klima and Bangma (1987), and *Dasyurus* is redrawn from Hill and Hill (1955). These drawings are not to scale; a newborn *Trichosurus* weights approximately 200 mg and is approximately 15 mm in length, whereas a newborn *Dasyurus* weighs approximately 12 mg and is less than 6 mm in length. Note the extreme altriciality in the *Dasyurus* embryo. Not only is the difference in state of the forelimb and hindlimb extreme, but the head is rudimentary. Arrows point to the opening of the external ear; note in *Dasyurus* the short distance and absence of any features between the nasal opening and the ear. The head in *Trichosurus* is fairly well differentiated. The large mass ventral to the head and neck in *Dasyurus* is undifferentiated mesenchyme, which is apparently important in support of the head and neck in the absence of sufficient musculature.

Hughes, 1987; Hughes and Hall, 1988). Figure 1 illustrates two extremes. Macropodids (kangaroos and wallabies) generally have a single young, which is relatively large and relatively well developed. Their gestation lengths and time to weaning tend to be relatively long. Dasyurids, at the other extreme, are ultra-altricial. The young may weigh as little as 10–15 mg at birth and are extraordinarily rudimentary in their morphology (Hill and Hill, 1955). Note for example, in Figure 1 the extreme difference between the fore- and hindlimbs and the poorly developed head and face in *Dasyurus*. Of interest, *Tarsipes*, the honey possum, is not monophyletic with the dasyurids, but its neonate is as, if not more, altricial than a typical dasyurid (Renfree, personal communication). Their young may weigh as little as 3–6 mg at birth (Tyndale-Biscoe and Renfree, 1987). This observation suggests that ultra-altriciality is not primitive within marsupials, but instead has evolved at least twice.

Despite the variation one sees in marsupials, neonates are characterized by several distinct features that distinguish them from other amniotes and signal major shifts in the timing of development of various structures. These features have been characterized by several workers and the unique morphology has been attributed to the functional requirements at this highly altricial stage (e.g., McCrady, 1938; Hall and Hughes, 1987; Hughes and Hall, 1988; Gemmell and Nelson, 1988a,b, 1992; Gemmell and Selwood, 1994). The most obvious shift is the overall anterior–posterior gradient in development of the animal. The anterior or cranial end is highly developed, whereas the structures of the posterior or caudal regions are distinctly rudimentary. The anterior–posterior gradient is evidenced in the enormous difference in the relative degree of development of the forelimb relative to the hind limb (e.g., Klima, 1987). The forelimb is used by the neonate to climb to the teat and possesses differentiated cartilages, muscle, and some bones. In contrast, the hind limb often contains only early anlagen of skeletal and muscular elements. The gradient of relative development is also apparent in axial structures, including the vertebral column, spinal cord, and spinal nerves. All tetrapods appear to exhibit some advancement of the forelimb bud over the hind limb bud, but in none is it as well developed as is seen in marsupials. Although this gradient has been noted for well over a century, the details have not been examined. It is not known, for example, if the heterochrony involves the generation of presomitic mesoderm, somitogenesis, or later differentiation of somites. Furthermore, the relation between limb bud appearance and general differentiation of the axis has not been investigated in marsupials. On the other hand the digestive, respiratory, urogenital, and cardiovascular systems are functional to various degrees at birth and also advanced relative to overall development. They do not show a significant anterior–posterior gradient; however, these systems all exhibit heterochronies and specializations of their own. For example, at birth, the lungs are responsible for some degree of oxygen exchange, but only consist of bronchi of various dimensions. Subdivision into alveoli occurs well after birth (Krause, 1988). At birth, the kidney is a functional mesonephros, and the final adult metanephros only appears at some time postnatally (Krause, 1988). Finally, several authors have noted that much of the development of the central nervous system occurs postnatally (e.g., Nelson, 1987, 1988; Saunders et al., 1989; Krause and Saunders, 1994).

Monodelphis domestica (family Didelphidae), the gray short-tailed opossum, is often used as an experimental model for marsupial development. Because it is the first marsupial to be targeted for genome sequencing, it is likely to be of increasing importance. It is small (adults weigh ~80–120 grams), relatively docile, and easily kept and bred year round in the lab (Fadem et al., 1982; VandeBerg, 1983). Development in *Monodelphis* will be discussed in detail in this review, as it can provide a good case study of marsupial development. *Monodelphis* embryos are born after approximately 14.5 days gestation; the neonate is somewhat intermediate in degree of development between the two embryos shown above. The primitive streak stage is on day 10, and

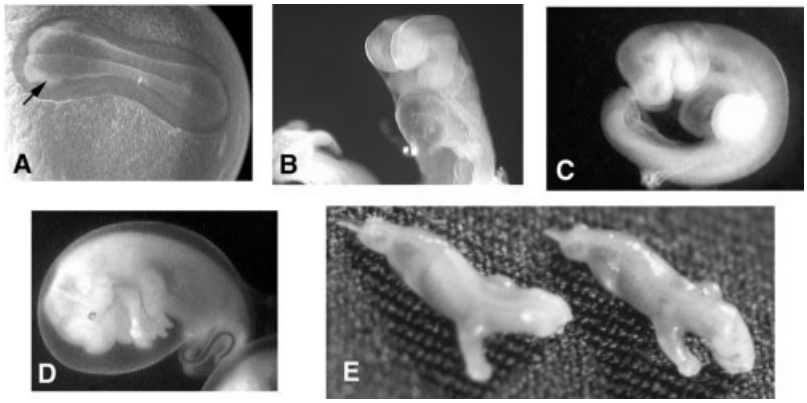

Fig. 2. An overview of development in *Monodelphis domestica*. **A:** Stage 23 embryo; approximately 10.2 days of gestation. At this stage, the embryo is a flat disc on the egg vesicle and neural crest has just begun migration (arrow indicates neural crest). **B:** Stage 26 embryo (approximately 10.7 days). The neural tube is still open anteriorly and the large mandibular and frontonasal processes may be seen. **C:** Stage 28 embryo, approximately 11.4 days gestation. The neural tube is closed by this stage and the embryo has developed a yolk sac placenta (not shown). **D:** Stage 33 embryo in its amniotic sac. This stage is one-half day before birth. **E:** Newborn *Monodelphis* (< 1 hr). The neonates are approximately 1 cm long.

they rapidly undergo organogenesis in the last 4.5 days of development (Mate et al., 1994). Newborns weigh approximately 100 mg and are approximately 1 cm long at birth. A female may have up to 13 young (although we have counted up to 19 embryos in utero). Newborns are fixed to the teat until approximately 12–14 days after birth and spend most of their time attached until approximately day 30 postnatal. Most senses are functional by approximately day 35 (eyes are open and hearing is functional), and at this time, they gain increasing locomotor skill. They begin to eat solid foods between days 45 and 50 and are weaned between days 50 and 60. They reach sexual maturation at approximately 5–6 months of age. Figure 2 shows stages in the development in *Monodelphis domestica*.

ORGANOGENESIS IN THE MARSUPIAL CRANIOFACIAL REGION

Several differences in development in the marsupial and eutherian craniofacial region have been noted by previous authors. In particular, it has been observed that, in marsupials, the tongue and oral apparatus are quite advanced, that there is a massive chondrocranium, but limited cranial ossification, that the secondary palate appears to close relatively early, that differences in the rate and pattern of dental development exist, that the jaw joint undergoes a "recapitulatory" transformation postnatally, and that the brain is relatively underdeveloped at birth. Smith and colleagues in a series of studies have examined craniofacial development in marsupials (Clark and Smith, 1993; Smith, 1994, 1996, 1997, 2001a,b, 2002, 2003; Nunn and Smith, 1998; Vaglia and Smith, 2003; van Nievelt and Smith, 2005a,b). This work has alternated between detailed model-system approaches using *Monodelphis domestica* as a case study, usually in comparison with the well studied murid rodents, with broader phylogenetic surveys. The studies with a broad phylogenetic base ensure that the patterns observed in the model-system approaches can be attributed to true differences between marsupials and eutherians (rather than between *Monodelphis* and mice).

The broadest survey is found in several papers (Smith, 1996, 1997; Nunn and Smith, 1998) in which the relative timing of several developmental events in a variety of marsupial and eutherian species was studied. This work incorporated a broad variety of taxa and also a wide variety of events in the craniofacial region. Twenty-eight different events were studied, including the development of elements of the brain and sense organs, muscles, bone ossification, and appearance other elements of the cranial skeleton. This work, therefore, attempted to provide an overview of the interaction of systems during organogenesis in marsupials, and a reasonably comprehensive assessment of heterochronies in the craniofacial region during this period. To make such comparisons, Smith made use of several embryological collections to provide data on taxa not easily obtained, including the Hill and Hubrecht collections, and also developed new techniques for comparing the sequence of developmental events in a large number of taxa. (Information on the current status of these collections may be found at the following Web page http://www.biology.duke.edu/kksmithlab/JPHill/hill_collection.htm.) These approaches highlighted the utility of examining heterochronies in developmental sequences, rather than restricting the concept of heterochrony to analysis of relative changes in size and shape (Smith, 2001c, 2002, 2003).

The results of these studies identify which events exhibit heterochrony relative to the overall sequence of the two clades. Therefore, the analyses first identify common patterns across marsupials and eutherians and then which events are early or late relative to the common sequence in either clade. Because the description is of relative timing, it is reciprocal, and a statement saying that event A occurs relatively early in marsupials, could also be stated as event A occurs relatively late in eutherians. However, as the overall developmental pattern during this period in eutherians more closely resembles that of other amniotes (Smith 2001b, unpublished), results are generally expressed to indicate shifts in marsupials.

These studies identified several features where the relative timing of development reliably distinguished marsupials and eutherians (Smith, 1996, 1997; Nunn and Smith, 1998). Structures that consistently developed relatively early in marsupials included the first ossification of the dentary, maxillary, premaxillary and exoccipital bones and the closure of the secondary palate. The elements that consistently occurred late in marsupials were all generally related to neural development: the evagination of the telencephalon, the contact between the olfactory nerve and bulb, the fill-

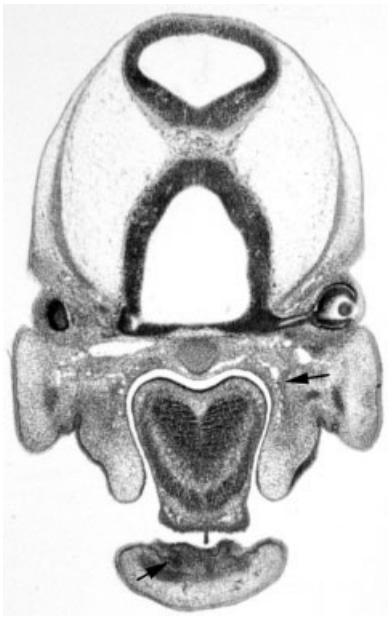

Fig. 3. Section through the head of a *Monodelphis* embryo one-half day before birth (stage 33). Note the well-developed tongue and ossification of the maxillary and dentary bones (arrows). At this stage, the brain is little more than an undifferentiated neuroepithelium and the eyes, too, are at an embryonic state. The palatal shelves are open, but will close soon after this stage and before birth.

ing of the lens vesicle by the primary lens cells, the differentiation of the thalamus and hypothalamus and the layering in the telencephalic cortex (Fig. 3). One additional trait was late in marsupials, the meeting of the membrane bones on the roof of the skull.

The overall conclusion from these studies was that, while there were some heterochronies in the development of individual elements within a system (e.g., the secondary palate does close early relative to other events in the cranial skeleton), by far the most significant difference between the two groups was the relative timing of differentiation of structures of the feeding apparatus relative to the differentiation of the central nervous system. Although previous authors have noted the early maturation of structures important in feeding, this series of studies showed that, when a broad phylogenetic sample was compared, the most notable feature was the whole scale shift in these two systems.

Smith (1997) hypothesized that this shift was due to the interaction of several independent factors. First, because the highly altricial neonate must have a fully functional oral apparatus, several structures of the skeletal-muscular system as well as many physiological systems must be in place at birth. These systems are absolutely critical to the survival of the newborn. At the same time, the period of organogenesis is exceedingly short in marsupials. As a consequence, there may be a limitation on cell resources and/or energy and raw materials. It has long been noted that the brain is an expensive tissue to build and maintain (e.g., Aiello and Wheeler, 1995), and Smith hypothesized that, because of the very short gestation period, development was under an energetic or material constraint. Embryonic resources were put into skeletal and muscular events rather than into structures that were both expensive and of apparently little immediate use to the embryo (i.e., the telencephalic cortex).

Model system approaches, which examined patterns in detail in *Monodelphis*, provide further information on individual elements. Clark and Smith (1993) studied ossification sequence and pattern in *Monodelphis domestica* and *Macropus eugenii*. Cranial ossification patterns were also studied by Nesslinger (1956) and Frigo and Woolly (1996) in other marsupial species and confirm that the basic patterns presented by Clark and Smith for *Monodelphis* and *Macropus* are general patterns for marsupials. Clark and Smith (1993) showed that the craniofacial skeleton ossified as two distinct units. The bones of the face are the first bones to begin ossification and also the first to complete ossification, whereas those of the neurocranium lag, with the exception of the exoccipital bone (which ossifies early as it serves as the attachment point of cervical muscles). "The face contains multiple ossification centers at a time when the neurocranium is still housed in membrane and cartilage and the bones of the face have approached each other to form a solid structure when the bones of the braincase are isolated elements. An excellent example of this pattern of growth is found in the ossification of the squamosal. The squamosal bone has two components, the zygomatic process, which contributes to the posterior bar of the zygomatic arch, and the squamous portion, which contributes to the sidewall of the braincase and also contacts the periotic. In *M. domestica* the zygomatic process is the first part of this bone to ossify, and by day 3, it has approached the jugal bone to complete the zygomatic arch. This portion is functionally a part of the facial skeleton. The squamous portion grows very slowly over the side of the braincase and makes its first contact with other bones 20–25 days postnatally when it touches the alisphenoid and the parietal. The frontal and parietal bones in both species exhibit similar patterns of relatively slow growth over the braincase" (Clark and Smith, 1993, p. 139).

Clark and Smith conclude that the slow and late ossification of both the dermal and endochondral elements of the braincase is almost certainly related to the period of extended brain growth. Several studies indicate a mechanistic relation between brain growth and development and the ossification of the neurocranium (e.g., Moss and Salentijin, 1969; Hanken, 1983; Showing, 1988; Herring, 1993), and it is likely that the relatively slow ossification of the neurocranium is in response to the long period of brain growth observed in marsupials.

One interesting feature of the marsupial neonate is the "recapitulatory" development of the jaw joint (Fig. 4). At birth, the jaw support is through the elements that form the primary jaw joint in nonmammalian tetrapods, the quadrate (incus), and articular (malleus). In *Monodelphis*, the condylar cartilage develops by approximately day 7 postnatal (P) and is relatively large. However, at this age, it does not sit in the glenoid fossa but abuts a thin wedge of bone from the squamosal. At this time, the post-dentary articulation (malleus and incus) is also large and there is, thus, a double jaw joint. By approximately day 20, a well-formed dentary squamosal joint is formed. The malleus and incus are still relatively large and retain a firm articulation with the braincase but are no longer connected to the dentary through Meckel's cartilage. They are incorporated into the middle ear in the next two weeks (Filan,

1991; Maier, 1993; Clark and Smith, 1993).

Smith (1994) studied the development of craniofacial musculature in *Monodelphis* and compared it with patterns reported for eutherians. She found that, unlike the cranial bones, for the most part all craniofacial muscles developed together on a similar time course. A few muscles, specifically the tongue and pharyngeal muscles, showed various stages of development (such as elongation and orientation of myoblasts, fusion of myotubes, and maturation of myofilaments) slightly earlier than other muscles (such as the ocular and facial muscles). However, by and large, the differences were minor (within a few days), and most muscles of the craniofacial region developed more or less simultaneously, even those that would not be required for function for several weeks (e.g., external ocular muscles). This finding was in marked contrast to the development of cranial bones, which showed gross functional and regional heterochronies.

The relative timing of muscle development was quite similar to patterns observed in mice and rats and many other species. The reasons for this more or less simultaneous development of craniofacial muscles are unknown (possible hypotheses include some kind of constrained regulatory program or the need to integrate with developing neuromotor systems). However, in the development of musculature, *Monodelphis* shows no notable heterochrony when compared with other mammals. In both *Monodelphis* and the eutherians thus far examined, the tongue is among the first muscle to align and mature. Therefore, one of the features often identified as an adaptation of marsupials—the early development of tongue musculature—is actually a feature that appears to be shared by all mammals (a similar pattern was observed in monotremes; Smith, unpublished observations). Intrauterine tongue movements are common in mammals and may be associated with proper facial and palatal development. Indeed tongue and pharyngeal muscles are among the first to be active in human fetuses—often showing motor activity such as the swallowing reflex between weeks 10 and 12 (Smith, 1992).

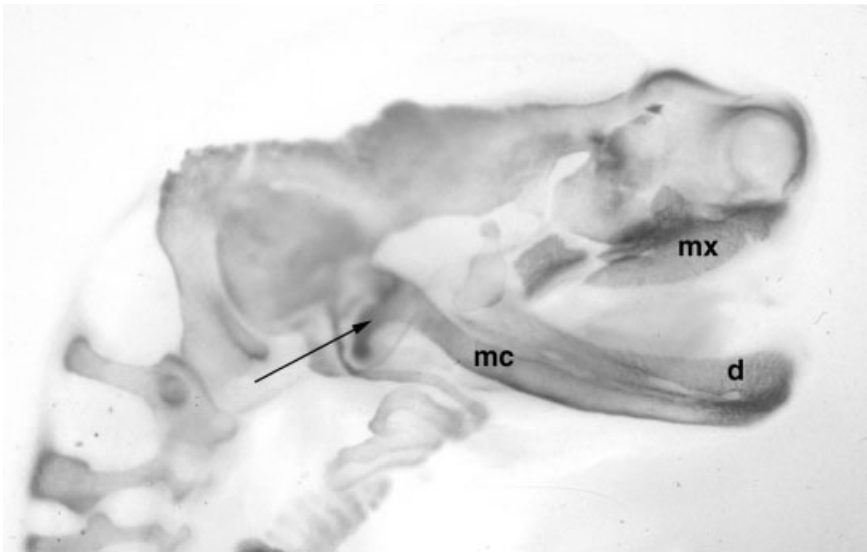

Fig. 4. A whole-mount stained and cleared head of a 2 day postnatal *Monodelphis* pup. Note that the jaw joint is formed by the quadrate and articular bones, that there is no dermal ossification around the cranial roof, and that the chondrocranium is extremely robust, especially in the nasal region and the sidewall of the braincase. mx, maxillary bone; d, dentary bone; mc, Meckel's cartilage; arrow points to the anlagen of incus (= articular), forming the primary jaw joint.

However, there are two very interesting differences between marsupials and eutherians (Smith, 1994). First, muscle differentiation is almost entirely prenatal in rodents and other eutherians, whereas it is largely peri- and postnatal in marsupials. Consequently in marsupials much of the fundamental organization and maturation of craniofacial muscles in marsupials occurs while the young are attached to the teat, suckling. It is possible that the difference is not as stark as it appears, as eutherians appear to perform many active oral movements during the later stages of fetal life. Nonetheless the marsupial newborn's life depends on a properly functioning oro-muscular apparatus during the period in which this apparatus is undergoing early stages in differentiation. This phenomenon is particularly marked in the highly altricial dasyurids where the jaw muscles at birth consist of little more than a few myotubes.

A second interesting difference is the fact that, although the time course of muscle development is quite similar in eutherians and marsupials, as shown above, the development of the cranial skeleton is quite different in the two. The skeletal and muscular systems in most species develop over the same period. But in marsupials, because so many skeletal elements are delayed in development, some muscles appear far in advance of the skeletal elements that will form their attachment points. For example, the first arch muscles that attach to the dentary, maxilla and zygomatic arch are attached to bone relatively early in their development, while the muscles attaching to bones of the neurocranium develop late attachments. The area of the attachment of the temporalis muscle, for example does not fully ossify until approximately 19 days after birth. While the late attachment may not have a significant impact on the muscles themselves (which are attaching to tendons), this observation raises interesting questions regarding the mechanical effects of the muscles on bone. It is well known that the mechanical environment in which a bone develops has a significant impact on that bone's development. In marsupials, the cranial skeleton faces a wide variety of mechanical influences during its initial stages of development.

Dental development in marsupials has been studied since 1897 when Hill (Wilson and Hill, 1897), one of the pioneers of detailed embryological and anatomical studies of marsupials, described dental eruption in *Perameles*. In this study, the notable fact that

marsupials appear to generally suppress one generation of dentition was examined in detail. In later years, several authors have studied this phenomenon, including Kirkpatrick (1978), Luckett (1993), and Luckett and Woolley (1996). These authors have demonstrated that the first generation of teeth (the deciduous dentition) is either absent or vestigial in all marsupials thus far examined, with the exception of a single locus, the fourth premolar, which is replaced. In some species, there is no evidence of any first generation teeth, whereas in others, the vestigial deciduous dentition may advance to dentine differentiation before regressing. Several authors, starting with Wilson and Hill (1897; Winge, 1941, Ziegler, 1971; Luckett, 1993), have concluded that the loss of the first dental generation is specifically related to the long period of attachment of the young to the teat. These authors suggest that the attachment of the young may lead to some kind of mechanical suppression of dental development. In addition, others (e.g., Cifelli et al., 1996) have suggested that the loss of the deciduous dentition is evidence in and of itself of a marsupial reproductive pattern and have used this trait to infer the reproductive biology of fossil organisms.

Van Nievelt and Smith (2005a,b) studied dental development in detail in *Monodelphis domestica*, with comparisons with other species, and concluded that the relation between the attachment of the young to the teat and the suppression of a dental generation was not clear cut. Specifically, they made two major observations. First, they found no evidence of suppression of odontogenesis during the period of fixation. During this time, the anterior dentition undergoes all morphogenetic stages, therefore, refuting any hypothesis of direct mechanical suppression of dental development due to teat attachment. Second, van Nievelt and Smith summarized data demonstrating that the suppression of deciduous dental elements is quite common in mammals and is not unique to marsupials. Many eutherians suppress one generation at a single locus and some suppress the entire first generation. Therefore, the marsupial reproductive pattern is neither a necessary nor a sufficient condition to explain the loss of a dental generation, as it appears to occur in many different taxa in relation to many different phenomena.

Interesting questions remain, however, on the reasons for the loss of deciduous teeth at most loci in marsupials and the relation of this pattern to marsupial life history, growth, and development. More recently, marsupial dentition has received attention as a potential model for molecular genetic studies of dental development (e.g., Jernvall, 1995). Unlike mice, marsupials possess a complex, heterodont dentition and may prove useful for understanding mechanics of dental differentiation.

Finally, details on the development of the brain and sense organs have been provided in many studies. These are beyond the scope of the current study, but ongoing work suggests that the relative delay in the CNS is not general but, instead, confined largely to the telencephalon, and to a lesser extent the diencephalon and mesencephalon. The olfactory bulb and the rhombencephalon are not delayed (when compared with the relative timing of events in *Mus*), as might be expected given functional requirements of the neonate (Cork and Smith, manuscript in preparation).

Studies summarized here largely cover the organogenic period. However, the origination of these heterochronies is unknown. There are two alternatives for the origins of these timing shifts. First, it is possible that these heterochronies represent shifts in the later differentiation and maturation of structures and are largely due to changes in relative growth. Alternatively, these differences may represent a deeper underlying heterochrony in the relative timing of first differentiation of the major regions and tissues of the face. To distinguish between these two alternatives, earlier events in craniofacial differentiation must be examined. Of particular interest is the early differentiation of the cranial neural crest.

DIFFERENTIATION AND MIGRATION OF CRANIAL NEURAL CREST

Neural crest cells make up much of the connective tissues of the facial region and appear to be critical in patterning muscular organization (e.g., Noden, 1983, 1984, 1988; Couley et al., 1993, 1998; Hall, 1999; LeDouarin and Kalcheim, 1999). Commonly in vertebrates, neural crest cells emerge after the neural folds have closed (e.g., Tosney, 1982; Hall and Horstadius, 1988; Epperlein and Löfberg, 1993; Hanken et al., 1997; Hall, 1999; Horigome et al., 1999; LeDouarin and Kalcheim, 1999; Falck et al., 2000), although in placental mammals and other vertebrates such as some anurans, neural crest emigration is initiated during neural fold elevation stages. In mice and rats, in which crest migration has been well studied, neural crest cells are generated at the future first arch region at the three-somite stage and first arch crest leaves the neural tube at approximately four to five somites, as the neural tube is beginning to close (Nichols, 1981, 1987). (In this review, I use the term "first arch crest" to refer to crest that arises anterior to the third rhombomere and, thus, to crest that will enter the frontonasal region as well as the first arch proper.) Post-otic crest begins to emerge at the six- to eight-somite stage, when the neural folds first begin contact, and the second arch crest appears at the eight- to nine-somite stage (Nichols, 1981, 1987; Tan and Morriss Kay, 1985, 1986; Morriss-Kay et al., 1993; Osumi-Yamashita et al., 1994, 1996; Serbedzija et al., 1992).

In *Monodelphis*, neural crest cells begin to leave the neural plate at the beginning of stage 22, approximately 10 days after mating (Smith, 2001a; Vaglia and Smith, 2003) and a few hours after primitive streak formation. The stage 22 embryo consists of little more than a broad, flat neural plate. There is virtually no morphological differentiation within this plate except for shallow notches at the side of the neural tube, the first indications of the pre-otic and otic sulci. First arch neural crest disengages in a broad region anterior to the pre-otic sulcus from the ventral surface of the very thin neural plate at the margin of the neural plate and ectoderm. It accumulates as a broad mass sandwiched between the flat neural plate and the ectoderm and the underlying mesoderm. This single mass of crest ap-

pears to give rise to all the neural crest of the future mandibular, maxillary, and frontonasal processes (Fig. 5).

By stage 24 (6–8 somites), a large mass of neural crest has accumulated in the first arch region and crest has started appearing in the second arch region. The pre-otic and otic sulci are well defined and appear to serve as boundaries between the various neural crest streams. Both optic pits and otic placodes are present at this stage, but there is no morphological indication of a midbrain/hindbrain or midbrain–forebrain boundary. Nor is there any contact of the neural folds at any point along the embryonic axis. At this stage, the neural crest lies as a mass of tissue ventral to the flat neural plate and dorsal to the paraxial mesoderm. The paraxial mesoderm shows no sign of subdivision or localized proliferation.

The first contact of the neural folds in the post-otic and cervical regions occurs a stage 25 (12–13 pairs of somites), although the anterior parts of the brain remain open until stage 28. At this time, rhombomeres are identifiable, although there is still minimal differentiation anteriorly. There is a large accumulation of mesenchyme in the first arch and frontonasal regions, and maxillary and mandibular processes are distinguishable for the first time. Neural crest continues to leave the neural plate from the region anterior to the pre-otic sulcus. The second arch is also well developed and neural crest appears in the post-otic region. By stage 27, neural crest cells appear to have more or less ceased disengaging from the neural plate in the first arch region. At this time, the neural folds are still open anterior to the otic region, and although there is minimal differentiation of the neural tube anterior to the optic vesicles, major regions are recognizable. The telencephalon is particularly delayed in development.

One consequence of the early departure of neural crest from the neural plate is a change in the spatial relations between neural crest and various tissues in marsupials, compared with other amniotes. The earliest populations of postmigratory crest lie sandwiched in a three-layer, flat composite, with the neural plate and ectoderm dorsal to the crest, and a single cell layer of mesoderm ventral to the crest (Fig. 5E). After crest migration, when the neural folds elevate and the paraxial mesoderm proliferates, the geometry changes so that the more typical spatial relations are achieved (e.g., neural tube and paraxial mesoderm medial to the crest and ectoderm lateral to crest).

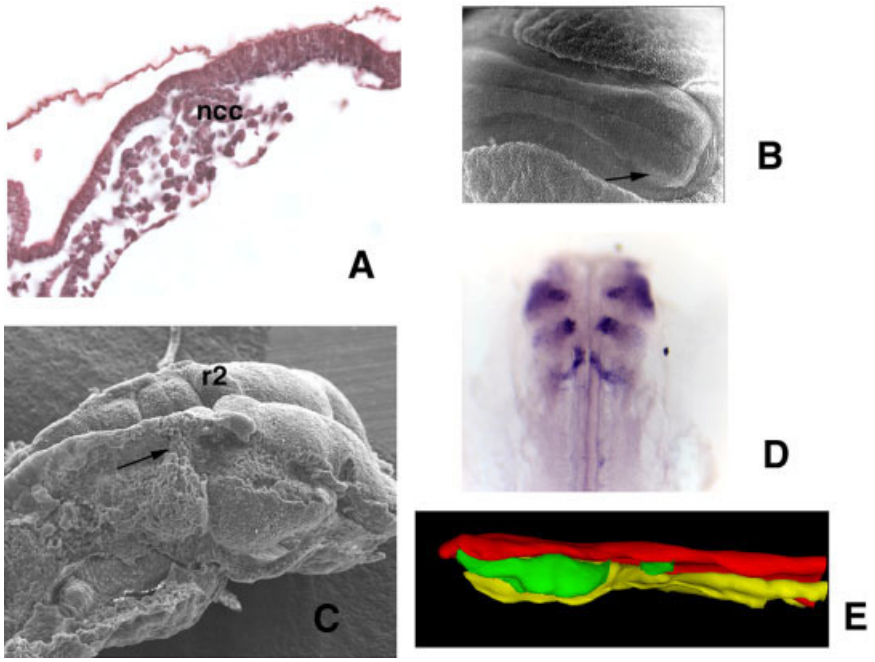

Fig. 5. Neural crest migration in *Monodelphis domestica*. **A:** Histological section through the first arch region of a stage 23 *Monodelphis* embryo. Neural crest cells (NCC) can be seen disengaging from the underside of the neural plate/ectoderm junction and migrating into the region between the ectoderm and endoderm. **B:** Photograph of a stage 23 embryo. The arrow represents accumulated crest. **C:** Scanning electron photomicrograph of a stage 25 embryo, showing migrating second arch neural crest (arrow) and rhombomeres. The second rhombomere (R2) is labeled. **D:** In situ hybridization of slug gene in stage 26 *Monodelphis*; dorsal view. Slug stains migrating neural crest. The streams to the first, second, and post-otic branchial arches can be seen. **E:** Three-dimensional reconstruction of neural crest (green) in a stage 23–24 *Monodelphis* embryo. The crest destined for the first arch and frontal nasal region accumulates as a broad mass between the dorsal neural plate and ventral paraxial mesoderm (a thin sheet, just a few cells thick in the cranial region at this stage). Second arch crest can also be seen behind a distinct crest free zone.

In summary, there is a striking difference between marsupials and most other vertebrate in the relative degree of development of the neural tube at the time that neural crest cells leave the neural plate. In most vertebrates thus far described, the neural tube is well developed at the time of neural crest emigration, and forebrain, midbrain, and hindbrain boundaries are apparent. In marsupials, at the onset of crest emigration the neural plate is flat and virtually featureless, except for the presence of shallow notches that mark the future pre-otic and otic sulci. No morphological landmarks exist in the region anterior to the pre-otic sulcus; thus the rostral hindbrain, midbrain, and forebrain are not distinguishable. Crest delaminates from the entire region anterior to the pre-otic sulcus as a single mass. The mass of crest collects beneath the flat neural plate and above a thin, undifferentiated sheet of paraxial mesoderm. The migration of most first arch neural crest is complete before the forebrain, midbrain, and rostral hindbrain may be distinguished by morphological boundaries.

Table 2 summarizes the relative timing of neural crest and neural tube differentiation in *Monodelphis* and in mice and rats. *Monodelphis* exhibits a mosaic of advanced and delayed features even at this very early stage of development. For example, the timing of neural crest migration is advanced in *Monodelphis* relative to both the differentiation of non-neural tissue (somites) as well as neural tissue (first contact of neural folds); other ele-

TABLE 2. Major Events in the Differentiation of the Neural Tube, Sensory Organ Anlagen, and Neural Crest in Murid Rodents and *Monodelphis domestica*[a]

Mouse, rat	Number of somites	Monodelphis domestica
	0	Pre-otic sulcus, otic sulcus, first arch neural crest begins migration
Pre-otic sulcus [1]	~1–2	
Otic sulcus [1]	~3	
First arch neural crest [2]	~4	Second arch neural crest, optic pits
First contact of neural folds [2]	~6–8	
Post-otic neural crest[3], Optic pits [2]	~6–8	Post-otic neural crest
Second arch neural crest [3]	~ 8–9	
	~12–13	First contact neural folds
	~15–18	Olfactory placode
Anterior neuropore closes [4]	~20	
Otocyst closes [4]	~25–29	Otocyst closes, anterior neuropore closes
Olfactory placode [4]	~30–34	

[a]Not only are events in *Monodelphis* accelerated relative to the differentiation of somites, but the migration of crest is early relative to other events in the differentiation of the neural tube and sense organs. The olfactory placode is also advanced in relative timing of differentiation in *Monodelphis*. References for *Mus* and *Rattus*: 1: Ruberte, Wood, and Morriss-Kay (1997); 2: Nichols (1981); 3: Tan and Morris Kay (1986); 4: Kaufman and Bard (1999). *Monodelphis* data from Smith (2001a).

ments such as the closure of the anterior neuropore are notably delayed in *Monodelphis*.

Therefore, the developmental shifts observed during the organogenic period appear to extend back to some of the earliest events in the differentiation of tissues of the face and central nervous system. While the ultimate causality of this shift is not yet understood, it is clear that marsupials differ from eutherians and other amniotes thus far studied. At the earliest "decision point" between the neural and facial tissue, the marsupial embryo allocates cells to the oral–facial skeletal tissues by means of the neural crest rather than the central nervous system. The differences between marsupials and placentals are not ones in relative rates of final differentiation of organs but include earlier and more fundamental changes in developmental pattern.

The discussion above was based on *Monodelphis* but suggested that this pattern was general for marsupials. Although there have been no published descriptions of neural crest appearance in other marsupials, neural crest was studied extensively by J. P. Hill and K. P. Watson in the early part of the 20th century. This work was briefly mentioned in a short study published after Hill's death (Hill and Watson, 1958). The unpublished material is extensive and includes detailed descriptions of the development of neural structures, including neural crest in embryonic series of macropodids, dasyurids, and peramelid marsupials. The work of Hill and Watson (1958) shows that the patterns described above for *Monodelphis* are, indeed, general for marsupials. It is currently being prepared for publication. (More information on Hill and Watson's studies can be found at the following Web page http://www.biology.duke.edu/kksmithlab/JPHill/hill_index.htm.)

NEURAL CREST AND CRANIOFACIAL PATTERNING

The advancement of neural crest differentiation relative to other elements of the face means that the context of neural crest differentiation and migration differs in marsupials relative to other vertebrates. This finding is significant because of neural crest's role in patterning the facial and branchial arch region. It is clear that proper patterning involves a complex interplay of signals from mesodermal cells and neural crest, the ectoderm of the facial and branchial arch region and the endoderm of the oral cavity and pouches (e.g., Prince and Lumsden, 1994; Köntges and Lumsden, 1996; Veitch, et al., 1999; Piotrowski and Nussien-Volhard, 2000; Trainor and Krumlauf, 2000, 2001). However, there is also compelling evidence that many important signals are derived from patterns initially laid down in the neural tube and that at least some of the patterning information in the facial region is carried by the neural crest.

For example, Noden (1983, 1988) showed that when premigratory first arch crest cells were transplanted into the region of the second arch, they transformed the morphology of this arch. In this case, duplicated first arch elements were produced in the region of the second arch. These experiments suggested that premigratory crest carried information that specified first arch patterning. More recently, Trainor et al. (2002) demonstrated that Fgf8 expression in the isthmus, the boundary between the midbrain and hindbrain, was critical in differentiating this first and second arch signal. Second arch structures are produced when the crest carries Hoxa-2 signals; the Fgf8 expression in the isthmus appears to down-regulate this expression in the first arch crest. If the isthmus is transplanted so that Hoxa-2 is not expressed in the second arch crest, then this crest will produce first arch structures.

Further evidence of the existence of patterning information in premigratory neural crest was provided by Schneider and Helms (2003). In a series of elegant experiments, they show

that when premigratory crest from a quail is transplanted into developing duck embryos, the crest is capable of transforming bill morphology into beak morphology (and vice versa). These data demonstrate that premigratory crest contains species specific information.

The region-specific patterning of the neural crest appears to relate in part to the fact that it arises from the neural tube, which possesses region-specific gene expression patterns (e.g., Keynes and Lumsden, 1990; Lumsden and Krumlauf, 1996; Lee et al., 1997; Irving and Mason, 1999, 2000; Shamin et al., 1999; Joyner et al., 2000; Mason et al., 2000; Gavalas et al., 2001; Martinez, 2001; Hildalgo-Sanchez et al., 2002; Trainor et al., 2002). Notably, in both mice and chick, the genes that appear to either signal or pattern regional specificity in the brain are expressed before neural crest leaves the neural plate.

It is in this light that the relative timing of neural crest migration in marsupials is of most interest, as morphological studies suggest that at least in anterior regions, neural crest migration is well under way before regionalization within the neural plate has occurred. In particular, first arch crest has largely migrated before forebrain, midbrain, and rostral hindbrain regions are distinguishable. It is unknown, however, if region-specific gene expression has been established. Two major potential alternatives for the relation between neural tube genetic differentiation, neural tube morphological differentiation, and neural crest differentiation in marsupials exist. One is that in the neural tube genetic differentiation is correlated with, and primarily related to, neural tube morphological differentiation, as it is in other vertebrates so far studied. If this is the case, then we would predict that expression of major patterning genes would occur at a similar stage of neural tube morphology in marsupials and other vertebrates. In this case, neural crest migration would occur largely before genetic differentiation and crest identity must be regulated independently. Alternatively, it is possible that genetic expression in the neural tube will be early relative to morphological indicators of neural tube differentiation and, therefore, be present in marsupials when neural crest differentiation begins. Studies designed to distinguish these alternatives are under way.

DISCUSSION, CONCLUSIONS, AND PROSPECTIVE FOR FUTURE WORK

Comparative studies of craniofacial development in marsupial and placental mammals may contribute to understanding several different kinds of questions. First, they provide a model system in which the developmental mechanisms producing an evolutionarily important event—the adaptation of the neonate for independent function at a highly altricial state—may be studied. The work cited here traces some of these changes in relative timing and rate of development at organ and cellular levels and points the way to critical genetic changes. It, therefore, provides a case study for the production of evolutionary change by specific shifts in developmental mechanisms at multiple levels of organization.

Although these data do little to resolve outstanding questions on the evolution of the marsupial and eutherian reproductive strategies, they do suggest that the developmental consequences of the reproductive strategies are significant. The shifts we observe in marsupials appear to extend back to some of the earliest events in morphological differentiation and seem to involve major changes in the relative timing of morphological, cellular, and genetic events. There is little evidence that development can be characterized as slight shifts in timing with a continuum linking marsupial and placental mammals.

Second, a marsupial–eutherian comparison provides us with a model system to test certain hypotheses on the relationships among various developmental events. Many events that are highly correlated during development in more typical model systems are shifted in relative timing in fundamental ways in marsupials. These kinds of natural timing shifts might be used to help test hypotheses on causality. Comparative tests have long been used by functional biologists, but are only recently being used in developmental biology. Properly applied, they can provide great insight.

For example, this kind of natural experiment has been useful in addressing the issue of modularity and/or integration in the craniofacial region (e.g., Raff, 1996). It has been hypothesized that highly integrated units will evolve in a correlated manner and that units that are subdivided into modules may possess greater evolutionary flexibility. We would hypothesize that units retaining conserved patterns of development in both marsupials and placentals, despite the overall change in timing of surrounding elements, may be integrated by important developmental mechanisms. For example, the neurocranium, whose ossification is highly related to, and integrated with, the growth and differentiation of the brain, develops relatively late in marsupials. In contrast, we see little relation between the ossification of bones and the development of the muscles that attach to them. Another example concerns the elements of the first arch. Alberch (1980) and Kay (1986) have suggested that, in mammals, first arch elements are particularly integrated in evolution and development. Smith (1996) discusses these hypotheses and shows that elements of the first arch appear to not be particularly linked, at least in terms of developmental timing. Some elements of the arch are highly delayed in marsupials, while others are among the first to develop, weakening the argument of tight integration.

Another important and general issue is the possibility that energetic or material constraints during the very rapid development of the embryo are causally responsible for the delay of neural, and in particular forebrain, development. Energetic or material constraints during development have been recognized in several different model systems (e.g., Aiello and Wheeler, 1995; Nijhout and Emlen, 1998; Nowicki et al., 2002; Fish and Lockwood, 2003; Nowicki and Searcy, 2004; Leigh, 2004) and further study of potential constraints on marsupial brain development may provide an interesting case study of this general phenomenon.

Finally, in ongoing studies we hope to understand the relation between patterning in the neural plate/tube

and patterning in the neural crest and peripheral tissue. It is not known whether the early segmental organization of the neural plate (the hindbrain as well as the forebrain and midbrain) is necessary for the proper patterning of the neural crest and entire pharyngeal arch region. The pattern observed in marsupials is particularly pertinent to this issue as all evidence indicates that neural crest differentiates and migrates before the segmental organization of the brain is established.

If in marsupials, neural crest cells migrate before local regionalizing signals appear in the neural plate, then it will demonstrate fairly conclusively that patterning signals from the neural plate carried by crest are not necessary for setting up peripheral pattern but that these signals may be regulated independently. Alternatively, if in marsupials region-specific genes are advanced in the timing of their expression and are expressed before crest migration, it will be strong evidence that they are critical to later patterning events and provide us with firm data on yet another level of heterochrony. In so doing, we hope to complete our understanding of the differences in pattern and mechanism of development in marsupial and placental mammals.

PROSPECTUS FOR FUTURE WORK

The heterochronies in craniofacial and indeed overall development evidenced by marsupials when compared with virtually any other model vertebrate, means that the study of marsupial development may help illuminate basic developmental processes. Already *Monodelphis domestica* is being used extensively by the biomedical community for work in development, genetics, and anatomy. The Southwest Foundation for Biomedical Research, which maintains the largest research colony of this organism, states that over 400 research papers have been published on *Monodelphis domestica* in the past 2 years (http://www.sfbr.org/pages/about_resources5.php). At least three relatively recent technical advances make the likelihood of major contributions arising from this organism high.

First, because of a variety of aspects of marsupial reproduction, obtaining marsupial embryos (as opposed to postnatal specimens) is quite difficult compared with many more commonly used model organisms. Many marsupials are highly seasonal breeders and many breed only once a year even in the laboratory. *Monodelphis* females are induced ovulators, and, therefore, must be in contact with males for a period before ovulating and becoming receptive. However, no plug forms to indicate copulation. In several labs, protocols to introduce males and females and film subsequent interactions have been developed that allow the documentation of mating so that precisely aged and staged embryos may be reliably obtained.

Second, *Monodelphis domestica* is the first marsupial whose genome will be sequenced. The initial shotgun sequence is complete, and genome assembly and SNP collection are under way (http://www.broad.mit.edu/mammals/opossum/). The genome of another marsupial, *Macropus eugenii*, the tammar wallaby, is also being sequenced in a joint effort by the United States and Australian governments (http://www.genome.gov/12512299). Once broader genome sequences are available, then it is anticipated that microarray analysis, genomic analysis and in situ hybridization studies of gene expression patterns will advance our understanding of the relation between genetic and morphogenetic events.

Finally, marsupial embryos have been successfully cultured for many years (e.g., Selwood and VandeBerg, 1992; Cruz et al., 1996; Selwood et al., 1997). Although there are no large scale programs using marsupial embryos in culture as an experimental model, the relative ease of culturing marsupials means future work on cell transplantation and even interspecies transplants are potentially feasible. At this time, marsupial embryo culture techniques are being used in fate mapping studies of population of cells in the neural plate and neural crest (Smith, unpublished observations).

Although at present, few labs are actively addressing issues of craniofacial development in marsupials, it is hoped that with further development of the above resources and techniques, studies of *Monodelphis domestica* will contribute much more to our understanding of comparative developmental patterns and mechanisms.

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