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Early Diencephalon Development in *Alligator*

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Key Words

Alligator mississipiensis · Development · Diencephalon · Evolution · Forebrain · Ontogeny · Pretectum · Reptiles

Abstract

Diencephalon development was investigated in a reptilian embryo, *Alligator mississipiensis*, beginning at a single compartment stage and continuing until internal subdivisions were present within major units. A variety of morphological techniques were used: immunocytochemistry, histochemistry, and cresyl violet staining. The diencephalon begins as a single unit. In the transverse domain, the diencephalon subsequently divides into two: the parencephalon and the synencephalon. The parencephalon then splits into the parencephalon anterior and parencephalon posterior. Still later, the synencephalon undergoes parcellation into the synencephalon anterior and synencephalon posterior. Subsequently, internal subdivisions occur in each of these four compartments. When the diencephalon has become subdivided into two compartments and continuing until internal subdivisions are present in each unit, a longitudinal border separating a dorsal, presumed alar plate, from a ventral, presumed basal plate, was seen. No clear cut subunits were reliably identified in the telencephalon or secondary prosencephalon during this period of early development in *Alligator*. Early diencephalon development in birds (chick) and mammals (humans) follows a similar pattern. Specifically, a single diencephalic compartment divides into two zones: the parencephalon and synencephalon. Subsequently, the parencephalon becomes subdivided into an anterior and posterior unit. Some studies, including the present one, have noted further parcellation of the synencephalon into an anterior and posterior component, whereas others have not. Notwithstanding differe nces as to whether the synencephalon is a single unit or not, these detailed analyses in reptiles *(Alligator)*, birds (chick), and mammals (humans), suggest that the initial pattern of early diencephalon development in amniotes is similar.

Introduction

A number of approaches have been used to unravel the organization and evolution of the forebrain in vertebrates [Nieuwenhuys, 1998b]. Varying degrees of success have been achieved through an analysis of neuronal cell types and aggregates and their respective properties in a wide variety of adult animals [Nieuwenhuys, 1998b; Striedter, 2005] usually selecting representative species in a given class. An alternative approach has been to analyze development, with the hope that certain features might be uncovered which would have remained obscure had only adult animals been examined [Puelles, 1995].

In analyzing the forebrain, the diencephalon was chosen rather than the telencephalon because its properties in nearly every aspect are seemingly less complex than that of the telencephalon [Sherman and Guillery, 2006]. It is believed that the diencephalon shares common characters in all vertebrates during early development and changes occur later in time which are specific for that group or species [Bergquist, 1952; Bergquist and Källén, 1954; Rubenstein et al., 1994]. However, some researchers

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have questioned whether such a phylotypic stage exists for body plans in vertebrates [Richardson et al., 1997]. It remains to be seen whether or not such a phylotypic stage is present for developing vertebrate brains.

Most developmental studies have focused on just a few species [Richardson et al., 1997] concentrating on mouse as a representative mammal and chick as an example for birds. However, because mammals and birds diverged early in phylogeny [Kumar and Hedges, 1998; Benton, 1999], using representatives solely from these two classes would not distinguish between similarities of the features in question based on inheritance from a common ancestor versus independent evolution of these traits [Pritz, 2005].

Crocodilians are the reptilian group most closely related to birds [Whetstone and Martin, 1979; Hedges, 1994]. As a representative of this order, the American alligator, *Alligator mississipiensis*, was selected because it is relatively available and its developmental stages have been well characterized [Ferguson, 1985].

This report details early diencephalon development in *Alligator* before overt compartmentalization begins and continues until the major histogenetic zones have become internally subdivided. Morphological techniques that proved successful in analyzing early hindbrain development in *Alligator* [Pritz, 1999] were applied to the forebrain. These observations form the basis of this report.

Two questions were addressed. First, what morphologic features identify divisions of the diencephalon at early stages of development in *Alligator*? Second, how do these observations in *Alligator* compare with early diencephalon development in other vertebrates? Only by documenting that the organization of the *Alligator* diencephalon early in development is similar to that found in birds, mammals, and other vertebrates can later divergences in morphology be of potential biological significance. Specifically, it was hypothesized that early diencephalon development in *Alligator* would follow the pattern described for birds [Vaage, 1969; Puelles et al., 1987; Larsen et al., 2001 – chick] and mammals [Müller and O'Rahilly, 1997 – humans]. If so, changes occurring later in ontogeny could be identified as the key events that produced the adult diencephalon in *Alligator* and perhaps other reptiles. These developmental events in *Alligator* could then be compared with similar ontogenetic changes in diencephalon development in birds and mammals. Armed with these stage specific morphologic data, the molecular events responsible for these findings could be investigated.

The present analysis did not attempt to determine whether the observed diencephalic subdivisions represented and satisfied the developmentally significant criteria enumerated by others [Keynes and Lumsden, 1990; Lumsden, 1990]. Similarly, these experiments did not investigate gene or transcription factor expression because it was first necessary to identify the individual units and their appearance during ontogeny before investigating other properties. Furthermore, unlike the hindbrain where rhombomeres can readily be seen even in unstained whole mount preparations [Pritz, 1999], identification of transversal diencephalic subdivisions proved much more elusive.

Materials and Methods

Procedures and protocols listed below were reviewed and approved by the Indiana University School of Medicine animal care committee. These details conform to the National Institute of Health guidelines.

Animals

Alligator eggs were obtained from the Rockefeller Wildlife Refuge in Grand Chenier, Louisiana. The location of the embryo was marked and the egg positioned with the embryo on top. Eggs were placed in a 2:1 mixture of vermiculite and water in an incubator at a temperature of 30°C.

Embryos were sacrificed between stages 2 and 16 [Ferguson, 1985]. Viable embryos were harvested by making an opening in the eggshell using fine scissors under magnification from an operating microscope. A variety of fixatives were used for the various morphological stains. However, best results for histochemical and immunocytochemical experiments were obtained with 100% methanol as the fixative in which embryos could be stored for many months at –70°C. Embryos were first staged [Ferguson, 1985] and then dissected free from surrounding tissues using a fine tungsten needle, microscissors, and jeweler's forceps. Except at the earliest stages, brains were divided at the isthmus and the forebrain and midbrain were processed as a single unit.

In brains stored at –70°C, best results for histochemistry and immunocytochemistry were achieved by gradual tissue re-warming: –20°C (30–60 min); 4°C (30–60 min); and then room temperature (≥30 min) prior to embedding. Brains stained for cresyl violet were placed in a variety of fixatives: 10% formalin; 4% paraformaldehyde; 4% glutaraldehyde; or Bouin's solution (0.9% picric acid, 9% formaldehyde).

Tissue Processing

Embedding and sectioning of tissue processed for peanut agglutinin histochemistry or immunocytochemistry was identical to the methods described previously [Pritz, 1999]. These techniques are summarized briefly.

Brains were embedded in gelatin or albumin-gelatin. Blocks that were processed for histochemistry or immunocytochemistry were placed overnight in 30% sucrose in sodium phosphate buffer (PBS; 0.1 M at pH 7.2). At least 4h before sectioning, blocks were

Abbreviations used in figures 1–9					
Acetyl Tub	acetylated tubulin	PP	parencephalon posterior	ap	presumptive alar plate
AChE	acetycholinesterase	R	rhombencephalon	bp	presumptive basal plate
CV	cresyl violet	S	synencephalon	c	caudal
D	diencephalon	SA	synencephalon anterior	l	lateral
M	mesencephalon	SP	synencephalon posterior	m	medial
P	parencephalon	s2–14.5	stage 2–14.5	r	rostral
PA	parencephalon anterior	T	telencephalon	v	ventral
PNA	peanut agglutinin	2°P	secondary prosencephalon		

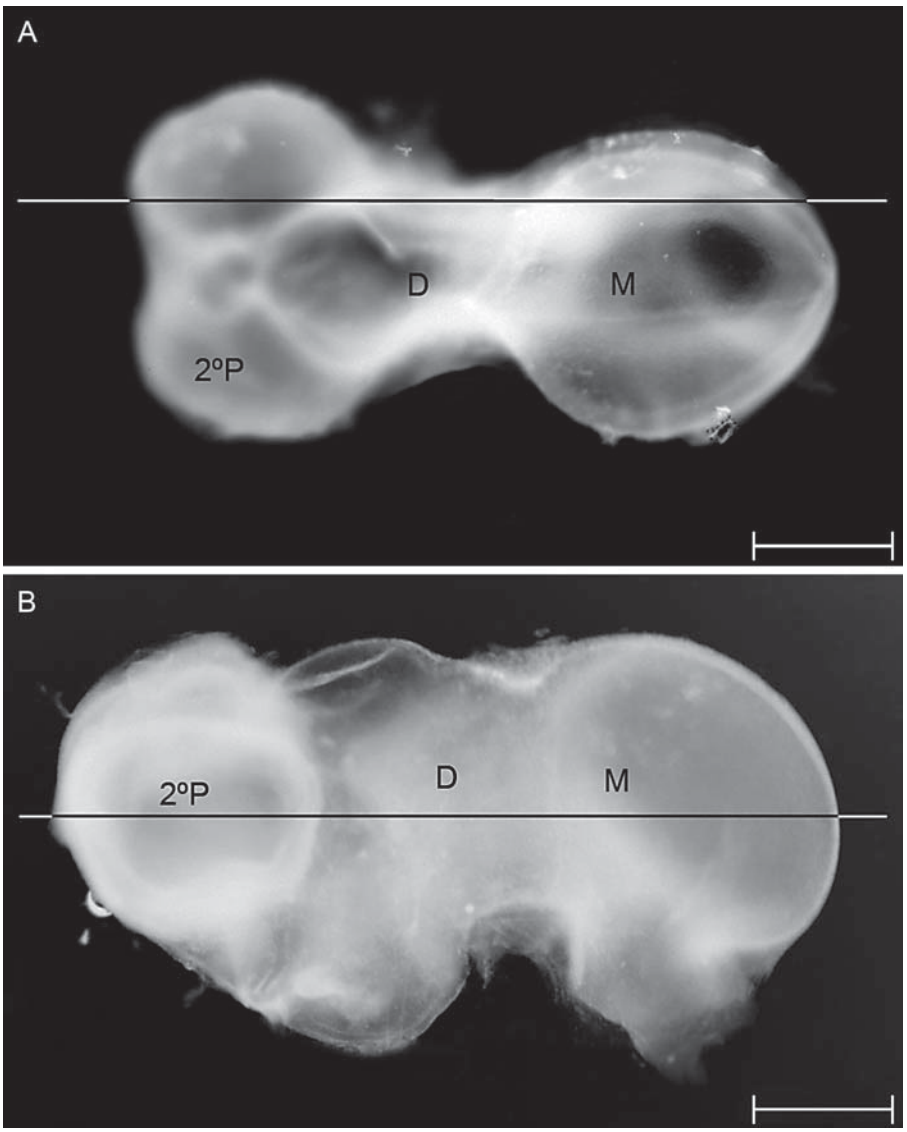


Fig. 1. Whole mount brains illustrating plane of section. Stage 12 brain viewed dorsally (**A**) illustrates the sagittal plane and the lateral photo (**B**) shows the horizontal plane. Scale bar = 1 cm.

placed in a solution of 2–4% formalin-30% sucrose-PBS. For cresyl violet staining, blocks were placed in 30% sucrose-10% formalin or 30% sucrose-4% glutaraldehyde solution overnight before sectioning. Frozen sections were cut sagittally or horizontally at 25–40 μm on a sliding microtome and collected in PBS. Sagittally sectioned brains were cut parallel to the rostrocaudal axis (fig. 1A) and brains cut horizontally were embedded with the dorsal brain surface as a base and then sectioned parallel to the plane illustrated in figure 1B. In a few cases, brains were sectioned transversely. With the exception of identification of the zona limitans interparencephalica, this plane of section was not useful at the stages of development examined in this study.

Methodology for peanut agglutinin histochemistry was identical to that described previously [Pritz, 1999]. Briefly, free floating sections were washed in three changes of PBS and then incubated in horseradish peroxidase (HRP)-peanut lectin (50 μg/ml) for 12–24 h. Tissue was then washed in three changes of PBS before incubation in a diaminobenzidine (DAB) solution consisting of 100 mg DAB in 10 ml of distilled water plus l ml of Triton X-100 plus 20 ml of 0.2 M cacodylic buffer (pH 5.6) plus 6 μl of 30% hydrogen peroxide for 30 s. Sections were then transferred to a solution of 70% alcohol for 1 min and then stored in PBS at 4°C until they were mounted out of distilled water onto chrome-alum coated slides.

Methods for immunocytochemical experiments were identical to procedures described in an earlier report [Pritz, 1999]. In short, free floating sections were washed in three changes of PBS and then incubated in the primary antibody in PBS containing 2% normal goat serum for 24 h followed by three changes in PBS and then incubated with the secondary antibody (goat anti-mouse IgG, Vector Labs, Burlingame, CA for primary antibodies raised in mouse or goat anti-rabbit IgG, Vector Labs, for primary antibodies raised in rabbit) at a concentration of 1:100 for 1 h. After three changes in PBS, sections were incubated in a peroxidase-antiperoxidase (PAP) complex appropriate for the primary antibody (mouse PAP or rabbit PAP; Chemicon, Temecula, CA) in PBS containing 2% normal goat serum at a concentration of 1:300 for 1 h. Sections were then washed in three changes of PBS before being reacted for 30 s in a DAB solution (see above) after which the reaction was stopped by transfer to a solution of 70% alcohol for 1 min before placement in PBS at 4°C until mounting. Mounted sections processed for either histochemistry or immunochemistry were dehydrated through a series of graded alcohols, cleared in xylene, and then coverslipped.

Immunoreactivity was observed with the following antibodies: monoclonal mouse anti-acetylated tubulin (1:500; Sigma, St. Louis, Mo., USA); mouse monoclonal anti-vimentin (1:50 or 1:100; Sigma); mouse monoclonal anti-acetylcholinesterase (1:50 or 1:100; Chemicon); and polyclonal rabbit anti-calretinin (1:500 or 1:1250; Chemicon). The following antibodies were unsuccessful in labeling forebrain compartments: monoclonal mouse anti-neural cell adhesion molecule (N-CAM) (Sigma); monoclonal mouse anti-glial fibrillary acid protein (GFAP; Boehringer Mannheim, Indianapolis, Ind., USA); and monoclonal mouse anti-growth-associated protein 43 (GAP 43; Sigma).

Controls for nonspecific staining were the following. One was substitution of normal rabbit or mouse serum for the primary antibody at a concentration equal to or greater than that of the primary antibody. A second was omission of the primary antibody from the reaction. Forebrain compartments were not visualized in this control tissue examined at these stages. Third, variation in antibody concentration produced more intense staining at higher, primary antibody concentrations.

Histological Analysis

Processed forebrain tissue was viewed under bright field illumination. Selected sections were drawn using a camera lucida drawing tube attached to a Leitz microscope or photographed using an Olympus microscope and a digital camera. Images were acquired in color, converted to black and white photos, and then imported into Adobe Photoshop 7.0 where the images were grouped in panels and labels were applied.

Results

The observations described below investigated forebrain subdivisions along two axes: rostrocaudal and dorsoventral. The former identified transverse diencephalic segments whereas the latter defined the presumed alar and basal plate of each respective division. This analysis began at the single segment stage and continued until internal subdivisions within a given primary compartment were observed. Morphological distinction between presumed alar and basal plate proved more difficult than the identification of transverse segments or their boundaries. Clear and reproducible divisions of the telencephalon or secondary prosencephalon were not observed. The rostral-most forebrain at stages after initial diencephalic compartmentalization was referred to as the secondary prosencephalon. The hypothalamus was included as part of the secondary prosencephalon and not as part of the diencephalon proper. Although some embryos were sectioned transversely during these stages of diencephalon development, observations in the sagittal or horizontal plane proved most useful. For naming of diencephalic subdivisions, the classical terms parencephalon and synencephalon were used [Rendahl, 1924; Puelles et al., 1987] and subsequently divided into anterior and posterior zones. The border separating the parencephalon anterior from the parencephalon posterior was termed the zona limitans interparencephalica following the nomenclature of others [Rendahl, 1924; Puelles et al., 1987].

A number of antibodies and histochemical markers were screened for their usefulness in identifying diencephalic subdivisions. Selection of these markers was guided by the observations of others (see below) and by the success of these stains in analyzing early hindbrain development in *Alligator* [Pritz, 1999]. For example, an anti-neural cell adhesion molecule which labeled diencephalic borders in chick [Figdor and Stern, 1993; Larsen et al., 2001] was unsuccessful in marking boundaries in

the present analysis. Similarly, peanut agglutinin histochemical preparations, which distinguish odd from even segments in chick [Figdor and Stern, 1993], stained the diencephalon in *Alligator* differently (see below). Furthermore, features that distinguished presumptive alar from basal plate were less distinct than transverse diencephalic zones or their respective borders. Frequently, this separation was highlighted by a staining pattern as opposed to a distinct line dividing the diencephalon longitudinally. Similarly, the pattern of longitudinal fibers stained by the antibody to acetylated tubulin early in compartmentalization gave the appearance of a dorsoventral boundary although the fibers themselves might not have represented this border. Nevertheless, a combination of histochemical and immunocytochemical markers coupled with the Nissl stained appearance in tissue which was examined both sagittally and horizontally over a number of developmental stages was felt to give a reliable and accurate assessment of early diencephalon development.

Single Segment Stage

At stage 2 [Ferguson, 1985], the telencephalon and diencephalon are unsegmented. The course and distribution of fibers visualized by acetylated tubulin immunoreactivity distinguished the diencephalon from the telencephalon rostrally and the mesencephalon caudally (fig. 2A). Clear borders between these three divisions were not nearly as distinct in Nissl stained material (fig. 2B). Regardless of the stain used, correlation of external shape and internal division was present (fig. 2). No longitudinal boundary separating presumed alar from basal plate was seen.

Two Segment Stage

Beginning at stage 3 and continuing through stage 4 [Ferguson, 1985], the diencephalon becomes subdivided into an anterior parencephalon and a posterior synencephalon. In fiber stained material using an antibody to acetylated tubulin, two wedge shaped areas separated by a fiber free zone were seen. Fiber sparse regions located posteriorly and anteriorly suggested borders with the mesencephalon and secondary prosencephalon respectively (fig. 3A). A similar appearance of the two diencephalic segments was seen in Nissl stained material (fig. 3B). In tissue processed for calretinin immunocytochemistry, a distinct border between the mesencephalon and synencephalon was observed. No clear boundary separating the parencephalon from the secondary prosencephalon was seen. Most of the parencephalon was calretinin immunoreactive (fig. 3C). For the first time during development, a dorsoventral boundary was observed. It was occupied by fibers (fig. 3A, B) and was marked by calretinin immunoreactivity. Using this latter antibody, the area inferior to this longitudinal division stained positively in the ventral parencephalon and negatively in the synencephalon (fig. 3C). In tissue reacted with an anti-

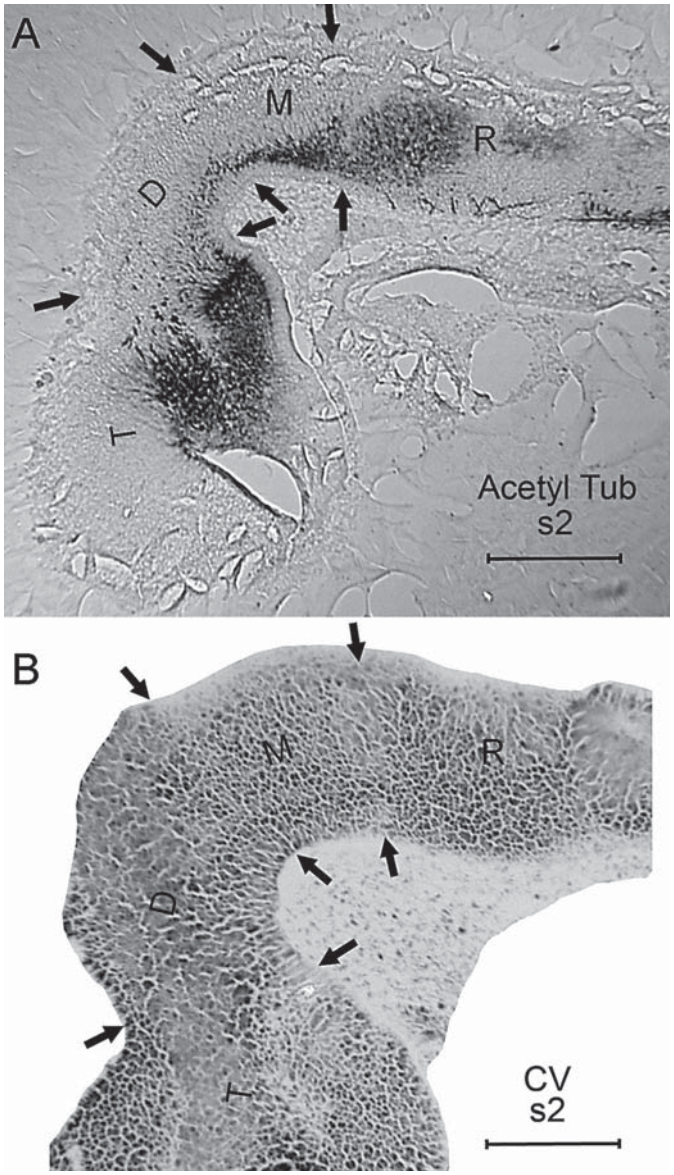
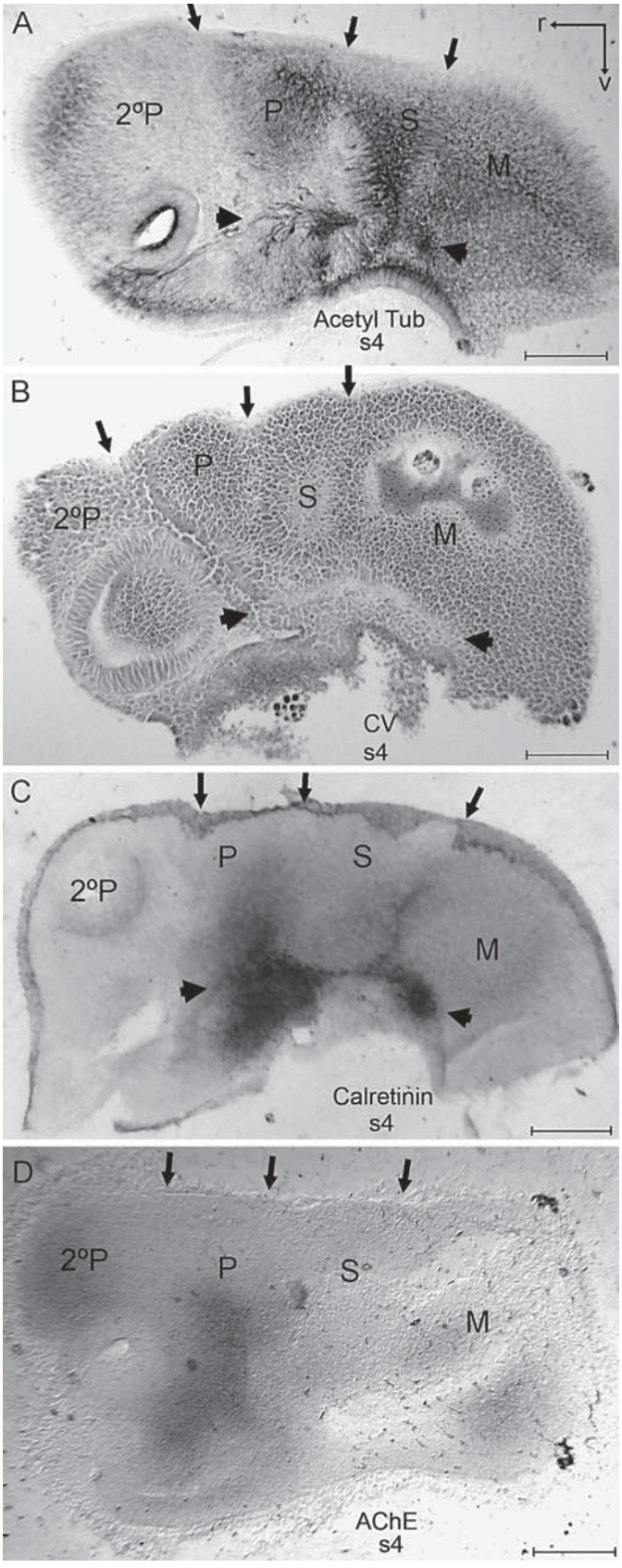


Fig. 2. Forebrain divisions at an unsegmented stage viewed sagittally. Stage 2 embryos stained with an antibody to acetylated tubulin (**A**) and with cresyl violet (**B**) show no transverse or longitudinal subdivisions in either the telencephalon or diencephalon. Scale bars = 200 μm.



body to acetylcholinesterase, the parencephalon was immunopositive but the synencephalon was not (fig. 3D). No longitudinal division separating presumptive alar plate from basal plate was observed with this latter antibody.

Three Segment Stage

Beginning at stage 5 and continuing through stage 11 [Ferguson, 1985], the diencephalon is composed of three divisions with the parencephalon subdividing into an anterior and posterior part. Calretinin immunoreactivity marked the boundary separating the parencephalon anterior from the parencephalon posterior (fig. 4A) as well as the border between the synencephalon and mesencephalon (fig. 4B). In an oblique sagittal section stained with an antibody to acetylated tubulin to visualize fibers, a band between the parencephalon anterior and posterior, the zona limitans interparencephalica, was partly seen (fig. 4C). Although distinct borders between the parencephalon posterior and synencephalon were not clear in tissue stained for calretinin (fig. 4 A and B) or acetylated tubulin (fig. 4C), fiber free zones were observed in Nissl stained material where three diencephalic segments were clearly identified (fig. 4D). A longitudinal boundary dividing presumed alar from basal plate regions was most distinct in material stained for calretinin (fig. 4A, B) and with cresyl violet (fig 4D).

Tissue sectioned horizontally (fig. 5) showed the relationship between neuromeric bulges and interneuromeric ventricular ridges. This was clearest in material stained with peanut agglutinin (fig. 5A) and cresyl violet (fig. 5B). Borders between the diencephalon and the secondary prosencephalon and the mesencephalon, respectively, and between the three diencephalic compartments (parencephalon anterior, parencephalon posterior, and synencephalon) were present on the lateral (pial) and me-

Fig. 3. Diencephalic divisions at the two segment stage viewed sagittally. Stage 4 embryos stained with an antibody to acetylated tubulin (**A**), calretinin (**C**), and acetylcholinesterase (**D**), and with cresyl violet (**B**) illustrate subdivisions of the diencephalon in both the transverse and longitudinal plane. Transverse separations (long arrows) illustrate parcellation of the diencephalon into a rostral zone, the parencephalon, and into a caudal segment, the synencephalon. The suggestion of a dorsoventral division extending longitudinally (short arrows) is seen in tissue stained with antibodies to acetylated tubulin (**A**) and calretinin (**C**) and with cresyl violet (**B**). Orientation in **A** is similar for all sections (**A–D**) shown. Scale bars = 200 μm.

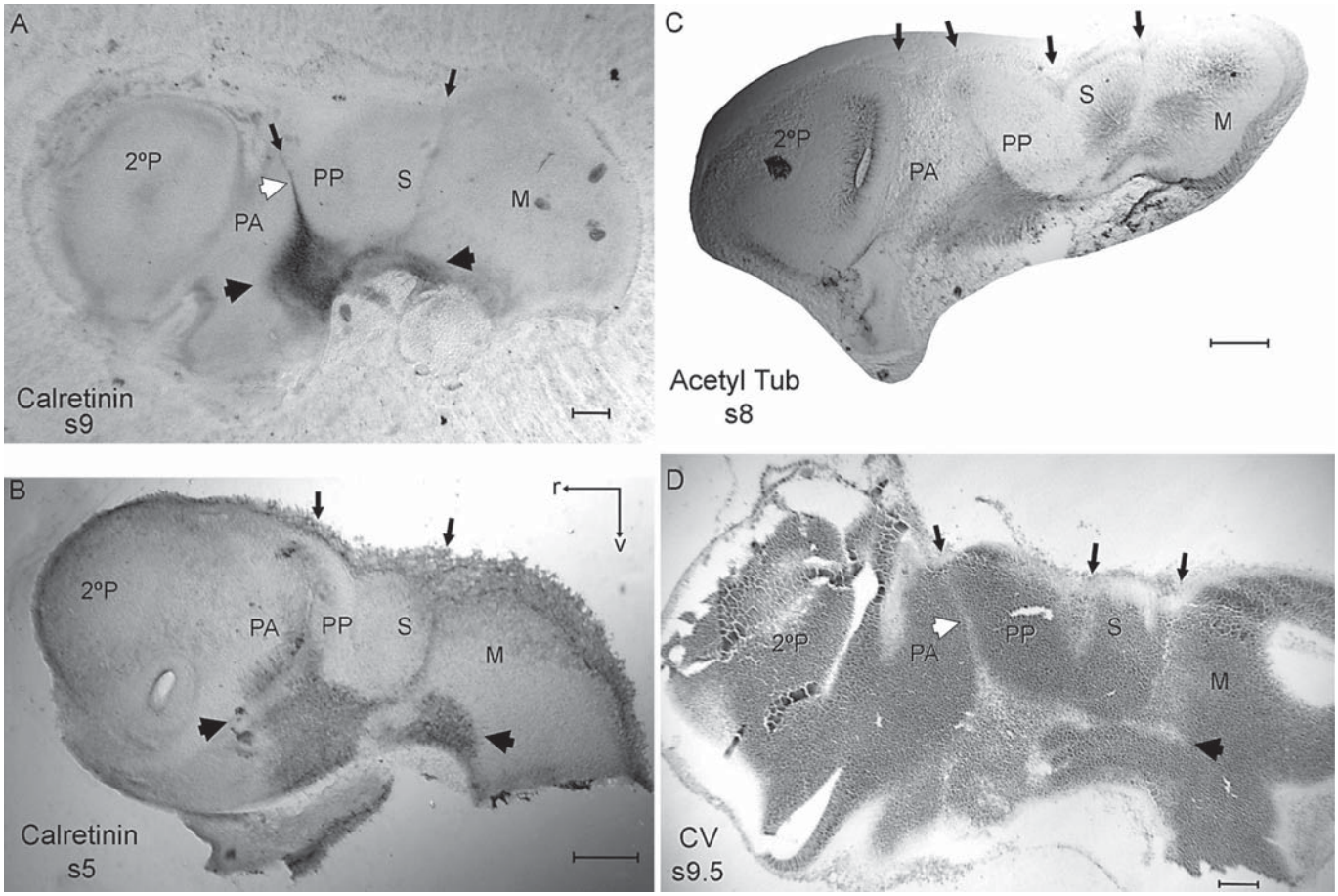


Fig. 4. Diencephalic divisions at the three segment stage viewed sagittally. Sections stained with an antibody to calretinin (**A**, stage 9; **B**, stage 5) and acetylated tubulin (**C**, stage 8), and with cresyl violet (**D**, stage 9.5) illustrate transverse subdivision (long black arrows) of the parencephalon into anterior and posterior compartments. The zona limitans interparencephalica (white arrow in **A** and **D**) is most clearly seen separating the parencephalon anterior from the parencephalon posterior. The suggestion of a dorsoventral border (short black arrows) is seen in some sections (**A**, **B**, **D**). Orientation of sagittal sections shown in **B** is the same for all sections (**A–D**). Scale bars = 100 μm.

dial (ventricular) surfaces (fig. 5A, B). The cell free area of the zona limitans interparencephalica, separating the parencephalon into anterior and posterior divisions, was distinct in cresyl violet stained material (white arrow, fig. 5B).

Four Segment Stage

Beginning at stage 11.5 [Ferguson, 1985], the synencephalon divides into an anterior and posterior portion. Thus, a total of four diencephalic segments are present and these divisions are more evident in horizontally as compared to sagittally sectioned material. At stage 15 [Ferguson, 1985], internal subdivisions were clearly seen. Only in sagittally sectioned material stained with an antibody to vimentin (fig. 6C) was a difference between the two subdivisions of the synencepalon suggested.

The zona limitans interparencephalica separating the parencephalon anterior and posterior was clearly seen in immunostained material using an antibody to acetylated tubulin (fig. 6A, 7A) and calretinin (fig. 6B, 7B–D) in which both cells and fibers seem to be labeled (fig. 7D).

Differential staining using an antibody to vimentin suggested a transverse border ventrally between the secondary prosencephalon and parencephalon anterior. A border separating the synencephalon posterior and mesencephalon was faintly seen in calretinin immunoreactive sections (fig. 6B). Some of these intra-diencephalic

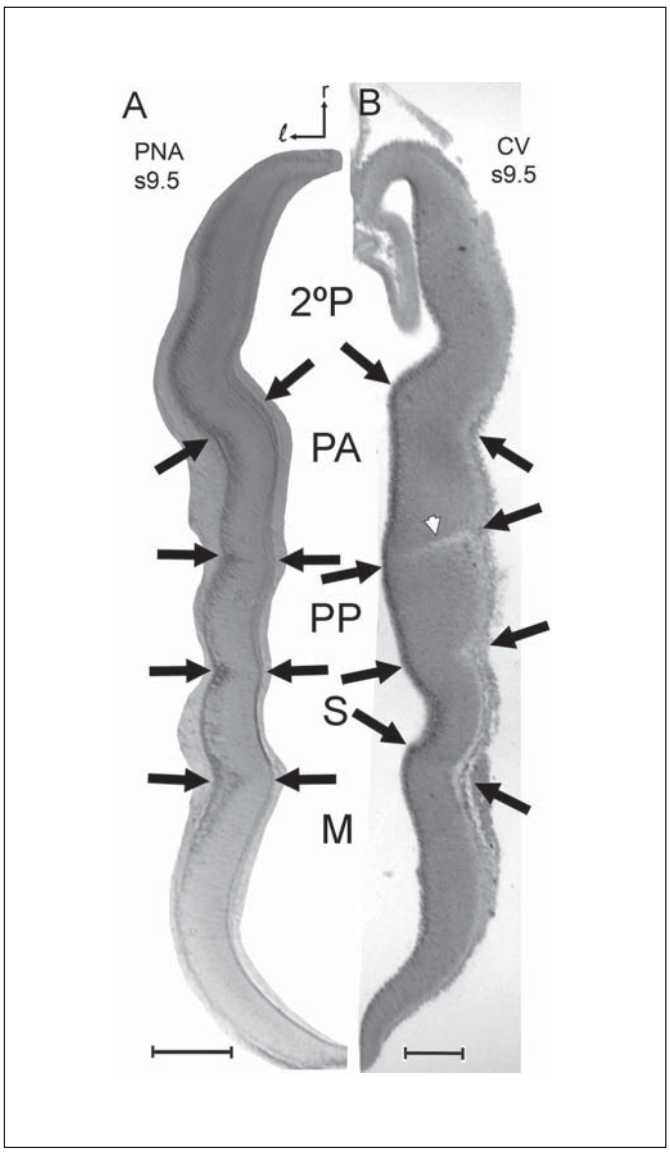


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Fig. 5. Diencephalic divisions at the three segment stage viewed horizontally. Embryos at stage 9.5 stained with peanut agglutinin (**A**) and cresyl violet (**B**) show subdivision of the parencephalon into anterior and posterior segments. The zona limitans interparencephalica is most clearly seen in the cresyl violet stained section (**B**, white arrow). The synencephalon remains unsegmented. Orientation of both sections is similar although **B** is reversed with respect to **A**. Scale bars = 200 μm.

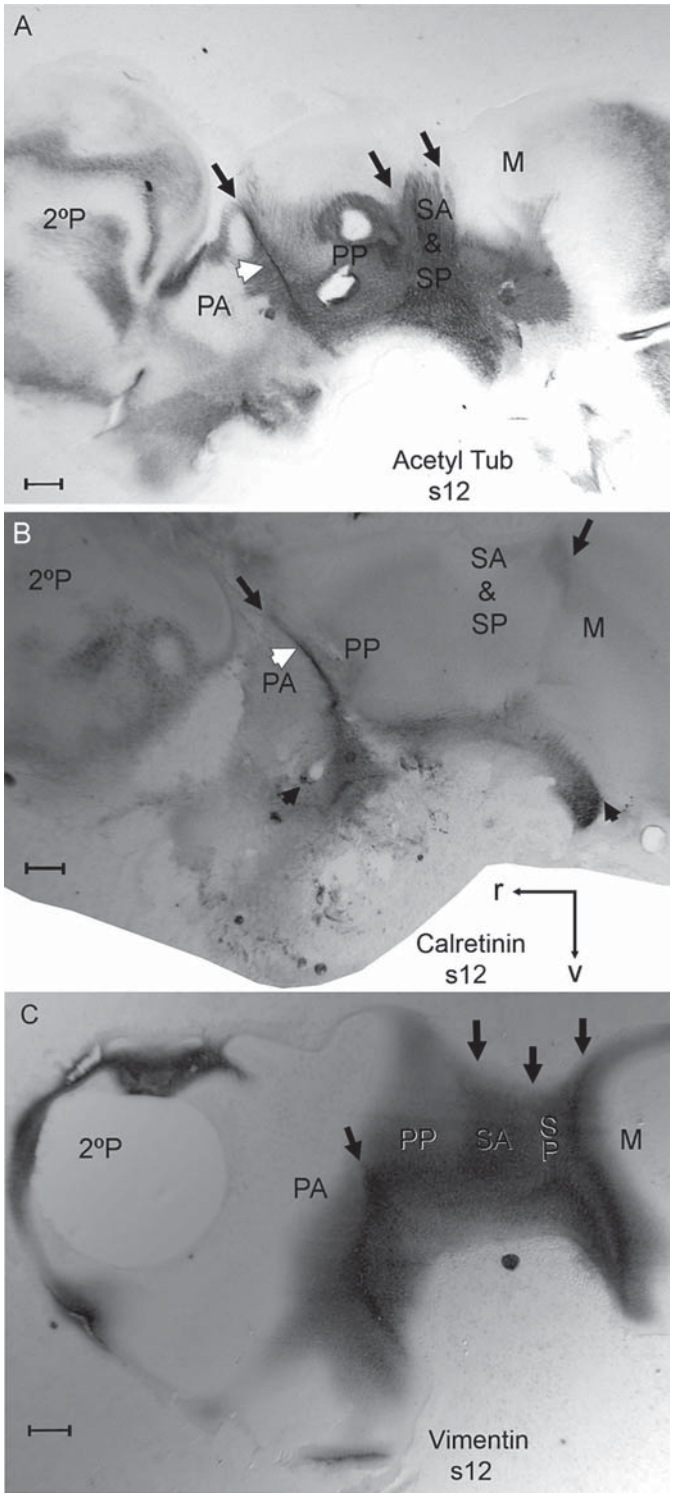

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Fig. 6. Diencephalic divisions at the four segment stage viewed sagittally. Embryos stained with an antibody to acetylated tubulin (**A**, stage 12); calretinin (**B**, stage 12), and vimentin (**C**, stage 12); are shown. While subdivision of the parencephalon into anterior and posterior segments is seen with all stains (long black arrows **A–C**), it is most clear in material stained with antibody to acetylated tubulin (**A**) and calretinin (**B**) where the zona limitans interparencephalica (white arrow in **A** and **B**) is distinct. Parcellation of the synencephalon is best visualized in tissue stained with an antibody to vimentin (**C**) where distinction of subdivisions is faint. Boundaries between the diencephalon and secondary prosencephalon and between the diencephalon and mesencephalon, respectively, vary depending on the stain used. A dorsoventral boundary is most clearly visualized in tissue processed using an antibody to calretinin (short black arrows in **B**). Orientation shown in **B** is similar for all sections (**A–C**). Scale bars = 100 μm.

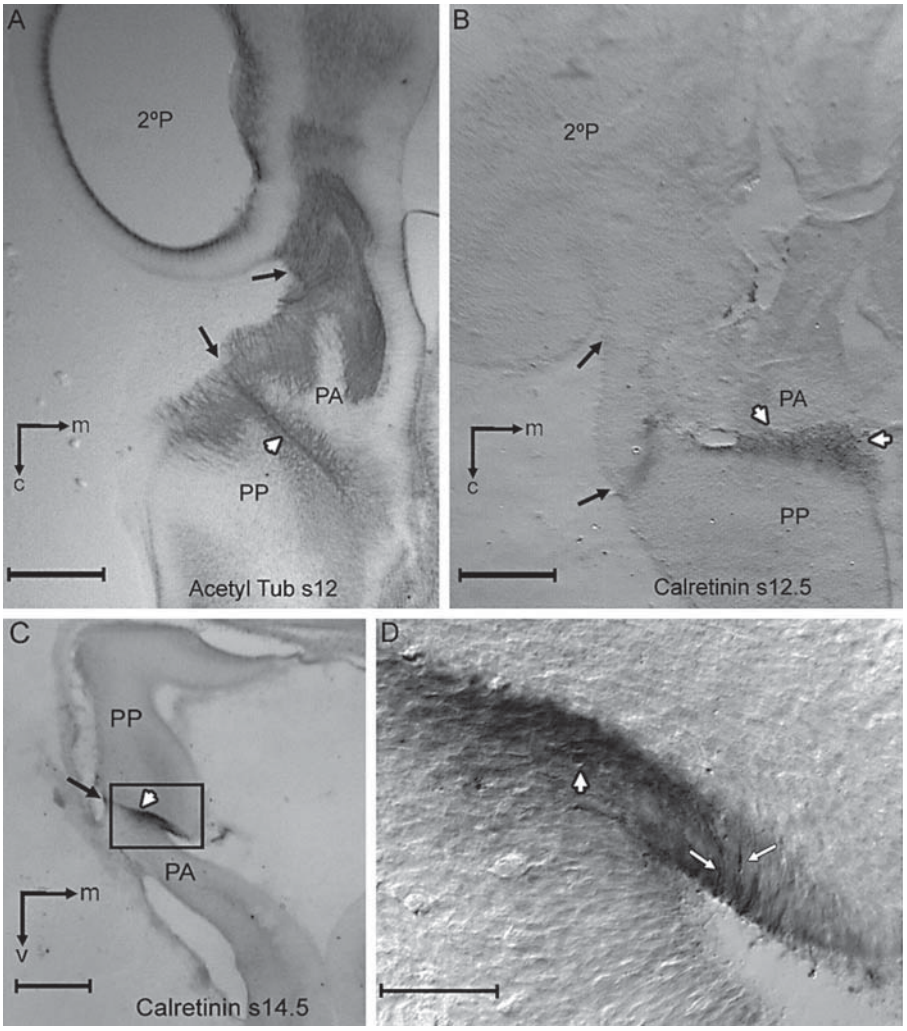

Fig. 7. Zona limitans interparencephalica. The appearance of the zona limitans interparencephalica separating the parencephalon anterior from the parencephalon posterior is shown in horizontal (**A** and **B**) and transverse (**C** and **D**) material stained with an antibody to acetylated tubulin (**A**) and calretinin (**B–D**). Transmission (**A–C**) and Nomarski differential interference (**D**) microscopy are illustrated. Higher magnification (**D**) suggests that calretinin immunoreactivity labels cells (short arrow) and fibers (long arrows). Bar scales = 250 μm in **A–C** and 50 μm in **D**.

borders were faintly observed in tissue stained with an antibody to vimentin (fig. 6C). A dorsoventral division in sagittally sectioned brains into presumed alar and basal plates was most clearly visualized by calretinin immunocytochemistry (fig. 6B) and less so in cresyl violet preparations.

All four diencephalic divisions were observed in horizontal sections. Although sharp borders marking diencephalic segments were not clearly seen, staining patterns that distinguished these compartments were apparent (fig. 8). Segments were most clear in peanut agglutinin stained sections. Using this histochemical stain, the thickness of the most lateral portion of the positively stained diencephalic mantle layer and constituent longitudinal bands distinguished not only diencephalic subdivisions but also the boundary between the anterior parencephalon and secondary prosencephalon rostrally and between the posterior synencephalon and mesencephalon caudally (fig. 8A). Segment demarcation was much less well visualized with an antibody to acetylated tubulin (fig. 8B) or cresyl violet staining (fig. 8C). With all of these three stains, the alar parencephalon posterior was morphologically distinct by its similar size and shape (fig. 8). On this basis alone, the alar parencephalon posterior could be distinguished from other alar territories. Differential staining properties at the ventricular and pial surfaces distinguished diencephalic segmental boundaries particularly those borders between the secondary prosencephalon and the parencephalon anterior and between the synencephalon posterior and the mesencephalon (fig. 8).

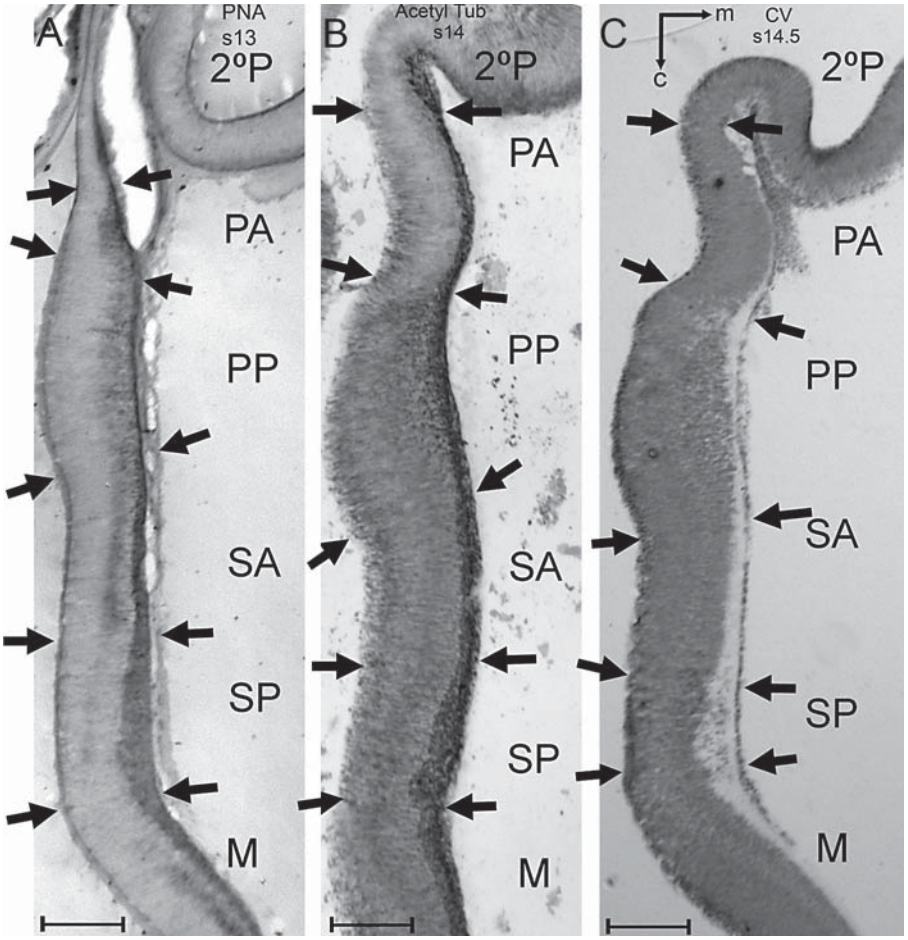

Fig. 8. Diencephalic subdivisions at the four segment stage viewed horizontally. Embryos stained with peanut agglutinin (**A**, stage 13); with an antibody to acetylated tubulin (**B**, stage 14); and with cresyl violet (**C**, stage 14.5) are shown. Borders between individual diencephalic segments as well as boundaries between the secondary prosencephalon and diencephalon and between the diencephalon and mesencephalon vary in clarity depending on the stain. Orientation (shown in **C**) is similar for all sections (**A–C**). Scale bars = 200 μm.

Compartment and Boundary Features

Features of diencephalic segments and their respective transversal borders changed over the course of early diencephalon development. Similarly, overt longitudinal differentiation between the presumptive alar and basal plate varied according to the stage of development. Although these details have been mentioned previously, they are summarized below and illustrated schematically (fig. 9).

At the two segment stage, immunoreactivity to calretinin (fig. 3C) and acetylcholinesterase (fig. 3D) was positive in the parencephalon and negative in the synencephalon. Similar to compartment markers, histochemical and immunocytochemical features of borders between segments varied over time. Immunoreactivity to calretinin distinguished borders between the diencephalon and mesencephalon regardless of whether two (fig. 3C), three (fig. 4B), or four (fig. 6B) segments were present. Both calretinin (fig. 4A, 6B, 7B–D) and acetylated tubulin positive (fig. 4C, 6A, 7A) immunoreactivity at the three and four segment stage marked the zona limitans interparencephalica and differentiated the parencephalon anterior from the parencephalon posterior.

No longitudinal demarcation between presumptive alar and basal plates was seen when the diencephalon was unsegmented (fig. 2). However, calretinin immunoreactivity distinguished a dorsoventral division at the two (fig. 3C), three (fig. 4A, B), and four (fig. 6B) segment stages. At the two segment stage, fibers visualized by acetylated tubulin immunoreactivity (fig. 3A) suggested a longitudinal division into a presumptive alar and basal plate.

Discussion

The present series of morphological observations examined early forebrain development in *Alligator*. These results focused on the diencephalon and its main com-

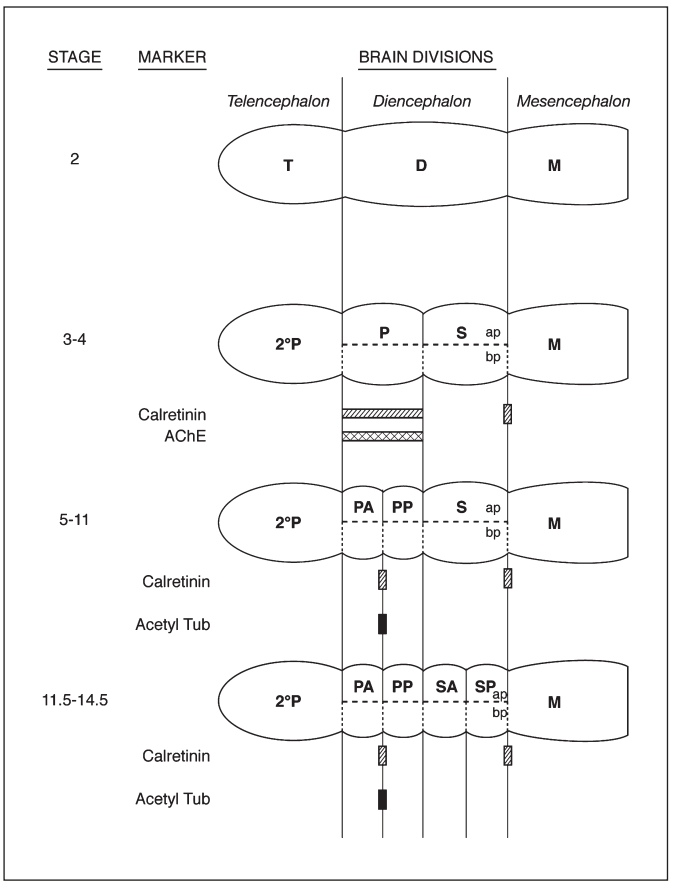

Fig. 9. Early diencephalon development in *Alligator*. Schematic sequence of selected features of diencephalon formation in sagittal images in which each representative stage has been drawn to the same length and proportions. Segmentation from a single compartment with subsequent subdivision into two, three, and four zones is illustrated as well as the morphological features that characterize each respective stage. Boundary (vertical bars) and compartment (horizontal bars) markers are shown based on data from sagittally sectioned material.

partments and primarily on alar (dorsal) areas. Controls for the immunocytochemical experiments coupled with the observations made on tissue processed for peanut agglutinin histochemistry and Nissl staining indicate that the identification of forebrain compartments and their respective boundaries are real rather than artifactual.

Although the antibodies used for the immunocytochemical experiments identified compartments and boundaries, exactly what they stained (neurons, glia, fibers) was not directly addressed. It was presumed that acetylated tubulin stained microtubules within fibers [Piperno et al., 1987] and vimentin labeled radial glia [Tapscott et al., 1981]; however, direct confirmation of this was not done. Similarly, whether antibodies to calretinin or acetylcholinesterase marked cells and /or glia was likewise not addressed. To do so would have required additional experiments, using for example, one marker for immunocytochemistry (e.g., antibody to calretinin) and a second for neurons or glia. Moreover, immunoreactivity varied over time making an answer to this question even more complicated. Data presented previously suggest that at later stages calretinin labels both cells and fibers (fig. 7D). Regardless, the goal of this study was to trace the development of the diencephalon from a single compartment into subdivisions until each segment became internally subdivided. The exact structure(s) that various antibodies labeled at different times during development should not detract from the present analysis. Furthermore, the observations made on material stained for peanut agglutinin and for cresyl violet provided independent support for the immunocytochemical findings.

The plane of section employed was critical not only for the observations made in this study but also for comparisons with similar experiments done in other vertebrates. Tissue sectioned transversally (perpendicular to either the sagittal or horizontal plane used in the present analysis) was useful only for documentation of the zona limitans interparencephalica (fig. 7C, D). The sagittal and horizontal planes of sections used in this analysis have been illustrated in a representative external view of an *Alligator* brain divided at the isthmus (fig. 1). Nevertheless, some distortion inevitably occurred related to freezing, tissue preservation, the amount of external tissue surrounding the brain, and differential brain growth during development resulting in some deviation from the planned plane of section. This could have influenced the interpretation of sectioned material. It was for this reason that the tissue was analyzed in both sagittal and horizontal planes to confirm observations and minimize misinterpretations. Even obliquely sectioned material proved useful in analyzing forebrain compartments and borders. In this way, the natural curvature of examined neural tissue would not give a false appearance to the observed diencephalic compartments.

In *Alligator*, clear and reliable subdivisions subsequent to formation of the most rostral forebrain area known as the secondary prosencephalon [Rendahl, 1924; Puelles et al., 1987] regardless of the plane of section (sagittal, horizontal, or transverse) were not seen. On the other hand, the developing diencephalon clearly transforms from an initial single compartment into various subdivisions relative to both the dorsoventral and rostrocaudal axes. The diencephalon first divides transversely into the paren-

Table 1. Diencephalic compartmentalization during early development in various vertebrates

Vertebrate group	Common name	Subdivisions	Author(s)	Year
Lampreys	river lamprey	3	Bergquist	1952
	sea lamprey	3	Bergquist and Källén	1953
	sea lamprey	3	Pombal and Puelles	1999
	sea lamprey	4*	Osorio et al.	2005
Shark/rays	shark	3	Bergquist	1952
	dogfish	3	Bergquist	1952
	marine ray	3	Bergquist	1952
	marine ray	3	Bergquist and Källén	1953
Amphibians	newt	3	Bergquist	1952
	frog	3	Bergquist	1952
Teleosts	zebrafish	3	Wullimann and Puelles	1999
	medaka	3	Kage et al.	2004
Reptiles	snake	3	Bergquist	1952
	sea turtle	3	Bergquist	1952
	sea turtle	3	Bergquist and Källén	1953
	lizard	3	Bergquist	1952
	alligator	3	Bergquist	1952
	alligator	4	Pritz	present report
Birds	pigeon	3	Bergquist	1952
	chick	3	Rendahl	1924
	chick	4	Vaage	1969
	chick	3	Puelles et al.	1987
	chick	4	Figdor and Stern	1993
	chick	4	Larsen et al.	2001
Mammals	hyrax	3	Bergquist	1952
	mole	3	Bergquist	1952
	mouse	3	Bergquist	1952
	mouse	3	Bulfone et al.	1993
	bat	3	Bergquist	1952
	hamster	3	Keyser	1972
	opossum	3	Bergquist	1952
	rhesus monkey	3	Gribnau and Geijsberts	1985
	humans	3	Bergquist	1952
	humans	3	Bergquist and Källén	1953
	humans	3	Müller and O'Rahilly	1997

* Although these authors proposed four diencephalic subdivisions, it is likely that the two most rostral ones would be combined into a single segment when compared with other authors' schema.

cephalon and the synencephalon. Subsequently, the parencephalon splits into an anterior and a posterior compartment with the appearance of the zona limitans interparencephalica. Still later in development, the synencephalon becomes divided into anterior and posterior subdivisions. Later in ontogeny, internal divisions are formed within specific compartments. In addition, when the diencephalon divides into two transverse zones and continuing throughout the period of development studied in this report, a longitudinal division along a dorsoventral axis identified a presumptive alar and basal plate. However, borders separating compartments within the alar plate were more readily recognized than those in the basal plate.

Numerous studies in a multitude of vertebrates have documented subdivisions in the diencephalon early in

Table 2. Onset of diencephalic segmentation in *Alligator*, chick, and humans

# Segments	Vertebrate Group				
	Alligator[1](s/d)	Chick[2] (s/h)			Humans[3] (s/d)
		Vaage[4] (38 °C)	Puelles et al. (37.8 °C)	Larsen et al. (37 °C)	
2	3/3	13/48–52	13/48–52	16/51–56	13/32
3	5/5	15/50–55	19/68–72	19/68–72	14/33
4	11.5/12.5	17/52–64	N/A	22/84	N/A

d = Days; h = hours; N/A = not applicable; s = stage; # = number.

[1] Based on staging scheme of Ferguson [1985] for eggs incubated at 30°C.

[2] Based on staging scheme of Hamburger and Hamilton [1951].

[3] Based on the staging scheme of Müller and O'Rahilly [1997].

[4] Data from Vaage [1969] were based on text description of living embryos, wax reconstructions, and fixed and partly sectioned material. These data are at variance with the summary figure (fig. 40).

Numbers in parentheses refer to incubation temperature. See text for further description.

ontogeny (see table 1). However, besides the present observations, investigations of early diencephalon development beginning at a single compartment stage are few. Most have used chick [Vaage, 1969; Puelles et al, l987; Larsen et al., 2001] whereas one study investigated humans [Müller and O'Rahilly, 1997]. Observations in these four studies were based on living embryos [Vaage, 1969]; fixed tissue whole mounts including wax-plate reconstructions [Vaage, 1969]; scanning electron microscope images [Larsen et al., 2001]; sectioned material using a variety of stains labeling cells [Vaage, 1969; Müller and O'Rahilly; 1997; Larsen et al., 2001]; including graphical reconstructions [Müller and O'Rahilly, 1997]; histochemical staining of acetylcholinesterase positive neuroblasts in whole mount and sectioned material [Puelles et al., 1987]; immunocytochemistry on whole mount and sectioned material [Larsen et al., 2001]; in situ hybridization [Puelles and Rubenstein, 1993, Larsen et al., 2001]; bromodeoxyuridine labeling [Larsen et al., 2001]; and dextran labeling [Larsen et al., 2001]. Although some common techniques were used (e.g., Nissl staining), the histochemical and immunocytochemical markers in the present experiments differed in part from those of others [Vaage, 1969; Puelles et al., 1987; Müller and O'Rahilly, 1997; Larsen et al., 2001]. Nevertheless, the present observations in *Alligator* are largely consistent with the observations in chick [Vaage, 1969; Puelles et al., 1987; Larsen et al., 2001] and humans [Müller and O'Rahilly, 1997] at least in documenting division of a single diencephalic proto-segment into the parencephalon and synencephalon and subsequent further separation of the parencephalon into an anterior and posterior component. The present study and others [Vaage, 1969; Larsen et al., 2001] described further parcellation of the synencephalon into an anterior and posterior portion whereas other studies [Puelles et al., 1987; Müller and O'Rahilly, 1997] have recognized only a single compartment. In humans [Müller and O'Rahilly, 1997], the diencephalon was divided into two parts: D1and D2. In the present analysis, only the D2 compartment [Müller and O'Rahilly, 1997] is comparable to the diencephalon of chick [Vaage, 1969; Puelles et al., 1987; Larsen et al., 2001] and *Alligator* [present study]. D1 in this schema [Müller and O'Rahilly, 1997] encompasses the optic chiasm and, in the definition of many [Nieuwenhuys, 1998a], would be considered a part of the secondary prosencephalon rather than part of the diencephalon proper [Puelles, 1995; Puelles and Rubenstein, 1993, 2003]. Although methodology for identification differs, the zona limitans interparencephalica separating the parencephalon anterior and posterior in chick [Rendahl, 1924; Puelles et al., 1987] lies in a similar topographical position and extent as observed in *Alligator* [present study]. In addition, although techniques differed, the approximate location of the separation between the presumptive alar and basal plate in chick diencephalon [Puelles et al., 1987] is similar in position to that seen in *Alligator* [present study].

A comparison between the onset of diencephalic segmentation and the vertebrate groups in which detailed descriptions of early development are available is present-

ed in table 2. Development in *Alligator* [Ferguson, 1985] as well as chick [Hamburger and Hamilton, 1951] varies depending on temperature. At 30°C, hatching occurs in *Alligator* after 62–67 days [Ferguson, 1985]. In chick, it occurs at 20–21 days [Hamburger and Hamilton, 1951]. At these early stages of diencephalon development, chick stages are approximated in hours based on the classical descriptions of Hamburger and Hamilton [1951]. However, according to their description, stages 14 to 35 eggs were kept at 39.4°C whereas eggs from the other stages were kept at 37.5°C [Hamburger and Hamilton, 1951]. Presumably, temperature and species of chick did not influence staging although these factors might have impacted the conversion to hours after egg laying. In *Alligator*, the time of egg deposition was occasionally imprecise. This did not influence staging but would have an effect on time after presumed egg deposition. At stage 8 and before, the day after egg laying and stage correspond; thereafter, stage and egg age vary. Based on data from Ferguson [1985], stage 11.5 occurs at day 12.5. My observations are in agreement with this. Bearing in mind these potential sources of variation, comparisons among *Alligator* [present study], chick [Vaage, 1969; Puelles et al., 1987; Larsen et al., 2001], and humans [Müller and O'Rahilly, 1997] provide a time frame for compartment and boundary formation in the diencephalon in relation to gestation (table 2). In mice, in which gestation is 19–21 days, a comparable sequence of diencephalon development is lacking. Unpublished observations state that 'diencephalic neuromeres appear at 9–11 days post coitum and are comparable to their chick counterparts' [Figdor and Stern, 1993, p. 633].

One difference between the present observations and those which described early diencephalon development in chick and humans is whether the synencephalon remains a single unit [Puelles et al., 1987; Müller and O'Rahilly, 1997] or divides into two [Vaage, 1969; Larsen et al., 2001]. The present observations in *Alligator* suggest that the synencephalon divides into an anterior and posterior segment. Furthermore, others have provided experimental data to support a division into anterior and posterior components [Figdor and Stern, 1993; Larsen et al., 2001]. Nevertheless, the vast majority of studies using a variety of techniques recognize only a single division (table 1). It is likely that methods other than the morphologic ones used in the present analysis will be required to settle this issue.

Although further diencephalic development in *Alligator* has yet to be investigated in detail, it is likely that these early alar subdivisions will follow a pattern similar to that described for other vertebrates. Viewed from this perspective, the parencephalon anterior is destined to become the ventral thalamus [Figdor and Stern, 1993; Nieuwenhuys, 1998a; Larsen et al., 2001] or prethalamus [Puelles and Rubenstein, 2003]; the parencephalon posterior is the presumptive dorsal thalamus [Rubenstein et al., 1994; Larsen et al., 2001] and epithalamus [Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Nieuwenhuys, 1998a] or thalamus [Puelles and Rubenstein, 2003]; and the synencephalon is the anlage for the pretectum [Nieuwenhuys, 1998a; Larsen et al., 2001; Puelles and Rubenstein, 2003]. When two divisions of the synencephalon are recognized, the anterior compartment will become the pretectum anterior whereas the posterior division is the presumptive pretectum posterior [Figdor and Stern, 1993].

The separation between the parencephalon anterior and the parencephalon posterior is occupied by the zona limitans interparencephalica. Although the zona limitans interparencephalica might correspond to part or all of the zona limitans intrathalamica, additional markers used by others [Figdor and Stern, 1993; Zeltser et al., 2001; Kiecker and Lumsden, 2004; Scholpp et al., 2006] will be required to confirm such a relationship. Nevertheless, several reports have suggested that this region itself is a separate compartment [Figdor and Stern, 1993; Larsen et al., 2001; Zeltser et al., 2001; Kiecker and Lumsden, 2004; Zeltser, 2005] although a general consensus has not been reached [Puelles and Rubenstein, 2003]. The purely morphological data obtained by the present observations cannot determine if this is indeed the case. In addition, specifics as to the status and morphology of the roof plate, epichordal strip, and floor plate [Vaage, 1969; Puelles et al., 1987; Puelles, 1995; Puelles and Rubenstein, 2003] were not examined in detail in this analysis.

The present series of morphological experiments traced diencephalon development in the transverse plane from a single segment into four compartments until individual zones undergo internal subdivisions. These components are most clearly seen in the presumptive alar plate and are less distinct in the presumptive basal plate region. A number of terms to describe these divisions have been employed previously and used interchangeably: compartments, zones, segments, subdivisions, regions, and areas. However, neuromeres and their forebrain counterparts, prosomeres, have been avoided because these terms have taken on different meanings and interpretation since their introduction into the English language literature by Orr in 1887. In his original description, Orr defined neuromeres by their morphological ap-

pearance stating that '…Each neuromere is separated from its neighbors by an external dorso-ventral constriction, and opposite this an internal sharp dorso-ventral ridge, – so that each neuromere (i.e., one lateral half of each) appears as a small arc of a circle. The constrictions are exactly opposite on each side of the brain…' [Orr, 1887, p. 335]. By this definition, the identified segments in the present report most clearly seen in horizontal sections (see fig. 5 and 8) are indeed neuromeres.

However, others researchers [Keynes and Lumsden, 1990; Lumsden, 1990] have suggested that several criteria need to be met if a neuromere is to be of developmental significance. The observations in the present analysis fulfill only one of these four features [Keynes and Lumsden, 1990; Lumsden, 1990]; namely, that compartments follow a segmental pattern. The other three criteria, which were not addressed in this paper, are the following. First, neuromeres are preceded or produced by a segmental pattern of cell proliferation. Second, borders between neuromeres should be barriers to cell mixing and, therefore, should create lineage restrictions. Third, putative regulatory genes should be expressed in patterns that relate to the neuromere(s) being investigated. These features have been studied most intensely in the developing chick hindbrain where rhombomeres satisfy these criteria [for review, see Lumsden, 2004; Kiecker and Lumsden, 2005]. Extending this approach to the forebrain in general, and to the diencephalon in particular, has resulted in somewhat different interpretations [Figdor and Stern, 1993; Larsen et al., 2001; Puelles and Rubenstein, 2003]. Although a recent review [Lim and Golden, 2007] has partly examined these differences, the following is a detailed summary of these three analyses of diencephalon development.

The first researchers to examine this problem using methodology to address the four criteria to determine whether neuromeres in the diencephalon were of developmental significance were Figdor and Stern [1993]. They used the following methods: scanning electron microscopy; histochemical and immunocytochemical stains with light microscopic analysis; fluorescent tracer injection into a single neuroepithelial cell and similar injections into small groups of cells within diencephalic neuromeres; and expression domains of several putative regulatory genes in chick. They concluded that the diencephalon was organized into four cellular domains that form in precise sequence with characteristic shapes in which boundaries between neuromeres represent lineage restrictions with certain putative regulatory genes corresponding to borders and individual diencephalic segments. Furthermore, specific fiber tracts formed boundaries between segments. In this scheme, the four diencephalic compartments were labeled D1 to D4, and corresponded to the parencephalon anterior (D1), parencephalon posterior (D2), synencephalon anterior (D3), and synencephalon posterior (D4). The hypothalamus was considered part of D1 in this analysis.

This problem was re-investigated in chick by Larsen et al. [2001] using many of the same techniques (morphology, gene expression, cell lineage restriction, acquisition of boundary specific phenotype) employed by Figdor and Stern [1993]. However, Larsen et al. [2001] obtained results different from those of Figdor and Stern [1993] and thus reached different conclusions regarding diencephalon compartment development [Larsen et al., 2001]. When the diencephalon was divided into two units, the parencephalon and synencephalon, only the synencephalon exhibited neuromeric characters at its interface with the midbrain. The synencephalic/parencephalic border did not restrict cell mixing nor did the parencephalon/secondary prosencephalon boundary. When the parencephalon became divided into anterior and posterior parts by the zona limitans intrathalamica, neither division exhibited neuromeric morphology nor did these compartments display lineage restriction at their boundaries. When the synencephalon became subdivided as determined by tissue morphology and differential gene expression, neither cell lineage restriction nor cell boundary phenotype were observed. In addition, S-phase nuclei (as determined by bromodeoxyuridine labeling), which are localized apically at rhombomere borders, were found at this location only in the zona limitans intrathalamica and at the synencephalon/mesencephalon border. They concluded that the diencephalon did not display a segmental pattern of development [Larsen et al., 2001]. On the other hand, recent fate mapping experiments in chick [Garcia-Lopez et al., 2004] found that both alar and basal parts of each of the diencephalic segments showed neuroepithelial cell restriction at inter-segmental boundaries. However, the interpretation of orthotopic mapping of cell fates is open to potential criticism from two sources. One limitation is the size of the grafts. The other is the potential change in cellular behavior that might occur as a result of injury to embryonic donor tissue and the subsequent integration of the graft [Kiecker and Lumsden, 2005].

The third interpretation, the prosomeric model, put forth by Puelles, Rubenstein and co-workers, was initially based on morphological observations in chick [Puelles et al., 1987] and subsequently extended to other vertebrates [Puelles and Rubenstein, 2003]. These observa-

tions were heavily influenced by gene expression mapping. As additional markers became available, re-evaluations of the territories and boundaries of prosomere 1 (synencephalon), prosomere 2 (parencephalon posterior), and prosomere 3 (parencephalon anterior) have occurred and these zones have been re-defined. This paradigm is subject to further modification as new data become available [Puelles and Rubenstein, 2003]. However, the prosomeric model was an approach to correlate morphological observations with gene expression data [Puelles and Rubenstein, 2003]. Other developmentally significant features of neuromeres have yet to be investigated by this group.

The present experiments, by their very nature, cannot determine which of these interpretations is most accurate. Nevertheless, the identification of the time course and pattern of formation of diencephalic subdivisions from a single compartment in *Alligator* into four transverse zones provides a roadmap for future investigations to examine other developmentally significant properties of these regions.

Divisions of the secondary prosencephalon in *Alligator* early in development were obscure whereas boundaries and compartments in the diencephalon were much more readily identifiable. The presence of secondary prosencephalic subdivisions in vertebrates has been a matter of dispute [for summary, see Nieuwenhuys, 1998a; Striedter, 2005]. Even those who have proposed segments within this part of the forebrain have subsequently modified their interpretation as additional data became available. Initially, based on the appearance of acetylcholinesterase positive neuroblasts, secondary prosencephalic subdivisions were not described [Puelles et al., 1987]. Later, investigations that examined certain gene expression patterns postulated transverse regions within the secondary prosencephalon [Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Puelles, 1995, 2001]. Most recently, molecularly distinct transverse regions of the secondary prosencephalon were best interpreted as secondary, non-neuromeric subdivisions because interneuromeric boundaries did not extend from the roof plate to the floor plate [Puelles and Rubenstein, 2003]. Accordingly, gene expression data rather than the purely morphological observations made here will be necessary if secondary prosencephalic transverse regions are to be identified in *Alligator*.

Regardless of whether or not the synencephalon subsequently becomes subdivided, the overall pattern of early diencephalon formation observed in mammals [humans: Müller and O'Rahilly, 1997] birds [chick: Vaage, 1969; Puelles et al., 1987; Larsen et al., 2001], and reptiles [*Alligator*: present study] is similar. These observations suggest that the initial building blocks of the diencephalon follow a common plan in all amniotes and perhaps in other vertebrates as well. It remains, however, to be determined how subsequent development produces different adult diencephalons in various species. Identification of key stage specific morphological changes that distinguish the *Alligator* diencephalon from that of closely and distantly related species should serve as a starting point to investigate what molecular events are responsible for these differences.

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