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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