Reelin Expression during Embryonic Brain Development in *Crocodylus niloticus*

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ABSTRACT

The expression of reelin mRNA and protein was studied during embryonic brain development in the Nile crocodile *Crocodylus niloticus*, using in situ hybridization and immunohistochemistry. In the forebrain, reelin was highly expressed in the olfactory bulb, septal nuclei, and subpial neurons in the marginal zone of the cerebral cortex, dorsal ventricular ridge, and basal forebrain. At early stages, reelin mRNA was also detected in subventricular zones. In the diencephalon, the ventral lateral geniculate nuclei and reticular nuclei were strongly positive, with moderate expression in the habenula and focal expression in the hypothalamus. High expression levels were noted in the retina, the tectum, and the external granule cell layer of the cerebellum. In the brainstem, there was a high level of signal in cochleovestibular, sensory trigeminal, and some reticular nuclei. No expression was observed in the cortical plate or Purkinje cells. Comparison with reelin expression during brain development in mammals, birds, turtles, and lizards reveals evolutionarily conserved, homologous features that presumably define the expression profile in stem amniotes. The crocodilian cortex contains subpial reelin-positive cells that are also p73 positive, suggesting that they are homologous to mammalian Cajal-Retzius cells, although they express the reelin gene less intensely. Furthermore, the crocodilian cortex does not contain the subcortical reelin-positive cells that are typical of lizards but expresses reelin in subventricular zones at early stages. These observations confirm that reelin is prominently expressed in many structures of the embryonic brain in all amniotes and further emphasize the unique amplification of reelin expression in mammalian Cajal-Retzius cells and its putative role in the evolution of the cerebral cortex. J. Comp. Neurol. 457:250–262, 2003. © 2003 Wiley-Liss, Inc.

Indexing terms: cerebral cortex; Dab1; VLDLR; ApoER2; in situ hybridization; reeler; brain evolution

Disruption of the *reelin* gene causes neuroanatomic abnormalities in reeler mice (D’Arcangelo et al., 1995; Lambert de Rouvroit and Goffinet, 1998). Reelin is a large glycoprotein that is secreted from a variety of neuronal cell types (D’Arcangelo et al., 1997; de Bergeyck et al., 1997). In mammals, reelin expression is highest in neurons of the developing marginal zone of the cerebral cortex and hippocampus, particularly in Cajal-Retzius cells, which are also characterized by specific expression of p73 (D’Arcangelo et al., 1995; Yang et al., 2000; Meyer et al., 2002b). In addition, reelin expression is robust in mitral cells of the olfactory bulb, external cerebellar granule cells, and retina but undetectable in some neuronal populations, such as neurons of the prenatal cortical plate and Purkinje cells (D’Arcangelo et al., 1995; Ogawa et al., 1995; Ikeda and Terashima, 1997; Schiffmann et al., 1997; Meyer et al., 1998, 2000). It is thought that reelin acts via the extracellular matrix on target neurons, instructing
them to assume their orientation. Target neurons bind reelin via two largely redundant receptors of the lipoprotein receptor family, VLDLR and ApoER2 (Hiesberger et al., 1999; Trommsdorff et al., 1999), and the signal is relayed via tyrosine phosphorylation of the adapter Disabled-1 (Dab1; Howell et al., 1997, 1999, 2000; Ware et al., 1997; for review see Rice and Curran, 2001).

Previous comparative studies of cortical development in reptiles suggested that the gene mutated in reeler mice played a role during cortical evolution, particularly in the synapsid lineage leading from stem amniotes to mammals (Goffinet, 1983; Goffinet et al., 1986). To assess this further, we studied reelin-expressing cells in the developing brains of representatives of nonmammalian lineages, namely, turtles (Bernier et al., 1999), lizards (Goffinet et al., 1999), and birds (Bernier et al., 2000). Here, we complete this survey with the analysis of reelin expression in the embryonic brain of a crocodilian, Crocodylus niloticus. In that species, the pattern of reelin expression is predictably reminiscent of that in birds, confirming the evolutionary relationships of birds and crocodiles within the archosaurian lineage. In the telencephalon, reelin-positive cells are prominent in the marginal zone, where they express p73, a marker of mammalian Cajal-Retzius cells, as well as in a subventricular zone at early stages, where they are p73 negative, a feature that has not been described in other species thus far.

MATERIALS AND METHODS

Animals

Three embryonic stages of C. niloticus corresponding to preplate, early, and intermediate maturation of the cortical plate were selected for study. They correspond to stages 19 (preplate), 21 (early cortical plate), and 23 (intermediate maturation of cortical plate) described by Ferguson (1985). After being cooled on ice, embryos were decapitated, and the heads were fixed by injection of 4% paraformaldehyde (in phosphate-buffered saline) around the brain. After overnight fixation at 4°C and rinsing in phosphate buffer, heads were immersed in sucrose (25% in phosphate buffer) at 4°C until they sank. The samples were embedded in OCT compound, frozen in dry-ice powder, and cut at 10 μm with a cryostat in a coronal orientation. Sections were collected on Superfrost Plus slides, air dried, and stored at −70°C prior to in situ hybridization. Bouin fixation followed by paraffin embedding was used for immunohistochemistry and routine histological staining. Animal care protocols were approved by the animal ethics committees of the University of Namur and University of Louvain.

Cloning of crocodilian partial reelin and p73 sequences

A 864-nucleotide segment of the crocodile reelin cDNA, corresponding to nucleotides 10,045–10,908 of the mouse reelin sequence (GI:6755311), was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA extracted from a crocodilian embryonic brain, using primers 5'-CAAGGTGACGACTGCTC-3' and 5'-CAATACTGCGATGTTA-3'. A 780-nucleotide segment of the crocodile p73 cDNA, corresponding to nucleotides 834–1,615 of the mouse p73 sequence (GI:730914), was amplified by RT-PCR, using primers 5'-CACCA TCTGTGACAATCTCATG-3' and 5'-CTGATGCCAGTT TGGACAC-3'. The amplicons were cloned in pCR2 using a TA cloning kit (Invitrogen, La Jolla, CA) and sequenced for verification.

In situ hybridization and immunohistochemistry

The reelin and p73 sequences were used for riboprobe preparation by in vitro transcription with T7 RNA polymerase (Promega, Madison, WI) in the presence of 125 μCi [α33P]UTP (Amersham, Arlington Heights, IL). The purification was performed by Quick Spin Columns (Roche, Basel, Switzerland). The hybridization protocol was carried out as described elsewhere (Simmons et al., 1989; Bernier et al., 1999). The sections were visualized on Biomax MR film, and films were directly photographed with a stereomicroscope for appreciation of the relative intensity of the signal at low magnification (see Figs. 1, 4, 6). Selected sections were dipped in emulsion (Amersham LM1), developed, and counterstained with toluidine blue for darkfield photomicrography (see Figs. 2, 3, 5, 7). Sense probes consistently yielded blank slides after comparable exposure times (not shown). Immunohistochemistry was carried out using monoclonal antireelin antibody 142 (Meyer et al., 2000).

RESULTS

Staging of crocodile embryos was performed according to Ferguson (1985). Because few data on brain development in crocodilians are available, neuroanatomical identification relied largely on recent reptilian brain atlases and on our previous analyses (Dubbeldam, 1997; ten Donkelaar, 1997; Bernier et al., 1999, 2000; Goffinet et al., 1999; Pritz, 1999). Developing structures that could not be identified are referred to as differentiating fields. Reelin mRNA expression was studied at three stages correspond-
The olfactory bulb and to a lesser extent the subventricular zone and connecting streams (Fig. 3). Interestingly, immediately subpial cells in the MZ with a morphology similar to that of reelin-positive cells were also labeled with the p73 probe. The p73 probe did not label any other neural cells, such as reelin-positive cells in the subventricular zone and connecting streams (Fig. 3). Almost all subpial cells were positive for reelin and p73. The olfactory bulb and to a lesser extent the superficial part of the basal forebrain contained neurons with high levels of reelin mRNA. Septal nuclei were heavily positive, except for the central component, which was weakly labeled (Fig. 1B). The striatal primordium was weakly labeled, with the exception of some reelin-positive cells that were dispersed in its subregional region. The retina contained a population of heavily labeled neurons in the ganglion cell layer, and the labeling of this zone decreased from the center to the periphery. A second population of more weakly labeled cells laid deeper in the tissue, at the level of the photoreceptor layer or the retinal germinall layer. In the diencephalon (Figs. 1D, 2E,F), several reelin-positive cell populations were found in differentiating fields. A focus of high reelin expression was present external to the basal hypothalamic VZ and closely associated with the optic chiasm. Several hypothalamic, medial ventral, and medial dorsal thalamic differentiating fields as well as the reticular thalamic nuclei and the anlage of the ventral lateral geniculate nucleus were strongly reelin positive, whereas lateral regions in ventral and dorsal thalamic regions were weakly labeled or negative, and the epithalamus was moderately labeled. Dispersed, moderately reelin-positive cells were present at the mesodiencephalic junction (Figs. 1E, 2G,H). By contrast, a much more intense signal was associated with the tectum (Figs. 1E,F, 2I,J). Although tectal VZs were negative, a strong reelin mRNA expression was present in the subventricular zone, surrounded by a negative layer and by a superficial layer of even more intensely labeled cells. The strong labeling in the subventricular zone extended into the torus semicircularis, but the center of that nucleus was weakly labeled. In the future cerebellum (Figs. 1F, 2K,L), two contingents of neurons were labeled, corresponding to the incipient external granular layer and to the more rostrally located cerebellar nuclei; differentiating fields including Purkinje cells and the VZ (except the lateral rhombic lip) were negative. A robust signal was found in the region of future isthmic nuclei (Figs. 1F, 2K,L). In the hindbrain, three main zones were reelin positive. A lateral zone corresponding of the sensory trigeminal nucleus, whereas intermediate and more medial positive zones were considered part of the reticular formation (Figs. 1E,F, 2M,N). In addition, a labeled ventromedial component may correspond to the future pontine nuclei. Other structures, such as cranial nerve nuclei, appeared weakly labeled or negative. The VZ of the rhombic lip was strongly reelin-positive, both in the cerebellum and in the rhombencephalon. Cranial nerve ganglia were negative or weakly reelin positive.

### Preplate stage

At the preplate stage, equivalent to preplate formation in the mammalian telencephalon, there was already widespread reelin mRNA expression in several areas (Fig. 1). In the cortical anlage (Figs. 1B, 2A–D), a large number of reelin positive cells formed a stream in subventricular zones around reelin-negative ventricular zones (VZ). The outer tiers of the intermediate zone and the region of the future cortical plate were negative. In the marginal zone (MZ), reelin-positive horizontal neurons were disposed close to the pial surface. In the dorsal ventricular ridge (DVR), a similar reelin-positive subventricular zone was found; in addition, labeled neurons with features reminiscent of cortical MZ cells were scattered throughout the primordium. At some places, particularly in the most medial field and at the border between DVR and striatum, streams of reelin-positive cells seemed to extend from the positive subventricular layer zone to the cortical MZ. Interestingly, immediately subpial cells in the MZ with a morphology similar to that of reelin-positive cells were also labeled with the p73 probe. The p73 probe did not label any other neural cells, such as reelin-positive cells in the subventricular zone and connecting streams (Fig. 3). Almost all subpial cells were positive for reelin and p73. The olfactory bulb and to a lesser extent the superficial part of the basal forebrain contained neurons with high levels of reelin mRNA. Septal nuclei were heavily positive, except for the central component, which was weakly labeled (Fig. 1B). The striatal primordium was weakly labeled, with the exception of some reelin-positive cells that were dispersed in its subregional region. The retina contained a population of heavily labeled neurons in the ganglion cell layer, and the labeling of this zone decreased from the center to the periphery. A second population of more weakly labeled cells laid deeper in the tissue, at the level of the photoreceptor layer or the retinal germinall layer. In the diencephalon (Figs. 1D, 2E,F), several reelin-positive cell populations were found in differentiating fields. A focus of high reelin expression was present external to the basal hypothalamic VZ and closely associated with the optic chiasm. Several hypothalamic, medial ventral, and medial dorsal thalamic differentiating fields as well as the reticular thalamic nuclei and the anlage of the ventral lateral geniculate nucleus were strongly reelin positive, whereas lateral regions in ventral and dorsal thalamic regions were weakly labeled or negative, and the epithalamus was moderately labeled. Dispersed, moderately reelin-positive cells were present at the mesodiencephalic junction (Figs. 1E, 2G,H). By contrast, a much more intense signal was associated with the tectum (Figs. 1E,F, 2I,J). Although tectal VZs were negative, a strong reelin mRNA expression was present in the subventricular zone, surrounded by a negative layer and by a superficial layer of even more intensely labeled cells. The strong labeling in the subventricular zone extended into the torus semicircularis, but the center of that nucleus was weakly labeled. In the future cerebellum (Figs. 1F, 2K,L), two contingents of neurons were labeled, corresponding to the incipient external granular layer and to the more rostrally located cerebellar nuclei; differentiating fields including Purkinje cells and the VZ (except the lateral rhombic lip) were negative. A robust signal was found in the region of future isthmic nuclei (Figs. 1F, 2K,L). In the hindbrain, three main zones were reelin positive. A lateral zone corresponding of the sensory trigeminal nucleus, whereas intermediate and more medial positive zones were considered part of the reticular formation (Figs. 1E,F, 2M,N). In addition, a labeled ventromedial component may correspond to the future pontine nuclei. Other structures, such as cranial nerve nuclei, appeared weakly labeled or negative. The VZ of the rhombic lip was strongly reelin-positive, both in the cerebellum and in the rhombencephalon. Cranial nerve ganglia were negative or weakly reelin positive.

### Early cortical plate stage

At the early cortical plate stage, there was no labeling in the VZs, except for a few foci in the diencephalon (Fig. 4). There was high reelin mRNA expression in several differentiating fields. At the rostral and basal pole of the telencephalic vesicle, the most strongly labeled structure was the olfactory bulb (Fig. 4B). Labeling was mostly associated with mitral cells, although a few cells located deep to the mitral cell layer were also positive. Strongly labeled, sparse cells were distributed at the subpial level, in the cortical anlage from the medial to the lateral part of the telencephalic vesicle (Fig. 5A,B), and in basal forebrain areas and around the DVR. All around the lateral ventricles, a moderate signal was associated with a subventricular zone immediately adjacent to the reelin-negative VZ; this zone was less

### Table 1. Relative Intensity of Reelin Expression in the Crocodilian Brain at the Three Stages Selected, Corresponding to Ferguson Stages 19, 21, and 23

<table>
<thead>
<tr>
<th>Brain structure</th>
<th>Stage 19</th>
<th>Stage 21</th>
<th>Stage 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory bulb</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Hippocampus (medial cortex)</td>
<td>MZ++</td>
<td>MZ++</td>
<td>MZ++</td>
</tr>
<tr>
<td>Dorsal cortex</td>
<td>MZ++</td>
<td>MZ++</td>
<td>MZ++</td>
</tr>
<tr>
<td>Lateral cortex</td>
<td>MZ++</td>
<td>MZ++</td>
<td>MZ++</td>
</tr>
<tr>
<td>DVR</td>
<td>Rostral</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Caudal</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basal forebrain</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Septal nuclei</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Striatum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Retina</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypothalamus (foci)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dorsal thalamic nuclei (r. retinans and triangularis)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reticular thalamus</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Ventral lateral geniculate nuclei</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ventral thalamic nuclei</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Epithalamus, habenula</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Meso diencephalic junction</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tectum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Torus semi circularis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Isthmic nuclei</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reticular formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trigeminal nuclei</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhombic lip</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>GC+++</td>
<td>ND</td>
<td>GC+++</td>
</tr>
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1MZ, isolated neurons in marginal zone; Sub-VZ, a zone surrounding the ventricular zone (VZ); DVR, dorsal ventricular ridge; GC, granule cells; ND, not done.
prominently labeled than at the previous stage (Figs. 4B,C, 5A,B). Apart from the sparse large and heavily positive cells mentioned above that were dispersed at the periphery, the DVR contained few positive cells. Strong labeling was associated with septal nuclei, particularly the peripheral component that surrounded a more weakly labeled core (Fig. 4C). The striatum was weakly labeled. The retina contained two layers of reelin-positive cells associated, externally, with the somata and inner processes of photoreceptor cells and, internally, with the ganglion cell layer (Fig. 5E,F). Only a subset of ganglion cells was positive, and there was a central-to-lateral gradient of labeling density that paralleled retinal maturation. In the diencephalic region (Figs. 4D,E, 5C,D), a few spots in the VZ were positive in the hypothalamic region, in areas facing the habenula and at the junction between dorsal and ventral thalamic primordia. The habenular complex expressed high levels of reelin mRNA, particularly in its medial component. Several strongly reelin-positive zones were located around the negative dorsal thalamic nuclei, such as the nucleus rotundus and triangularis, and particularly at the junction between dorsal and ventral thalamic fields. Ventral thalamic nuclei were moderately labeled. The ventral lateral geniculate nuclei and reticular nuclei were strongly labeled. Some rostral and intermediate hypothalamic differentiating fields expressed high levels of reelin, and reelin-positive cells were scattered at the mesodiencephalic border. In the midbrain (Fig. 4F), the VZs were negative, but labeling was associated with cells located externally to the VZs and in differentiating fields, particularly in the tectum and torus semicircularis. In the tectum (Fig. 5G,H), in addition to the positive subventricular zone, a zone of strong expression was present in the external tiers. Positive cells scattered in differentiating fields of the midbrain and hindbrain tegmentum could not be related to any anatomically defined nuclei and were tentatively considered to belong to the reticular formation (Fig. 4F). In the hindbrain, moderate labeling appeared to be associated to the trigeminal complex, as at the previous stage, whereas cranial nerve nuclei were negative, and a moderate or low labeling was associated with cranial ganglia.

**Intermediate architectonic development stage**

In the forebrain, the olfactory bulb was heavily positive (Fig. 6B), with the highest density of grains associated with mitral cells and moderate labeling of smaller neurons...
located more deeply than mitral cells. Immunohistochemistry confirmed heavy reelin expression in mitral and in more deeply located cells, perhaps a subset of granule cells or displaced mitral cells (see Fig. 8C,D). From the posterior and lateral aspect of the olfactory bulb, a population of large neurons was scattered in basal and basolateral forebrain areas (Fig. 6C), including the presumptive amygdala. These large positive cells were reminiscent of those found at the periphery of the DVR and in cortical MZs. The epithelial lining of the ventricles was negative, and the subventricular zone, well labeled at previous stages, was almost negative. As at previous stages, a strong signal was associated with scattered neurons in the MZs in medial, dorsal, and lateral cortical fields (Figs. 6C, 7A,B); grain density covering these cells was, however, clearly inferior to that found in olfactory mitral cells and even in the retina. Immunohistochemistry confirmed the presence of reelin in scattered subpial horizontal neurons (Fig. 8A,B). In the DVR, a dorsal tier was mostly negative, but moderate and diffuse expression was detected in the lower

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**Fig. 3.** Preplate stage (Ferguson stage 19). Comparison of reelin and p73 expression. **A-C:** Bright- and darkfield views of a coronal section at the level of the telencephalon and rostral diencephalon. Reelin-positive neurons are found in the cortex (CX) and basal forebrain (BF) as well as in the diencephalon, particularly the hypothalamus (Hyp), and the retina (Ret). **C,D:** Bright- and darkfield views of a coronal section in the cerebral cortex, showing reelin expression in subventricular zones (sVZ) and in horizontal neurons in the marginal zone (MZ). **E,F:** Bright- and darkfield views of a coronal section in the diencephalon. Reelin-positive neurons are present in the hypothalamus (Hyp), reticular thalamic nuclei (RTh), and epithalamus (Hb), whereas the DTh, VTh, and Rot were negative. **G,H:** Bright- and darkfield views of a section in the rostral hindbrain, with reelin expression in several differentiating fields at the mesodiencephalic function (MDJ), in the reticular formation (RF), and in the trigeminal complex (nVs). **I,J:** Bright- and darkfield views of a section in the tectum, with heavy, layered reelin expression in tectum (Te) and torus semicircularis (TSC). **K,L:** Bright- and darkfield views of a section at the level of the cerebellum, showing reelin expression in the incipient granule cell layer (GC) and in differentiating isthmic nuclei (IN). **M,N:** Bright- and darkfield views of a section in the rhombencephalon. There is strong reelin mRNA expression in the reticular formation (RF) and in the rhombic lip (RL). Scale bars − 1 mm in A–C, 10 μm in D,E.
tiers, in a pattern evocative of that described for chick, including areas that might correspond to the chicken area L (Bernier et al., 2000). Strong expression remained associated with septal nuclei, more with peripheral neurons of the lateral component than with the medial, weakly labeled core (Fig. 6C). The striatum was weakly labeled. The retina contained three reelin-positive layers. The ganglion cell layer was heavily labeled, and almost all cells were positive. The photoreceptor layer remained moderately labeled, and a moderate signal was associated with an intermediate layer of interneurons. In the diencephalon (not shown), the habenular nuclei, particularly the medial component, were moderately labeled. In the dorsal thalamic complex, specific nuclei, such as the nucleus rotundus, the dorsal lateral geniculate nucleus, and the nucleus medialis or area triangularis, were reelin negative. In contrast, there was robust labeling in a series of nuclei that surrounded the nucleus rotundus, including dorsolateral and dorsomedial nuclei. In the ventral thalamic complex, heavy labeling was associated with the ventral lateral geniculate nucleus, whereas other components, such as nucleus ventrolateralis and ventromedialis, were weakly labeled or were not labeled. In the rostral region, ventral and paraventricular regions of the hypothalamus, presumably including preoptic areas, were strongly and diffusely labeled, whereas lateral and dorsal hypothalamic components contained fewer reelin-positive cells. In the midbrain (Fig. 6D,E), the tectum was the most strongly labeled structure (together with cerebellar granule cell layers). Tectal reelin-positive cells were distributed in three layers (Fig. 7C,D). An inner layer of expression was found between the negative VZ and another external reelin-negative zone. A rather diffuse layer of moderately positive neurons extended at an intermediate level, and a strongly labeled layer was found at the superficial level. A robust signal was also seen in the subventricular component of the torus semicircularis bordering the negative VZ; this positive neuronal stream was continuous with the inner positive zone in the tectum. By contrast, the center of the torus semicircularis was weakly labeled. At the border of tectum, midbrain tegmentum, and posterior diencephalon, several strongly labeled neurons could not be readily identified. Positive, large neurons were scattered in tegmental fields of the mid- and hindbrain; they were considered to be part of the reticular formation because of their large size and lack of other obvious anatomical identity (Fig. 6E). In the cerebellum, the external granular layer, particularly its inner component of differentiating granule cells, was strongly reelin positive, whereas Purkinje cells were negative (Figs. 6F, 7D).
7E,F). In the brainstem (Figs. 6F, 7G,H), various parts of the reticular formation were strongly labeled. Cranial nerve nuclei were reelin negative, and cranial ganglia moderately positive.

**DISCUSSION**

The present work completes our previous studies (Schiffmann et al., 1997; Bernier et al., 1999, 2000; Goffinet et al., 1999; Meyer et al., 2000) of reelin expression in the embryonic amniote brain, with the exception of *Sphenodon punctatus*, unfortunately not available for investigation. Compared with that in mammals (Ogawa et al., 1995; Ikeda and Terashima, 1997; Schiffmann et al., 1997; Alcantara et al., 1998; Meyer and Goffinet, 1998), the developmental pattern of reelin expression in the brain of reptiles and birds reveals lineage-specific features, particularly in the telencephalon, as well as many similarities. The present discussion will consider these differences and similarities in relation to brain evolution and in terms of the putative mechanisms of action of reelin.

Our results show that some features of reelin expression appear specific to the crocodilian telencephalon. Crocodilian embryos have a unique, prominent stream of reelin-positive cells in subventricular zones at early stages, which abates with further development, indicating that these cells either leave that region, disappear, or downregulate reelin expression. Although this layer is separated from external reelin and p73-positive cells by a reelin-negative zone, there are places where both populations come in contact, suggesting that some cells may migrate from the subventricular zone to the MZ. reelin...
expression in subventricular zones is ideally placed to influence radial precursor cells in the VZ, including neurons that migrate rostrally to the olfactory bulb, and it is interesting in this respect that cells in the VZ express the Dab1 adapter and reelin receptors (Meyer et al., 2002a) and presumably have the capacity to respond to reelin. However, although a subventricular reelin-positive region is also present in chick, it is much weaker than in the crocodile and was not detected in turtle or lizard. In rodents, reelin expression in the subventricular zone is very weak, almost undetectable, but the high expression in the MZ coupled with processing (Lambert de Rouvroit et al., 1999) could provide enough active protein in the VZ. Furthermore, in all species, reelin in the MZ could act locally on the radial expansions of VZ cells.

Although the overall areal organization of the embryonic telencephalon of crocodiles and birds is quite similar, cortical fields are larger in crocodiles than in chick, and the number of reelin-positive cells in cortical MZs is proportionately larger. In the crocodile, the inner border of the embryonic cortical plate is not sharply defined, a feature vaguely reminiscent of that in turtles. However, there are few reelin-positive cells within the crocodilian cortical plate, whereas they are relatively abundant in turtles (Bernier et al., 1999). Reelin expression in the crocodilian cortex is confined to the MZ and differs from that in squamates, especially lizards, in which reelin-positive cells are found not only in the MZ but also below the cortical plate, with a typical bilaminar pattern (Goffinet et al., 1999). Finally, reelin expression in the embryonic crocodilian cortex differs from that in mammals in two respects. First, as discussed above, no subventricular reelin expression has been described thus far in mammals, and, second, p73-positive MZ neurons express much higher levels of reelin in mammals than in crocodiles. These variations in reelin expression parallel qualitative differences in the laminar organization of the cortical plate (Goffinet, 1983; Goffinet et al., 1986). As with its avian homolog, the crocodilian telencephalon is characterized by the contrast between, on one hand, a relatively large size and high neuronal density, two reelin-independent properties, and, on the other hand, a poor architectonic organization, which is a reelin-dependent feature. This would indicate that reelin has probably contributed little to the architectonic laminar evolution of the cortex in the archosaurian lineage (crocodiles and birds), in contrast to its role in lepidosaurian (lizards) and synapsid (mammalian) lineages (Bar et al., 2000).

In contrast to the lineage-specific aspects considered above, reelin expression reveals a conserved pattern in all
amniotes, including 1) strong expression in olfactory mitral cells and horizontal neurons in cortical MZs; in some thalamic nuclei, such as ventral lateral geniculate and reticular thalamic nuclei (or zona incerta in mammals); and the external granule cell layer of the cerebellum; 2) moderate expression in septal nuclei, basal forebrain, a few hypothalamic nuclei, tectum, and reticular formation of the mid- and hindbrain; and 3) absent or low expression in striatum; Purkinje cells; and, in mammals and birds, inferior olive. This conserved canvas was most probably present in the common ancestor of turtles, lepidosaurians (lizards), archosaurians (birds and crocodiles), and mammals, the so-called stem amniote, and defines a homology (Northcutt, 1981; Butler and Hodos, 1996). This homology suggests that reelin fulfills a common developmental function in amniotes, and recent observations in fish may extend this putative function to nonamniotes (Costagli et al., 2002). Thus far, mutations of reelin have been identified only in man and rodents. In this case, the phenotype is dominated by the cerebellar and cortical malformation. This led to the hypothesis that reelin is involved chiefly in the control of lamination, but the widespread expression found in the embryonic brain in all amniotes suggests that it may serve a more widespread function and control architectonic development in the whole brain. Although the formal assessment of the action of reelin in nonmammalian vertebrates awaits inactivation of the gene or protein, some reasonable ideas can be proposed based on comparative expression data. Subtle anomalies of the tectum are present in reeler mice. Insofar as many reptiles (especially crocodiles and lizards) and birds are characterized by highly elaborate tectal architectonics, reelin deficiency in these species would result in a drastic tectal malformation with severe phenotypic and behavioral consequences. Simi-

Fig. 7. Intermediate cortical development stage (Ferguson stage 23). Selected examples of reelin mRNA expression. A,B: Bright- and darkfield views of a rostral section through the telencephalic wall. Reelin expression is confined mostly to large, scattered neurons in the marginal zone (MZ), whereas other layers are reelin negative; V, lateral ventricle. Scale bar = 100 μm. C,D: Bright- and darkfield views of a section through the tectum. Reelin expression is found in three cellular layers: an external zone corresponding to the future stratum opticum (III), an intermediate zone (II), and an inner zone of subventricular neurons (I); the ventricular zone (VZ) is negative. Scale bar = 100 μm. E,F: Bright- and darkfield views of a coronal section in the cerebellum, with heavy reelin expression in granule cells (GC). PIA, pial surface. Other cell types are reelin negative. Scale bar. G,H: Bright- and darkfield views of a coronal section in the brainstem. Large reelin-positive neurons are scattered in the reticular formation (RF). A cranial nucleus (VIII) is weakly labeled. Scale bars = 100 μm in A–F, 1 mm in G,H.
larly, the olfactory bulb is slightly abnormal in reeler mice, and observations on cell migration to the bulb suggest a novel function of reelin (Hack et al., 2002). This malformation does not result in patent behavioral deficit but could prove more deleterious in species, such as crocodiles or turtles, in which the main and accessory olfactory bulbs are more highly developed than in mammals. Finally, the apparent contrast between the widespread expression of reelin and the moderate phenotype of reeler mice may simply reflect differences between artificial conditions in the laboratory and the ecological situation under which evolutionary pressures operate. For example, presym pathetic neurons migrate abnormally in reeler mice (Yip et al., 2000), but this does not drastically affect autonomic functions, in that mutant animals survive and even breed as homozygotes. However, it is reasonable to assume that such anomalies would most probably prove detrimental in the wild and that the pattern of reelin expression does indeed reflect some evolutionary advantage that remains to be defined further.

Our data point to another interesting homology between subpial Reelin-positive neurons in crocodiles, other reptiles, and birds and mammalian Cajal-Retzius cells (CR cells), a topic of obvious importance for the evolutionary biology of the cerebral cortex. In mammals, turtles, lizards, and birds, subpial Reelin-positive cells are among the first born cortical neurons, and our observation of Reelin-positive neurons in the MZ at an early stage of cortical development in Crocodylus niloticus shows that these cells are generated early in all amniote lineages. Furthermore, our observations that almost all subpial neurons are labeled with Reelin and p73 probes indicate that the p73 gene is coexpressed with Reelin in these cells, as in mammalian CR cells (Yang et al., 2000; Meyer et al., 2002b), whereas p73 is not expressed by other neuronal populations, particularly not by the reelin-positive subventricular cells. Although the coexpression of reelin and p73 remains to be studied further in other species, our data suggest strongly that early-born, reelin- and p73-positive neurons in the reptilian (and avian) MZ are truly homologous to mammalian CR cells but that a unique, drastic amplification of reelin expression occurred in the latter at some point during synapsid evolution.

Although the comparison of expression patterns can hardly elucidate the mode of action of reelin, it might provide some hints. Observations in reeler mice show that both reelin-dependent and reelin-independent patterning events occur during development and that the action of reelin extends to almost all brain regions, including the spinal cord and retina (Rice and Curran, 2000; Yip et al., 2000), and is not restricted to laminated structures as was previously thought (Lambert de Rouvroit and Goffinet, 1998). In the mouse and the lizard, and probably other species as well, structures with reelin-dependent layering (e.g., cortical plate, Purkinje cell plate, mouse inferior olive) are characterized by clear-cut segregation of the expression of reelin and the components of the reelin receptor complex (Dab1, VLDLR, ApoER2) in different, adjacent cells. On the other hand, in structures that are less affected by reelin deficiency, such as retina, olfactory bulb, and spinal cord, expressions of reelin and Dab1 are not as clearly segregated, giving the impression that reelin deficiency has more deleterious consequences where sources and targets of reelin are adjacent but separate. In all amniotes studied, reelin-producing cells are often surrounded by abundant extracellular matrix, as though the presence of reelin would prevent target neurons from invading reelin-rich areas. These observations have led to the proposal that reelin may act as a stop signal for migrating target neurons (Pearlman et al., 1998). In that reelin does not seem to influence axonal growth cones directly (Jossin and Goffinet, 2001), the avoidance of reelin-rich zones is probably not due to an interference with leading edge extension but could be explained if reelin regulates negatively nucleokinesis, the progression of the nucleus into the leading process (Morris et al., 1998; Lambert de Rouvroit and Goffinet, 2001). Thus far, no data have been provided to support or disprove this idea.

Fig. 8. Comparison of ISH and immunohistochemistry. A,B: In the cortical MZ at stage 23, large reelin-positive neurons (arrowheads) are revealed with the antireelin antibody 142 (A) and by ISH (B). C,D: In the olfactory bulb (stage 23), reelin immunoreactivity and ISH signal are present in mitral cells (arrows) and in neurons located below the mitral cell layer (arrowheads). Scale bars = 10 μm.


