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Radial Glia Defines Boundaries and Subdivisions in the Embryonic Midbrain of the Lizard *Gallotia galloti*

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ABSTRACT

We have studied the organization of the midbrain radial glia in embryos of *Gallotia galloti* using the fluorescent lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and the antibodies H5 and RC2. Our goal was to verify if the radial glia takes part in the midbrain boundaries formation and if it defines different zones. Our exam reveals two clear limits, anterior or mesencephalic–diencephalic (m/d) and posterior or mesencephalic–rhombencephalic (m/r), that can be defined as the borders where the midbrain radial glia processes end. Moreover, fasciculate radial glia processes characterize these limits totally or partially. They coincide with gene expression limits and with cytoarchitectonic limits defined by other criteria. Six different subdivisions, five alar and one basal, can be defined according to radial glia distribution, fasciculation, and immunohistochemical features. The ventral part of the alar region is defined by an RC2-positive bundle of radial glial cells. This bundle supposes a trustworthy landmark to point out the tectal/tegmental boundary. We hypothesize that this pattern of midbrain radial glia represents a basic model in amniota. J. Comp. Neurol. 473:162–176, 2004.

Indexing terms: DiI; H5; RC2; development; brain regionalization; mesencephalon; reptile

The reptilian midbrain can be represented schematically as a sphere leaning on an inverted wedge. The sphere corresponds to the tectum and the wedge to the tegmentum. In the tectum, a dorsal half or dorsal tectum (TO) and a ventral half or ventral tectum can be distinguished. The latter is subdivided from front to back in three regions: griseum tectalis (GT), intermediate area (IA), and torus semicircularis (TS). Diaz et al. (2000) has made a detailed description of these areas in the adult lizard *Gallotia galloti*. On the basis of cytoarchitectonic, immunohistochemical, and hodological criteria, similar subdivisions have been recognized in other vertebrates, although some disparity in terminology and identity of certain areas can be detected (Kappers et al., 1967; Repérant, 1975; Gamlin and Cohen, 1988; Caballero-Bleda et al, 1992; Medina et al., 1993, 1994; Puelles et al., 1994; Puelles et al., 1996; Nieuwenhuys, 1998) However, embryological studies describing their formation are very scarce, especially in reptiles. Classic contributions of Palmgren (1921), Bergquist (1953), Bergquist and Kallén (1954), and Senn (1970, 1979) established useful bases for understanding their structure. Palmgren (1921) distinguished four longitudinal columns (dorsal, medial, lateral, and ventral) and several smaller parcellations. But unfortunately he included very few reptilian specimens. Bergquist (1953) divided the subtectum into dorsal and ventral areas and two transversal segments. Senn (1970, 1979) conceived the midbrain structure subdivided in three main layers: periventricular, central, and superficial. Current researchers have developed new studies using some of these interpretational systems (Hergueta et al., 1993; Medina et al., 1994; Diaz et al., 2000). On the other hand, a few immunohistochemical studies on radial glia distribution

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in the midbrain of reptiles are available (Monzon-Mayor et al., 1990; Kálmán et al., 1997).

In addition, the embryological approach of Palmgren (1921) permitted him to establish rostral and caudal limits of the mesencephalon. The first one, or mesencephalic–diencephalic limit (m/d), was identified behind the posterior commissure and parallel to its fibers, whereas the caudal one, or mesencephalic–rhombencephalic limit (m/r) was defined as a clear tissular "hiatus" that spans, dorsoventrally, between both areas. These limits have been corroborated further by other authors in embryos and adults of different vertebrates (Rendahl, 1924; Vaage, 1969; Keyser, 1972; Puelles et al., 1987; Mastick and Easter, 1996; Diaz et al., 2000; Garcia-Calero et al., 2002).

Recently, the understanding of the cellular and molecular mechanisms that regulate mesencephalic development have greatly advanced. The number of studies analyzing gene expression patterns in normal and genetically or chirurgically disturbed brains has increased steadily. It is now known that, in the early neural tube, the presumptive midbrain territory occupies the anterior two thirds of the so-called mesencephalic vesicle (Martinez and Alvarado-Mallart, 1989). Its rostral border is marked by the early expression of the En gene (Araki and Nakamura, 1999) and also by the caudal expression of the Pax6 gene (Puschel et al., 1992; Mastick et al., 1997). Its caudal limit corresponds to that of the Otx-positive region (Millet et al., 1996), that is, the mesencephalon is formed in the tube region where the En and Otx expression overlap (Martinez and Alvarado Mallart, 1990; Araki and Nakamura, 1999). Subsequent to mesencephalic determination by both genes (and also by Pax2/5), the alar plate will be specified by Pax3 and Pax7 genes (Matsunaga et al., 2001), whereas tegmentum specification depends of Shh (sonic hedgehog protein; Nomura and Fujisawa, 2000; Argawala et al. 2001; Nakamura, 2001). Both processes explain the dorsoventral subdivision of the mesencephalon. The anterior–posterior regionalization has also been related to the expression of some genes as En, Ephrin A5, or Eprhin A2, and to the tectal connectivity gradient (Matsuno et al., 1991; Marin and Puelles, 1994; Liu and Joyner, 2001; Nakamura, 2001). It is generally accepted that early limits defined by gene expression are definitely transformed during neural tube development coinciding with those defined by cytoarchitectonic criteria, but cells or cellular structures involved in their formation are unknown.

Radial glia cells (RG) have been defined as a subtype of astrocytic glial cells having their somata in the ventricular zone (VZ) and with a large lateral process reaching the external surface of the brain wall. This surface is formed by the endfeet of these cells and is known as the external limiting membrane (elm). They express specific molecular markers as vimentin, RC2, and glial fibrillary acidic protein (GFAP) that can be shared by other subtypes of astrocytics cells. Besides studies on the genes role in brain regionalization, data involving radial glial cells in this process are becoming numerous: First, as they are mediators in cellular migration, they take part in the spatial distribution of migratory cells (Rakic, 1972; Hatten, 1999). Second, because it has been reported that they contribute to the formation of certain cerebral boundaries (Stoykova et al., 1997; Götz et al., 1998; Redies et al., 2000; Yoshida and Colman, 2000). And third, because they could be precursor cells whose capacity to generate neurons and glia change from zone to zone, according to their molecular features (Fishell and Kriegstein, 2003; Malatesta et al., 2003). The aim of this article is to show whether these cells participate in the subdivision and formation of boundaries in *Gallotia galloti* lizard midbrain. To this end, we used 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) lipophilic dye and H5, RC2, and GFAP antibodies as radial glia cells markers. We have studied the distribution, orientation, and framework of these cells through embryonic development. We hope that data derived from this study, give a useful model to understand the radial glia role in regionalization of more complex brains.

Abbreviations

C	cerebellum
D	diencephalon
elm	external limiting membrane
fr	fasciculus retroflexus
GT	griseum tectalis
IA	intermediate area
LR	laterorostral mesencephalic nucleus
M	midbrain
m/d	mesencephalic/diencephalic limit
m/r	mesencephalic–rhomboencephalic limit
nIII	oculomotor nerve
pc	posterior commissure
PT	pretectum
R	rhombencephalon
RFA	alar reticular formation
RFB	basal reticular formation
Tg	tegmentum
TO	dorsal optic tectum
TS	torus semicircularis
V	ventricle
VZ	ventricular zone

MATERIALS AND METHODS

Animals

Lizard embryos of *Gallotia galloti* were used in this study. The developmental stages were determined according to the normal development of *Lacerta vivipara* table of Dufaure and Hubert (1961). They were anesthetized on ice; their heads were cut and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 48 hours.

DiI labeling

A volume of 3–5 μl of the fluorescent dye DiI (Molecular Probes, Eugene, OR) dissolved at 2.5 mg/ml in dimethylformamide, was injected into the ventricle by using a glass micropipette. This device was inserted in the middle of the mesencephalic lobules. Brains were incubated at 37°C for 3 to 5 days, then sectioned on a Vibratome (Leica VT1000M) at 60–100μm, mounted with glycerin/phosphate buffered saline (1:1), and examined with a rhodamine fluorescent filter in a Leitz microscopy.

Immunohistochemistry

The brains were cryoprotected in 20% sucrose solution and frozen. Coronal, sagittal, and horizontal sections (thickness 20–30 μm) were obtained in a cryo state and mounted on glass slides. Sections were treated with 0.5% H_2O_2 (10 minutes) and blocked in 0.1% bovine serum

albumin (BSA) solution. These sections were incubated overnight at room temperature with the monoclonal antibodies against radial glia H-5 (1:20), RC-2 (1:10; both of Hybridoma bank, IA), or GFAP (1:50; Sigma, St. Louis, MO). Primary antibodies were visualized by using biotinylated antimouse antibodies (1:100 donkey anti-mouse IgG for H-5 and GFAP, Jackson Immunoresearch, Baltimore, MD; goat anti-mouse IgM for RC-2, Sigma) and streptavidin–peroxidase complex (Jackson Immunoresearch). Peroxidase reaction was developed using diaminobenzidine. Some parallel sections were stained with 0.5% cresyl violet.

Histological analysis

Lizard embryos were fixed with Bouin's fixative solution at room temperature for 18 to 24 hours. Heads were cut and subsequently dehydrated and embedded in paraplast. Serial sections (7–10 μm) were prepared and stained with 0.5% cresyl violet (Sigma) and occasionally some other Nissl stainings.

RESULTS

General description

Cytoarchitectonic landmarks. An examination of the picture shown in Figure 1 reveals the main subdivisions of *Gallotia galloti* embryonic midbrain. Under the TO, we can see the GT, IA, and TS in rostrocaudal order. The first and the latter can be characterized by their thickness and particular layer organization. The IA lies between both and has a periventricular strata with a thick plexiform layer and a dense cellular layer apparently not stratified (Fig. 9D). This layer partially coincides with the nucleus laminaris of the torus semicircularis described in ten Donkelaar (1998). In the scheme of Figure 1B, we draw the ventral border of the mentioned areas passing under the cellular layer parallel to the ventricle in Figure 1A. The alar/basal limit is unclear in sagittal Nissl sections, but in the transversal section of Figure 9D, it could be drawn like a line passing between RFA and RFB (respectively, alar and basal reticular formations, in according with Diaz et al., 2000). The space where RFA and laterorostral mesencephalic nucleus (LR) are located, corresponds to blank band over Tg in the scheme (Fig. 1B). Diaz et al. (2000) named this space lateral band. Following conventional landmarks, we outlined the midbrain rostral limit behind posterior commissure, ending in the cephalic curvature in front of nIII (oculomotor nerve). The caudal limit follows a "clear band" partially occupied by blood vessels (Fig. 1A), from a superficial point between TS and cerebellum (C), to the cephalic curvature behind nIII.

Radial glia organization. *Gallotia galloti* sagittal brain sections stained with DiI show that a belt of long radial processes defines the tectum ventral part. In the frontal area (GT) most processes are oriented rostrally, while in the back part (TS), the dominant orientation is caudal (Fig. 2A,B). Both zones are thicker than the intermediate part and protrude toward the ventricle, especially the caudal one. A small region lies between them, i.e., the IA, in which radial processes are perpendicular to the section plane (Fig. 2B). Above these areas is the TO. More densely joined processes define the boundaries of some areas with neighboring regions. Thus, a palisade of fasciculate radial glia is interposed between GT and pc (Figs. 2B, 3B, 8A–D) and between TS and cerebellum (Figs. 2B, 4C). Between tectum and tegmentum, images like Figure 2B suggest a band of glial fibers coming from the ventricular to external surface (cut by the section plane, and located under IA initials in the picture). If this idea is correct, corresponding transversal sections must show a band of radial processes extending from medial to lateral wall surfaces. Effectively, such a band can be seen in transversal sections of Figure 9A–C,E, it is an unfasciculated band of radial glia processes. In the basal region, the Tg rostrally limits with the synencephalon or pretectum and caudally with the rhombencephalon. A fasciculated palisade of radial glia defines the caudal limit of Tg (Figs. 4C,E,F, 10B,C). However, the rostral one does not have a similar structure (Fig. 10B). Despite this finding, differential features of tegmental and pretectal radial scaffolding can be appreciated.

The main features of radial glia pattern shown here are clearly seen from embryonic day (E) 33 on, but some characteristics can be traced from E31 onward. Ventricular radial cells in this stage express RC2, a monoclonal antibody considered specific of radial glia lineage. So, we assume that the radial pattern reported here is a radial glia pattern. Now, we will give more relevant details of this pattern in each domain.

Limits

In sagittal sections of brains in which DiI injection have only involved the mesencephalic ventricle, radial glia processes do not go beyond a certain anterior transversal limit. In parasagittal sections, this limit draws a straight line forming an obtuse angle on passing from alar to basal region (Fig. 3C). Most alar processes are orthogonal to the limit, while the basal one is parallel to it. This rostral frontier can be seen even when the DiI dyes the diencephalon, and in transversal and horizontal sections as well (Fig. 3B). The set of images obtained indicates that the limit passes just behind pc and that some glial processes spread by the limit, forming a fascicle (Fig. 2B). Radial glia of pc and GT limiting zones immunostained with H5 and RC2 antibodies have a different framework from early stages (Fig. 6B–D,F). Around hatching stages, only medial zones are positive for H5, GFAP, and RC2 (Fig. 7B,C). A glial bundle RC2- and H5-positive is interposed between pc and GT, silhouetting pc from ependyma to elm (Fig. 8A). This bundle is present from E31 (Fig. 8C) but only in the alar zone. In those stages both areas are H5- and GFAP-positive, but RC2 expression is almost negative in pc and positive in GT. In the basal zone, we have not been able to observe a similar bundle at any stage.

In superficial sagittal sections stained with Nissl or DiI techniques, a clear discontinuity can be observed between midbrain and hindbrain (Fig. 4A,B). In similar sections immunostained for H5, the caudal border appears marked by an arch-shaped line, strongly immunopositive, that passes caudally to torus and cephalic curvature (Fig. 4C). This line is formed by fasciculation of adjacent processes, as examination at upper magnification reveals. Processes of the TS show up perpendicularly or obliquely to the line, while those of the tegmentum are parallel to it. In deeper cuts, fasciculated processes of these limits run dorsoventrally from VZ to elm (Fig. 4F) and cytoarchitectonic images show a clear discontinuity between tegmentum and rhombencephalon, marked by differences in neuropile, radial processes density, and VZ thickness (Fig. 4D,E).

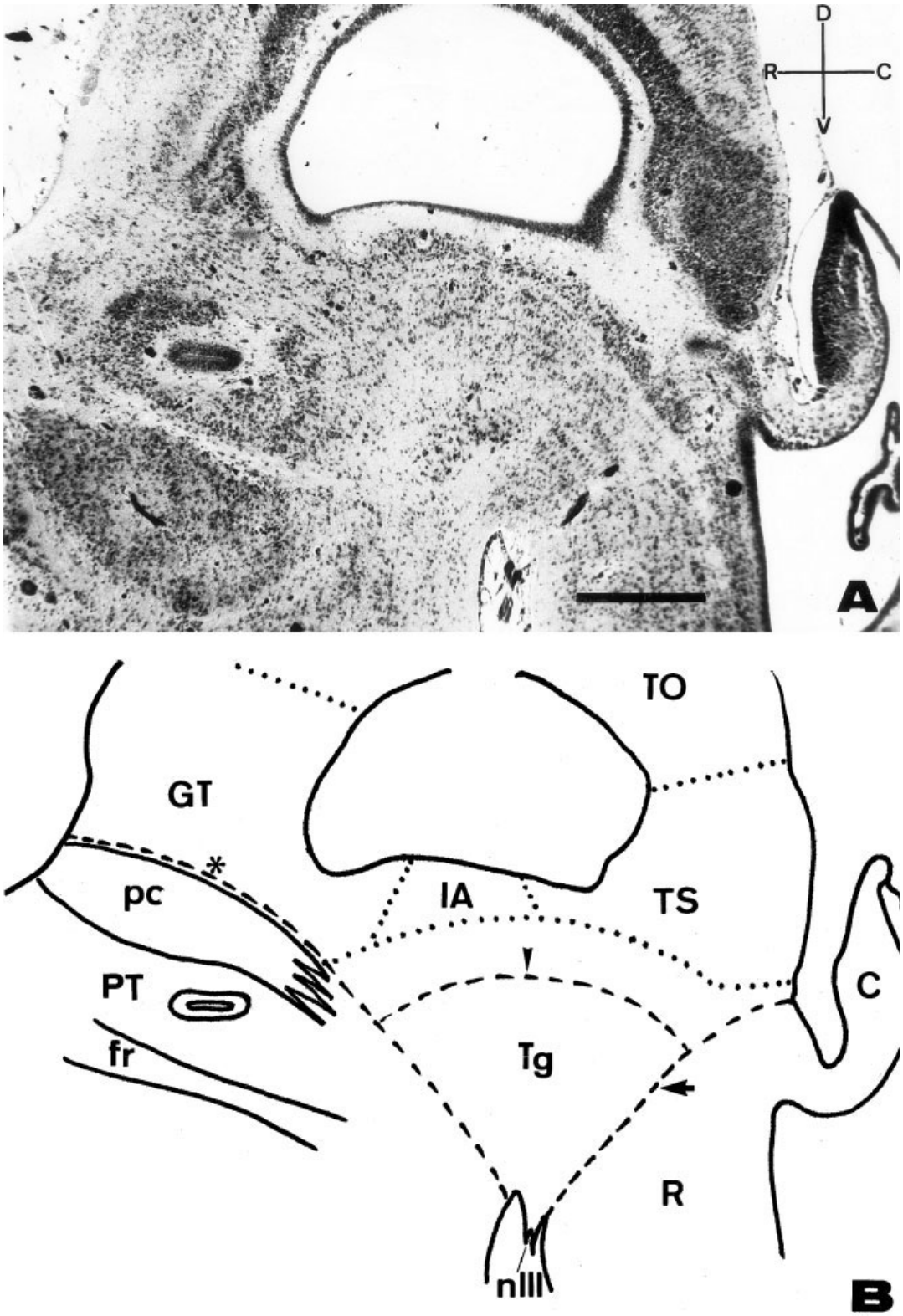

Fig. 1. Cytoarchitectonic organization and main subdivisions in the embryonic midbrain. **A:** Sagittal section at embryonic day 37 stained with cresyl violet. Axes in this and following figures indicate the orientation of the pictures in relation to the embryonic axis: D, dorsal; V, ventral; R, rostral; C, caudal. **B:** Scheme of the midbrain subdivisions. Dashed lines draw the rostral (asterisk), caudal (arrow), and alar/basal (arrowhead) limits of the midbrain. Spotted lines delimit internal midbrain subdivisions. For abbreviations, see list. Scale bar = 250 μm in A (applies to A,B).

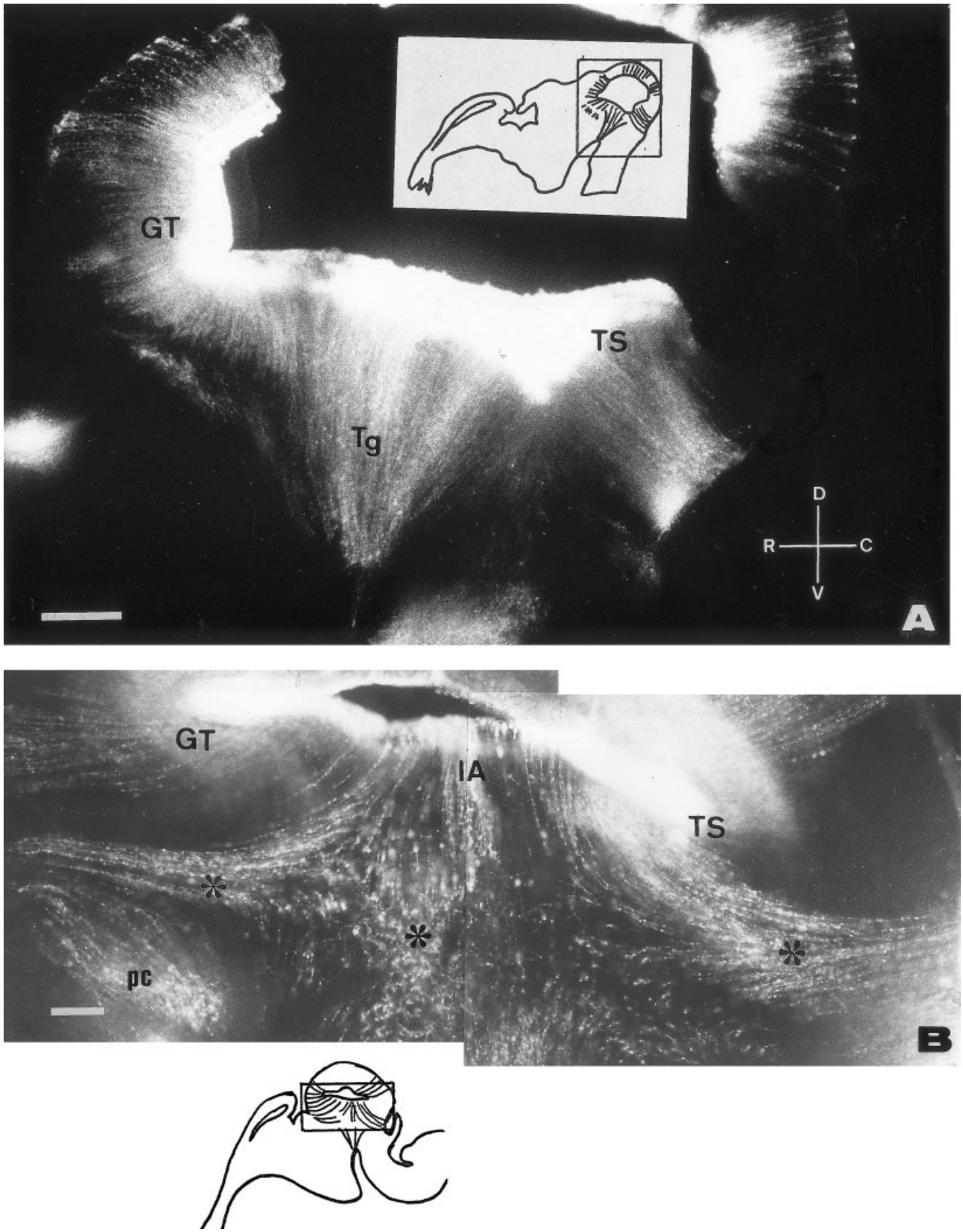

Fig. 2. General layout of the lizard embryonic midbrain injected with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI). **A,B:** Sagittal sections at embryonic day (E) 35 (A) and E37 (B). The orientation and distribution of the radial ventricular processes enable us to recognize different midbrain subdivisions. Insets in A and B show a sagittal view of brain in the respective stages; the rectangles box shows the picture region. Asterisks indicate radial glia processes in the ventral alar region. For abbreviations, see list. Scale bars = 200 μm in A; 100 μm in B.

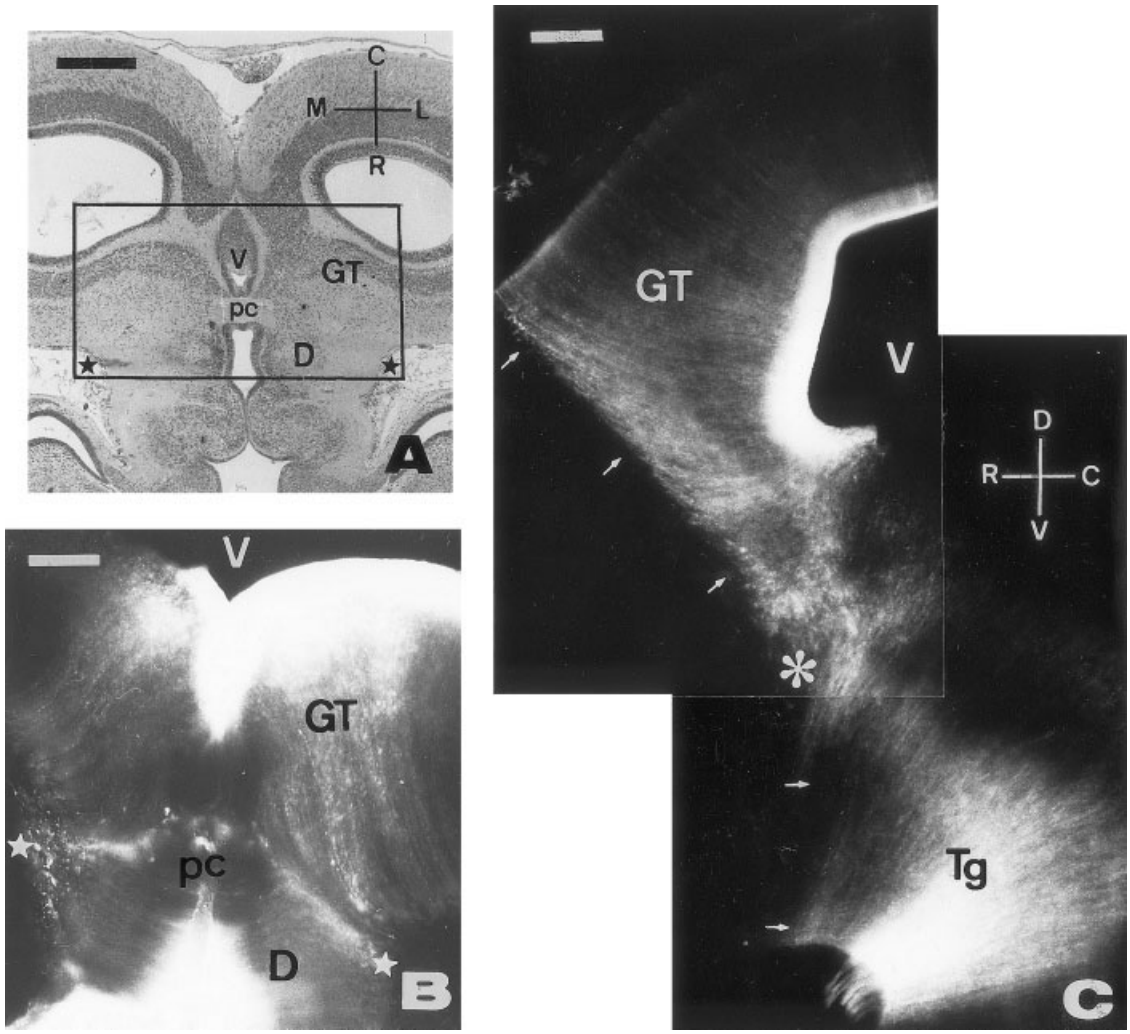

Fig. 3. The rostral midbrain limit is defined by the border where the midbrain radial ventricular processes end. **A,B:** Similar horizontal sections at embryonic day 35 stained with cresyl violet (A) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) (B). Rectangle in A delimits the zone shown in B. Stars mark the external point of the rostral limit (between pc and GT). **C:** Sagittal section stained with DiI; small arrow points out a rectilinear rostral boundary (m/d limit). The asterisk marks the approximate situation of alar/basal limit. For abbreviations, see list. Scale bars = 250 μm in A; 100 μm in B,C.

Transversal sections show that this limit coincides with the superficial sulcus between midbrain and rhombencephalon and a corresponding ventricular ridge (not shown).

Subdivisions

In the dorsal tectum, the radial glia distribution is homogeneous. Their processes are straight, parallel among them and orthogonal to the dorsal tectal surface (Fig. 5). They have an initially smooth surface, which later bristles with spiny projections (Fig. 5C). The radial processes meet in small and regularly spaced fascicles with two or three units each at least. The fasciculation degree varies along time and space (Fig. 5B,C).

In the ventral tectum anterior region, the GT, the majority of glial processes have a general rostral orientation (Figs. 2B, 3B). It is clear in sagittal and horizontal sections after E33, when GT turns laterally with respect to its original position (Fig. 6A,C,E). The framework of radial processes in GT is different from those of adjacent pc and TO areas. At the GT/pc interface limiting radial processes tend to group densely (Fig. 6D,F). Around hatching, the radial glia interposed between GT and pc forms a positive RC2 medial palisade, which goes from VZ to the external dorsal surface silhouetting the pc (Fig. 8A,D). At this stage, both GT and pc are H5- and GFAP-positive (Figs. 7B,C, 8A), but RC2 expression in pc is very scarce (Fig. 8D).

In the posterior tectal region, corresponding to TS, glial processes are orientated caudally (Fig. 2A,B). The glia cell somata are situated in the toral VZ, and their processes finish in the caudal surface of ventral tectum. This area widens as development advances, protruding toward the ventricle and external surface. In parallel, its radial processes curve dorsally, as shown in sagittal and transversal cuts (Figs. 2B, 4C, 9A,B).

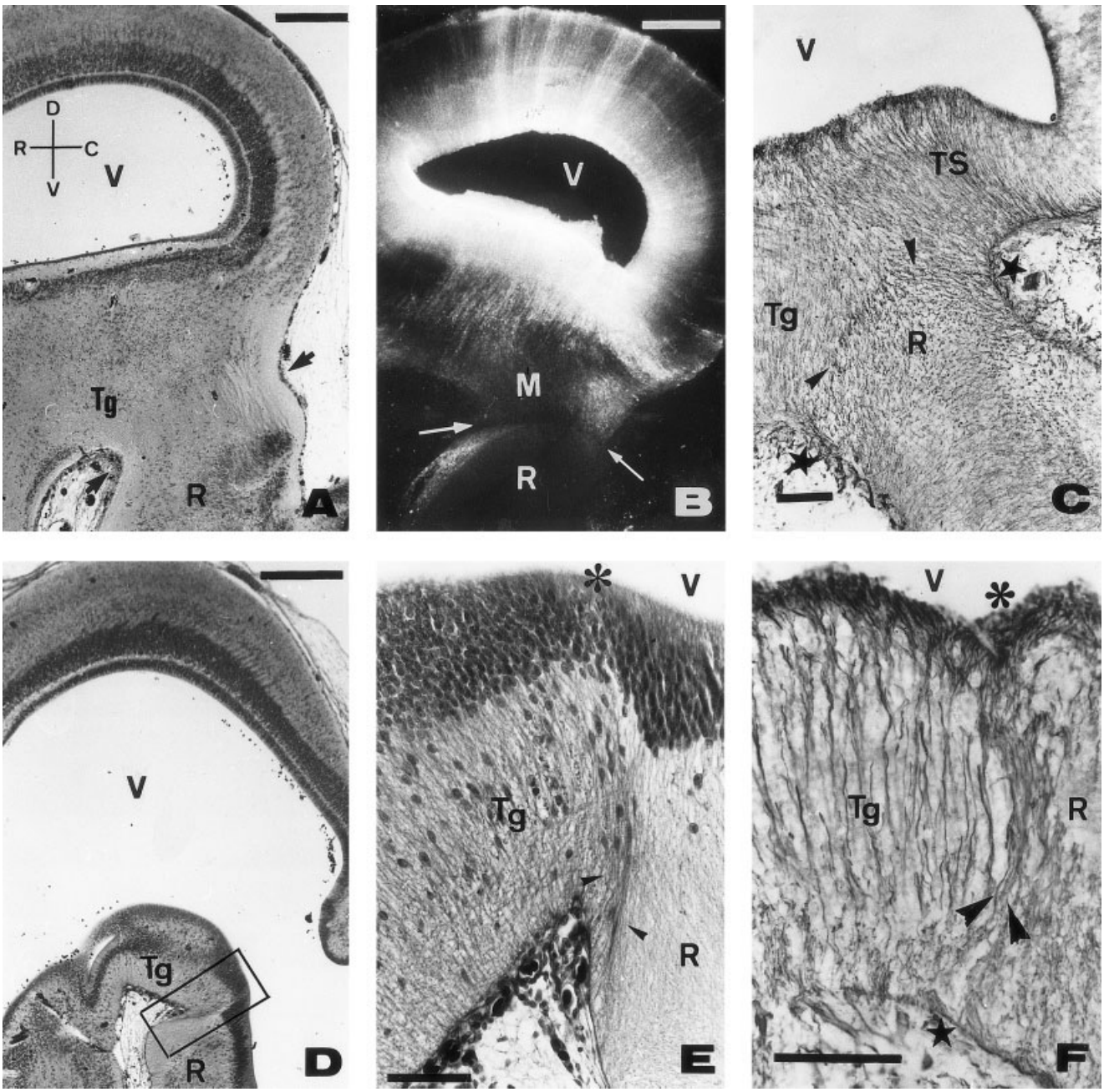

Fig. 4. The caudal midbrain limit in sagittal sections. **A–C:** Superficial sections respectively stained with Nissl, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI), and H5 immunostaining. Arrows in A, B, and stars in C point out the external position of the limit in superficial sections. Arrowheads in C point out an arched line of fasciculated radial glia processes at the limit. **D–F:** Medial sagittal sections stained with cresyl violet (D,E) and H5 immunostaining. Arrowheads point out fasciculated radial glia processes at the limit. Asterisks mark the ventricular position of the limit (coinciding with a small protrusion). For abbreviations, see list. Scale bars = 250 μm in A,B,D; 125 μm in C; 50 μm in E; 100 μm in F.

The IA is interposed between GT and TS as a transitional region (Figs. 1A,B, 2B). It has a periventricular stratum formed by a thick plexiform and a dense cellular layer around ventricle (Figs. 1A, 9D). In adult brain sections stained with Nissl technique, the periventricular cellular layer in this area is thinner than TS and thicker than GT periventricular cellular layer, and present a different curvature (Diaz et al., 2000). Similar features can be seen from middle developmental stages. Their radial glia processes extend laterally with a certain ventral inclination, ending on both sides of the ventral tectum. Thus, in the sagittal and horizontal sections, they appear truncated (Fig. 2B). Some authors identified this area like nucleus laminaris of the torus

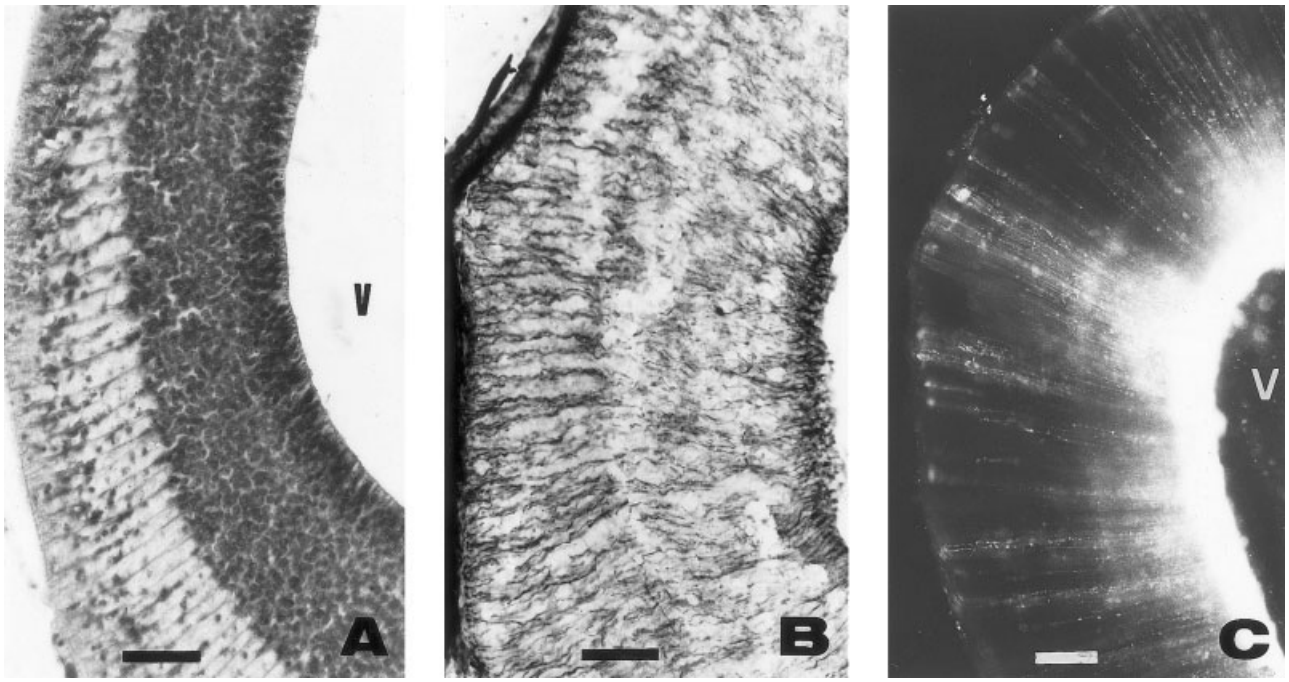

Fig. 5. Radial glia processes in the dorsal optic tectum are parallel and are associated in small and regularly spaced fascicles. **A:** Lateral portion from a horizontal section of embryonic day (E) 34, stained with cresyl violet. Migrating cells form radial lines. **B:** Caudal region from an E35 sagittal section immunostained with H5 antibody. **C:** The 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) staining of an E37 transverse section. For abbreviation, see list. Scale bars = 50 μm in A,B; 100 μm in C.

semicircularis (ten Donkelaar et al., 1987; ten Donkelaar 1998).

A thick bundle of radial glia processes ventrally surround the torus and IA (Fig. 2B). This bundle can be seen in transversal sections of H5 immunostained series from E37 on, under TS and IA (Fig. 9A–C). We have no clear evidence of this bundle under GT, although some DiI-stained processes bordered this structure (Fig. 2B). Whereas in transversal cuts the bundle extends from VZ to elm, in sagittal sections, it looked like a spotted region under the areas mentioned (not shown). That finding is because the section plane cuts its radial processes. The bundle is especially clear in the hatching stage, where it continues being RC2-positive, contrary to neighboring regions (Fig. 9C). This finding gives us a useful and objective reference to establish the alar/basal mesencephalic limit avoiding usual incertitude, at least between IA/TS and Tg.

In the tegmental zone (Tg), radial glia processes go toward the cephalic curvature, following straightly dorsoventral paths (Fig. 2A). This characteristic is seen from the first stage studied, i.e., E31 (Fig. 10A), and continues along all developmental stages (Fig. 10B,C). In some sagittal cuts, radial processes come up from the ventral tectum ventricular zone and seem to follow, without solution continuity, to the cephalic curvature through the tegmentum (Fig. 2A). However, when DiI staining is less intense, a separation is observed between dorsal (tectal) and ventral (tegmental) processes (Figs. 2B, 10C). In H5 immunostained series, fasciculation in tegmental processes is greater than adjacent ventral tectum (Fig. 10B).

Markers expression

Dynamic expression of H5 and RC2 antigens can be resumed as follows: Both are detected from the first stage selected (E31) in somata, processes, and endfeet of a subpopulation of ventricular radial cells (Figs. 8D, 10A). Expression increases to E34. However, from this stage, RC2 decreases, while H5 expression keeps constant until stages E37–E38. At hatching both antibodies only mark specific radial glia areas, like TS, GT, and pc, (Figs. 7B,C, 9C,E) but with differences in the expression pattern, as mentioned before.

DISCUSSION

Limits

The mesencephalic rostral limit (m/d) has been defined classically as “that which passes behind the posterior commissure” (pc; Palmgren, 1921). Larsen et al. (2001) have shown in chicken that the m/d limit is a clonal restriction boundary and joins typical features of a limit as differential expression of certain molecules, axon path, interruption of interkinetic nuclear migration etc. In the adult *Gallotia galloti*, this limit is not exactly evident, because the mesencephalic and pretectal nuclei do not appear separated by any clear tissular “hiatus” (Diaz et al., 2000). A similar pattern is observed in other vertebrates. However, in *Gallotia* embryos, a sharp interruption of tectal layers is observed at the posterior commissure level. Furthermore, in chick/quail chimera brains, we have observed that the pretectal area, rostral to the GT, is a diencephalic formation (unpublished results). Therefore, we do know

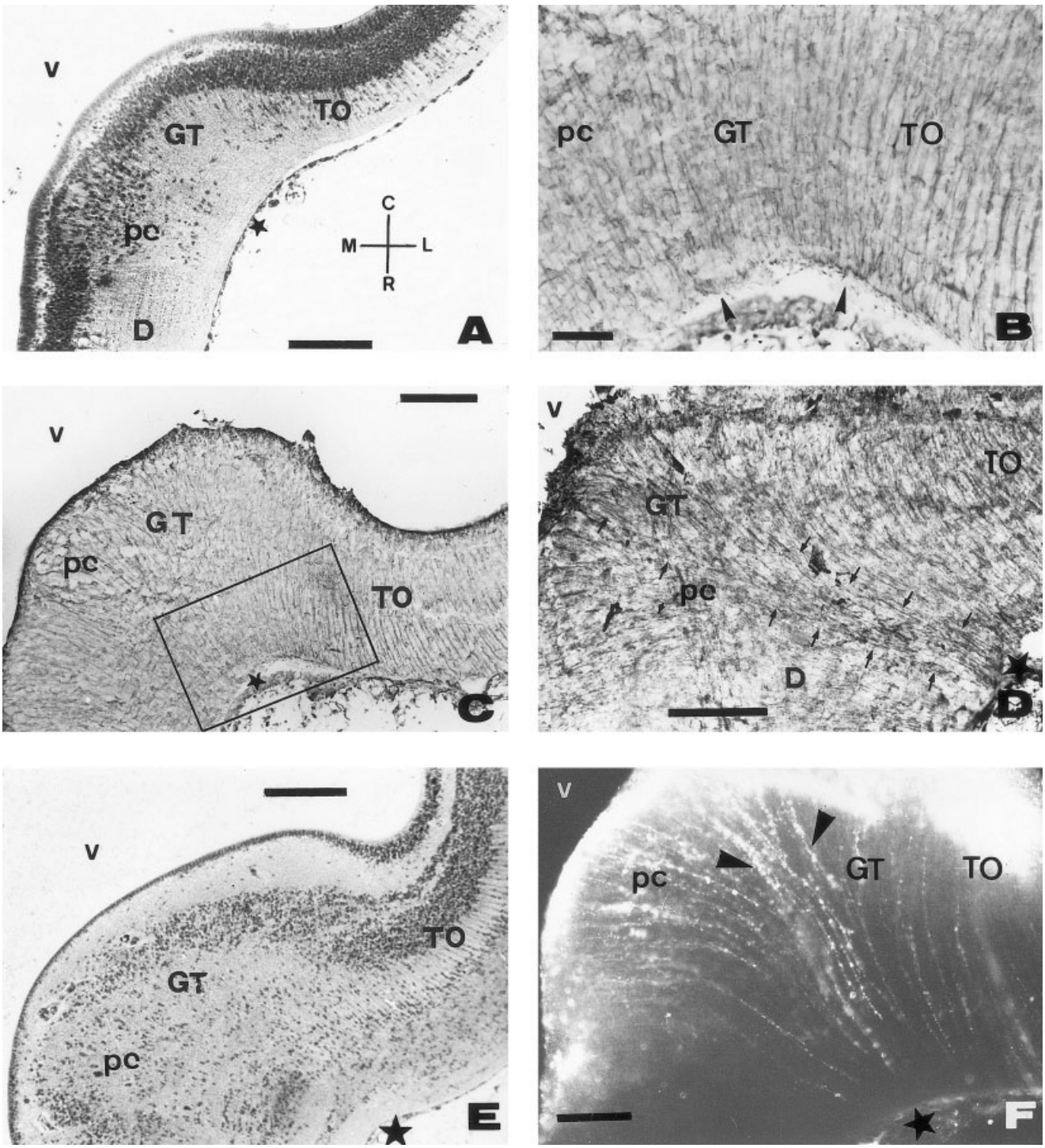

Fig. 6. Griseum tectalis and adjacent zones in horizontal sections of embryonic day (E) 33– E36. Differences in density, thickness, and fasciculation of radial glia processes can be appreciated among pc, GT, and TO. **A–C:** Horizontal sections of E33 stained with cresyl violet and H5 immunostaining, respectively. Enlargement shown in B is boxed in C. Different thickness between A and B are probably due to fixation and inclusion protocols. **D–F:** E35/E36 H5 immunostaining, cresyl violet and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) stain, respectively. Comparing A, B, and C, the GT territory seems to bend laterally. Behind pc, a denser bundle of radial glia processes can be seen in this stages (small arrows in D, arrowheads in F). Stars mark the external limit between pc and GT. Arrowheads in B marks the external surface of GT territory. For abbreviations, see list. Scale bars = 150 μm in A,C,D,E,F; 50 μm in B.

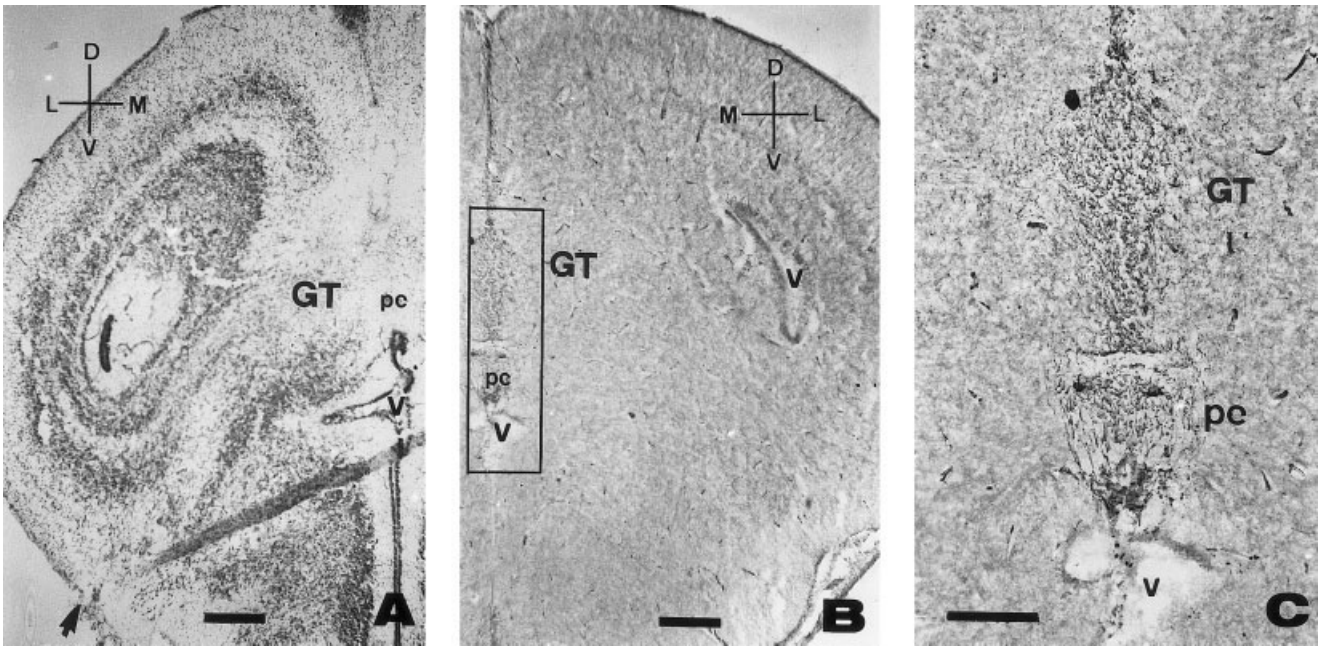

Fig. 7. **B,C:** Transverse sections in the hatching stage of the rostral midbrain showing glial fibrillary acidic protein expression in the medial zone of GT and pc. The rectangle in B is enlarged in C. **A:** Contralateral parallel section is stained with cresyl violet. The arrow points out the external limit between GT and pc region. For abbreviations, see list. Scale bars = 250 μm in A,B; 150 μm in C.

where the limit is, but what is its physical appearance? Which type of molecules or cells characterize it? Are we dealing with a "line" or a "fence"? The results of this work allow us to define the limit as "the border where mesencephalic radial glia processes end," and this frontier coincides with the caudal border of pc. They also indicate that the m/d alar limit is formed by the confluence of the mesencephalic and diencephalic radial glia. And that, in the alar area, the radial processes of the interface m/d fasciculate forming a glial bunch between pc and GT, giving "physical entity" to the limit. On the other hand, in the basal region, we do not observe a similar fasciculation between the tegmental and diencephalic processes. This finding supports the idea of anterior/posterior axis continuity of the basal longitudinal column (Rendahl, 1924), reactivated by the finding of genes expressed in longitudinal columns domains in the tegmentum and basal synencephalon (Sanders et al., 2002). In any case, we consider that basal rostral frontier can be defined by the border where the tegmental processes of radial glia end. Of interest, this border coincides with classic landmark proposed for this limit.

In *Gallotia galloti* embryonic brains stained with Nissl techniques, a clear histological discontinuity is observed between the midbrain and hindbrain, located behind nIII (separation of longitudinal cell columns, wall constriction, etc.). In this work, we show that, just in this area, distal tracts of radial glia processes fasciculate forming an H5-positive glial palisade that runs dorsoventrally in sagittal cuts. The position of this line coincides with that given for the adult *Gallotia galloti* m/d limit in base of cytoarchitectonic criteria (Diaz et al., 2000) and with that established by Palmgren (1921) for other vertebrates. It also coincides with the anterior border of the fgf8 expression domain at early stages (Crossley and Martin, 1995). Therefore, our data establishes the cellular identity of this limit. Protagonism of radial glia in limit formation has been reported in other models (Heyman et al., 1993; Stoykova et al., 1997; Redies et al., 2000). In the *Xenopus* rhombencephalon, each limit shows a specific radial glia subpopulation interposed between two rhombomeres from early stages (Yoshida and Colman, 2000). But in the midbrain of *Gallotia galloti*, a fasciculation between radial glia neighbor processes belonging to two adjacent areas seems to happen. Our data point out that this fasciculation, as those of m/d limit, seems to be progressive and proceeds from out- to inside. Furthermore, radial frameworks of two adjacent regions in this study have specific characteristics, while in the *Xenopus* rhombencephalon, organization and molecular features are identical between different rhombomeres. It is possible that we find ourselves faced by two different limit-establishing models. In fact, some other differences between limits of rhombencephalon and anterior regions have already been shown (Larsen et al., 2001).

Subdivisions

The different midbrain subdivisions recognized here on the basis of radial glia and grisea distribution, suggest a subdivision pattern similar to the model conceived by Palmgren (1921) and used by Diaz et al. (2000) in their study of *Gallotia galloti* adult midbrain. Both models recognized four longitudinal columns in the subtectal region, named dorsal, lateral, basal, and median bands in the *Gallotia* midbrain pattern. The region corresponding to the basal and median bands in the midbrain adult model (Diaz et al., 2000) was not subdivided in our model, because a specific study of the radial glia organization in this

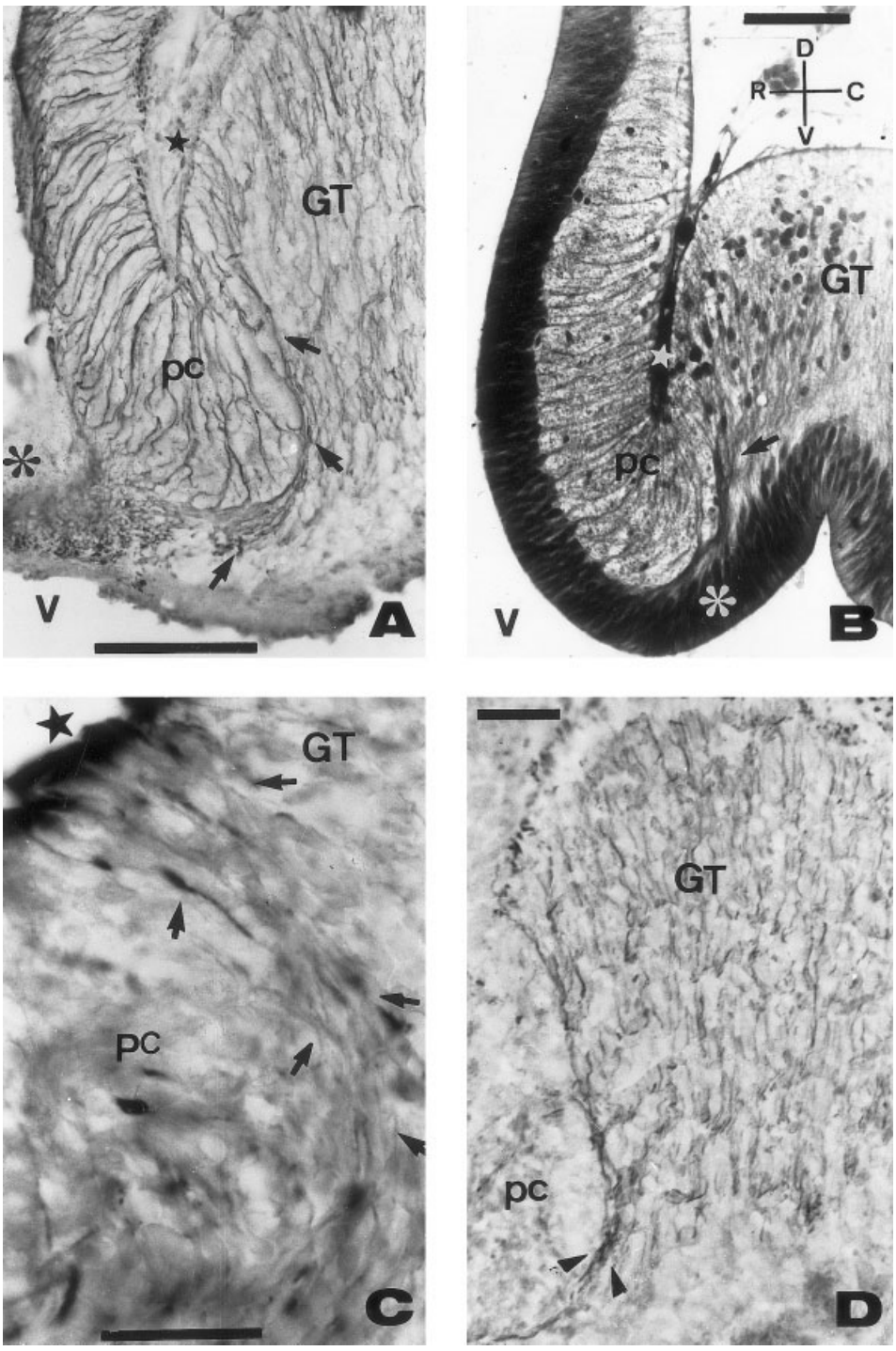

Fig. 8. Fasciculated radial glia processes in the alar m/d limit. **A,D:** Floor of pc and GT medial region, respectively, immunostained with H5 and RC2 antibodies (hatching stage). **B:** Aspect of the same region stained with cresyl violet. **C:** Detail at embryonic day 31 immunostained with RC2. Arrows or arrowheads point out the fasciculated bundle between pc and GT. Asterisks mark the ventricular zone where this bundle is born. Stars mark the external ending of the bundle. For abbreviations, see list. Scale bars = 100 μm in A,C; 50 μm in B,D.

zone will be dealt with separately. Of interest, the proposed pattern is according to the new data, coming from gene expression studies, that reveal an arcuate plan in the ventral mesencephalon (Argawala and Ragsdale, 2002; Sanders et al., 2002).

The dorsal band is the zone occupied by GT, IA, and TS. The cytoarchitectonic criterion to delimit these areas is based on their stratification pattern, described in detail by Diaz et al. (2000). We have observed stratification differences in these areas from stage E33 stage. Differential molecular features seen in radial glia of this zone, such as RC2 expression in GT and TS but not in IA, they cannot explain the stratification differences among these subdivisions, because they arise too late. But, we have observed that the reelin distribution pattern in *Gallotia galloti* embryos presents important differences among these ar-

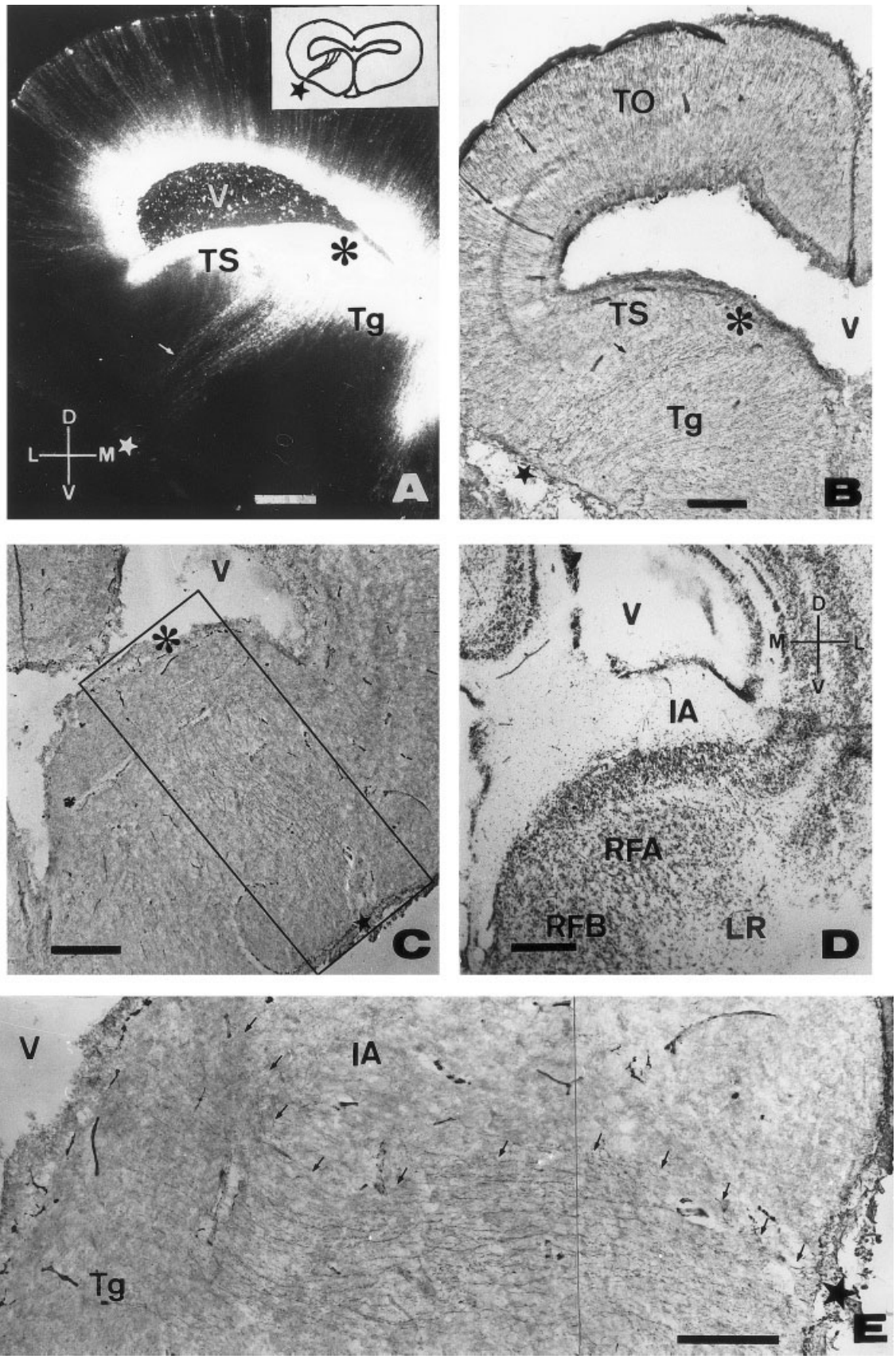

Fig. 9. The alar/basal limiting region. **A,B:** Transverse sections at embryonic day 37 showing a bundle of radial glia processes (small arrows) under the TS. In A, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) staining. In B, immunostaining with H5 antibody. **C,D:** Transverse sections of hatching stage through IA. In C, RC2 immunostaining. In D, parallel section stained with cresyl violet shows two nuclei situated in the bundle pathway (RFA and LR). **E:** The radial glia bundle is RC2-positive in this stage. The rectangle in C shows the location of this band (under IA in this level). For abbreviations, see list. Scale bars = 200 μm in A,B; 250 μm in C,D; 125 μm in E.

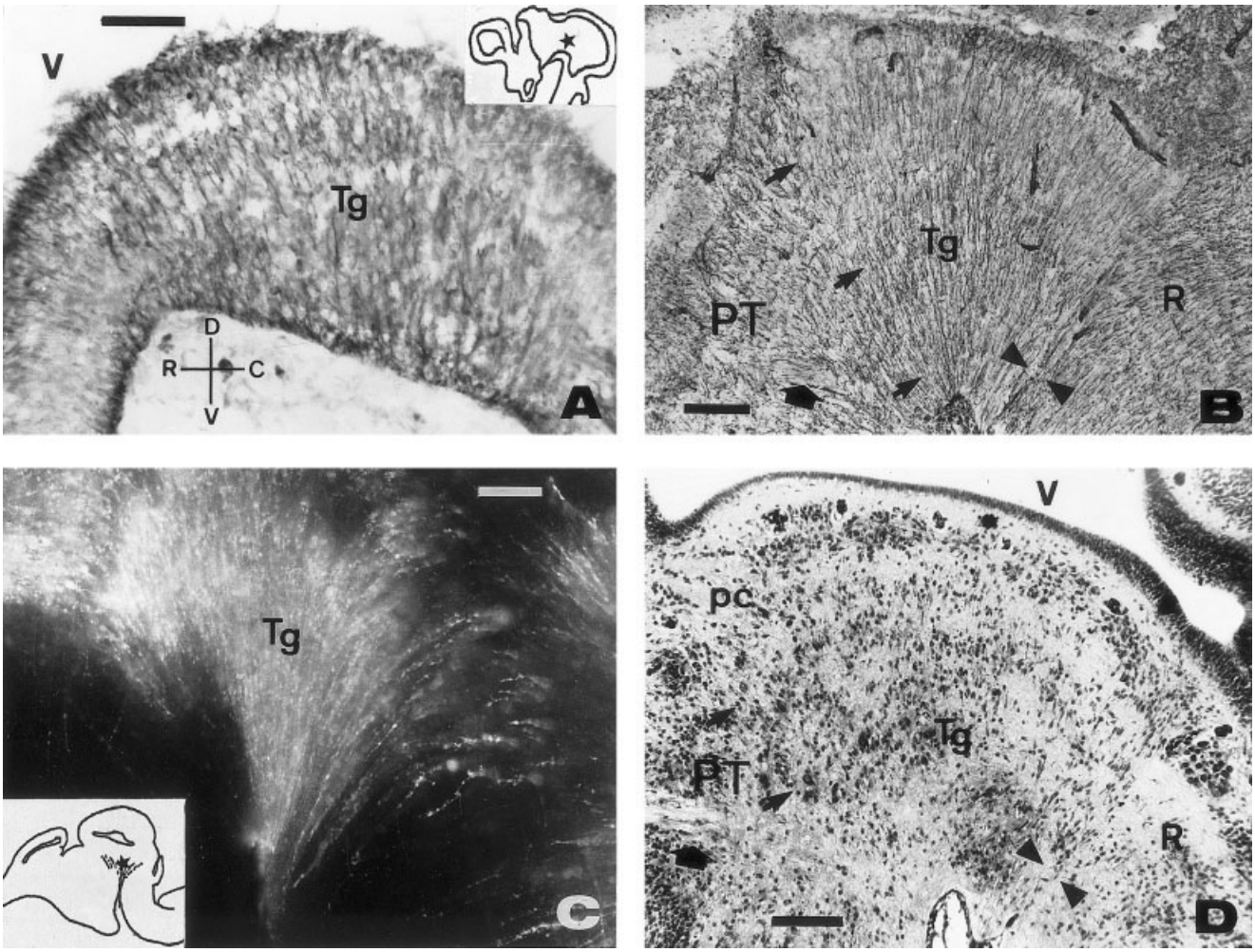

Fig. 10. Radial framework in the tegmentum. **A:** RC2-positive processes in a sagittal section of embryonic day (E) 31. **B:** Sagittal section of tegmentum at E37 immunostained with H5 antibody. Between Tg and R, fasciculated radial processes (arrowheads) form the boundary. A similar limit cannot be seen between GT and D (small arrows). A large arrow points out the fasciculus retroflexus in the diencephalon. **C:** Radial glia processes stained with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) in a sagittal section of tegmentum in E37. **D:** Cresyl violet section at E37, similar to B. For abbreviations, see list. Scale bars = 50 μm in A; 100 μm in B–D.

eas (unpublished results). This molecule is secreted by migrating cells and is involved in cellular migration (Goffinet, 1997). Therefore, intrinsic features in reelin expression of migrating neurones could participate in tectal differences. Furthermore, other processes, such as cellular proliferation or connectivity seem to contribute to it (Matsuno et al., 1991).

The most widely recognized midbrain subdivision in amniotes is the alar/basal or tectal/tegmental. Gene expression studies show that this subdivision is originated at early stages and is protagonized by the Shh protein (Nomura and Fujisawa, 2000; Argawala et al., 2001; Nakamura, 2001). However, until now, no clear landmark was available to point out the alar/basal limit. Several contradictions and omissions identifying alar or basal nuclei found in the literature could be due to that fact (refs. in Diaz et al., 2000). In this article, a dense radial bundle is shown passing under the ventral tectum and ending behind TS. The situation of this bundle coincides with the area described in adult *Gallotia galloti* as a lateral band, in which several ventral alar formations are located (Diaz et al., 2000). Therefore, this glial bundle constitutes the ventral margin of the alar plate, and its inferior border draws the alar/ventral limit. We think that this structure is shared by the embryonic midbrain of other vertebrates, because they have equivalent nuclei in this area. In fact, a nonmentioned similar band is present in the turtle mesencephalon, confused with neighboring GAFP-positive processes (Kálmán et al., 1997). Selective markers such as RC2 in hatching stage must be used to distinguish clearly this bundle.

Markers

Because RC2 is a specific marker of the astroglial lineage (Misson et al., 1988), its expression in *Gallotia galloti* E31 seems to point out a radial glia presence at much earlier stages than GFAP expression in this species allows us (Monzon-Mayor et al., 1990). Positive RC2 cell mor-

phology at these stages and similarity of its spatial pattern with that of posterior stages seem to confirm this expression. At E31, neurogenesis is intense in the neuroepithelium and mesencephalic ventricular cells are still highly proliferative. This finding makes us think that positive RC2 embryonic radial glia cells at E31 in *Gallotia galloti* could be neural precursors. This role has been shown for RC2-positive cells in the mouse telencephalon (Hartfuss et al., 2001). There, RC2-positive precursors coexist in distinct subpopulation differentiated in GLAST expression (astrocytes-specific glutamate transporter) and BLBP (brain lipidic-binding protein). Some of these precursors characterize the neurogenesis phase. Complementary assays in vitro corroborated that positive RC2 cells could be considered as multipotential neuronal precursors. However, although a percentage of its cells can be neuronal precursors, early pattern of radial ventricular cells in *Gallotia galloti* can be considered a radial glial pattern, given that it stays through development.

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