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ORIGINAL ARTICLE

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Reissner's substance expressed as a transient pattern in vertebrate floor plate

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Abstract The function of the floor plate in dorso-ventral patterning of the developing nervous system and in the guidance of commissural axons is well established. However, several morphological aspects concerning the exact localization of its rostral and caudal end and the regional and temporal specialization are still controversial. We present new insights revealed by the expression of Reissner's substance in the floor plate during early neurogenesis of zebrafish, *Xenopus*, chick and rat. We used a polyclonal antiserum raised against Reissner's substance, which is a secretory product of radial glia in the roof plate of the adult vertebrate brain. In early embryonic stages the rostral boundary of floor plate immunoreaction vary in the different vertebrates. Immunoreactive cells are not only present in the epichordal region (rat) but also in prechordal areas of the midbrain (chick) and forebrain (zebrafish and *Xenopus*). During further development, Reissner's substance expression disappears first in the most rostral areas and later also in the spinal cord. However, immunopositive labelling in the isthmus region at the mes-metencephalic boundary, described originally as the flexural organ, is most extensive and detectable during a long period of embryonic development. It is proposed that the gradual restriction of Reissner's substance expression to the isthmus reflects the complex differentiation processes in this region also in later embryonic development. Furthermore, the expression pattern in zebrafish indicates that Reissner's substance could play a role in axonal decussation.

Key words Flexural organ · Subcommissural organ · Immunocytochemistry · CNS development · Vertebrate embryos

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Introduction

Despite the broad diversity of the vertebrate bauplan that has emerged with the course of evolution, the molecular patterning underlying early embryonic development appears to have been largely conserved. Through comparative studies of the spatial and temporal expression of orthologous genes it is not possible to better understand the mosaic of ancestral, modified and new specific elements of molecular patterning. The establishment of neuronal connections between the right and left body sides already appears at the origin of bilaterians. Both in the midline of the insect nervous system and in the floor plate of vertebrates conserved, polarising signals occur, which control the cell phenotype and the pathfinding of commissural and longitudinal axons. The present work shows the expression of the Reissner's substance (RS) as a general transient immunohistological pattern in the floor plate of vertebrates.

In the adult nervous system of vertebrates the roof plate in the region of the last diencephalis prosomer is formed by a RS secreting glial complex. This structure was named as the subcommissural organ (SCO) because of its anatomical position below the posterior commissure (Dendy and Nicholls 1910). The SCO represents a phylogenetically old and an ontogenetically early differentiated glial region. It's cells are characterized by their radial orientation, an original intermediate filament pattern (cytokeratin 8/18 and vimentin), the possession of at least one cilium, and secretory activity (Viehweg and Naumann 1996). The specific secretion, the RS, is released apically into the ventricle, but also basally into the extracellular matrix (Kimble and Møllgård 1973; Lösecke et al. 1986). On account of its molecular nature and as a result of fluid flow the RS aggregates to a threadlike structure in the ventricle, the Reissner's fibre, which can be observed in the central canal of the whole spinal cord (for review, see Leonhardt 1980; Rodríguez et al. 1992; Meiniel et al. 1996).

The biochemical analysis of RS is mainly based on research of bovine Reissner's fibre. In addition to low molecular components, one high molecular glycoprotein of multiple molecular weight is suggested. For the latter two

partial gene sequences could be reported by screening of a cDNA library of bovine SCO (SCO-*spondin*: Gobron et al. 1996; *Reissner's fibre-GlyI*: Nualart et al. 1998). Both gene sequences encode a modular protein with numerous protein- and receptor-binding sites that are typical for extracellular matrix proteins. The amino acid sequence reveals the presence of thrombospondin type I repeats, low density lipoprotein receptor type A repeats and a cysteine-rich domain that is homologous to human MUC 2 and von Willebrandt factor. Southern blot hybridization of bovine *SCO-spondin* has revealed orthologous genes within the chordates, from *Ciona intestinalis* (Urochordata) to the vertebrates, including the human (S. Gobron, personal communication). Furthermore, the occurrence of anti-RS immunoreactivity in *Asterias rubens* (Viehweg et al. 1998) and in *Schisterocerca gregaria* (Lichtenfeld et al. 1998) indicates an even earlier evolution of this gene.

In addition to the secretory glial complex in the roof plate of vertebrates, Olsson (1956) first described comparable secretory cells in the floor plate during the development of anamniotes. During a limited embryonic period these cells are also involved in the Reissner's fibre formation. They form the so-called flexural organ (FO), which is situated in an area of the plica ventralis that is caudally difficult to delimit with conventional histology. Contrary to the permanent secretory activity in the SCO in most vertebrates, these "floor cells" lose this ability during embryonic development.

In recent years numerous morphogenetical molecules have been reported in the vertebrate floor plate. Undoubtedly, this embryonic structure plays a pivotal role in the early dorso-ventral patterning of the neural tube and later in the guidance of commissural and longitudinal axons. In this relation it seems important that effects of RS on neuronal aggregation and neuritic outgrowth could be demonstrated in in vitro assays (Monnerie et al. 1997). It suggests a function as an extracellular matrix protein during development. However, the expression of RS in the floor plate (Naumann 1986; Schöbitz et al. 1993; Rodríguez et al. 1996; López-Avalos et al. 1997; Yulis et al. 1998) originally described as the FO (Olsson 1956) has been neglected in this connection until now.

The present study focuses on the floor plate during the neurogenesis of zebrafish, *Xenopus*, chick and rat as examples of the four vertebrate taxa. An antiserum directed against the RS was used to perform an immunocytochemical investigation, searching for regional and temporal variations in the expression pattern. Our results are discussed with reference to other floor plate studies, and in terms of possible RS function.

Materials and methods

Test objects

All golden zebrafish embryos (*Brachydanio rerio*) used in this study were collected by natural spawning and staged according to Kimmel et al. (1995). We observed eight to ten embryos for each of six developmental stages from about 14 h to hatching, early larvae and adults.

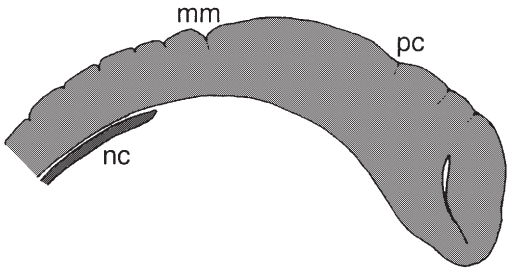

Fig. 1 Schematic drawing of a midsagittal brain section of 15 h zebrafish embryo (compiled from data in von Kupffer 1906; Kimmel 1995). A morphological subdivision of the rostral part is hardly distinguishable. The di-mesencephalic- and the mes-metencephalic boundaries are evident only on the dorsal side (*mm* mes-metencephalic boundary, *nc* notochord, *pc* posterior commissure)

Females of the south african clawed frog (*Xenopus laevis*) were stimulated to lay eggs by injecting human chorionic gonadotropin (Sigma). Embryos were staged according to Nieuwkoop and Faber (1967). We observed eight to ten embryos for each of ten developmental stages from about 20 h to hatching. Larval stages and adult animals were also investigated.

White Leghorn hen (*Gallus gallus*) eggs were incubated at 38.5° C and embryos were staged according to Hamburger and Hamilton (stage HH; 1951). We investigated eight groups of five embryos each from embryonic day E2 until E19.

Albino Wistar rats (*Rattus norvegicus*) were used in this study. In order to obtain particular stages of development, females were paired overnight with males and inspected for vaginal plugs on the next day. Embryos were taken from the uterus of anaesthetized females between embryonic day 10 (E10) and birth. They were staged according to Henneberg (1937). We observed three to five embryos for each of ten developmental stages.

Preparation of embryos for immunocytochemistry

The different developmental stages of the four species were fixed by immersion in Carnoy's mixture, containing 100% ethanol, chloroform and glacial acetic acid (6:3:1) for 1 to 3 days at 4° C. Tissue samples were permeabilized in propylene oxide (Serva) for 1 h, rehydrated in a graded ethanol series and washed in TRIS-buffered saline (TBS: 50 mM TRIS-HCl, 137 mM NaCl) pH 7.4.

The larger embryos of chickens and rats, *Xenopus* larvae as well as adult brains, were embedded in 0,9% agar in TBS and sec-

Fig. 2a–f Expression pattern of RS in the central nervous system of zebrafish embryos. **a**–**c** The initial anti-RS labelling of the floor plate is visible at 15 h. **a** Sagittal view of the most rostral labelled cells (*arrow*) in the prechordal region. The rostral tip of the notochord (*asterisk*) is localized caudal to the immunopositive cells. **b**, **c** Frontal sections of the floor plate in different regions. Whereas the floor plate in the rostral region consists of several cells (**b**), the more caudal floor plate is only one single cell wide (**c**). **d**–**f** Anti-RS labelling in the floor plate and the SCO of 48 h embryos. **d**, **e** Sagittal view of the head shows the localization of the RS-secretory cells in the roof plate at the di-mesencephalic boundary (*SCO*) and several radial cells ventrally at the mes-metencephalic region (*FO*) in whole mount (**d**) and paraffin section (**e**) Note the apical released material from the dorsal SCO and the ventral FO is condensed to form the immunoreactive Reissner's fibre that extends caudally over the whole spinal cord. **f** Immunoreactive floor plate cells at the mes-metencephalic boundary representing the FO. Both the radial cells as well as transversal crossing neurites are labelled (*C* primitive central canal/ventricular system, *CF* commissural fibres, *EM* external limiting membrane, *EY* eye, *FO* flexural organ, *RF* Reissner's fibre). *Bars* **a** 20 µm, **b**, **c**, **e**, **f** 10 µm, **d** 100 µm

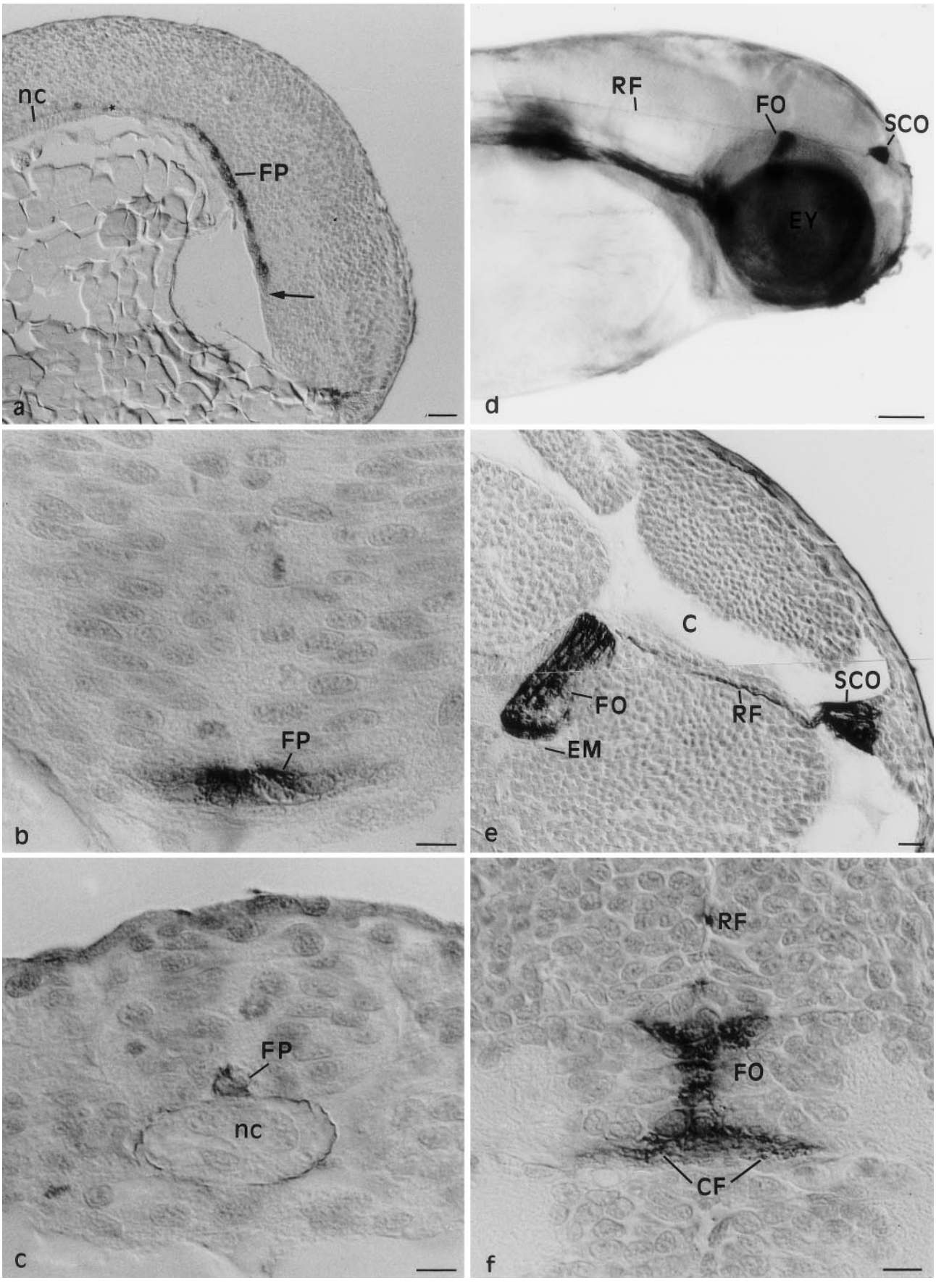
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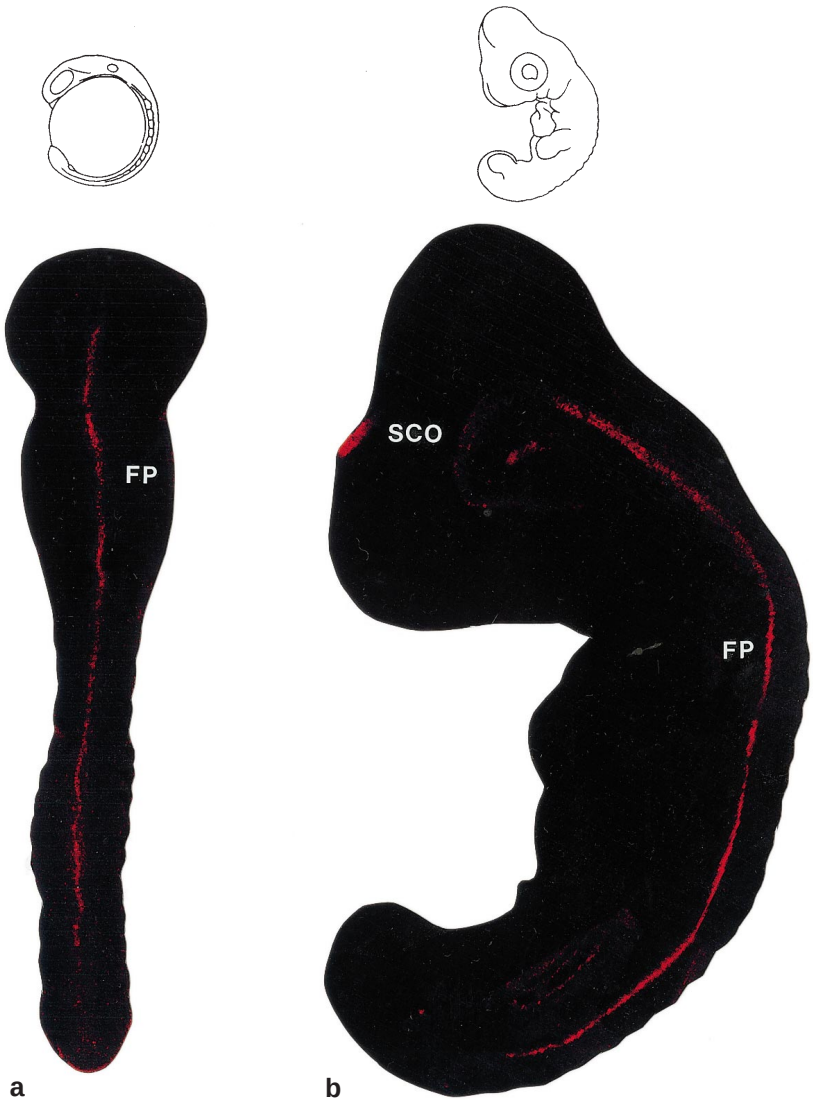
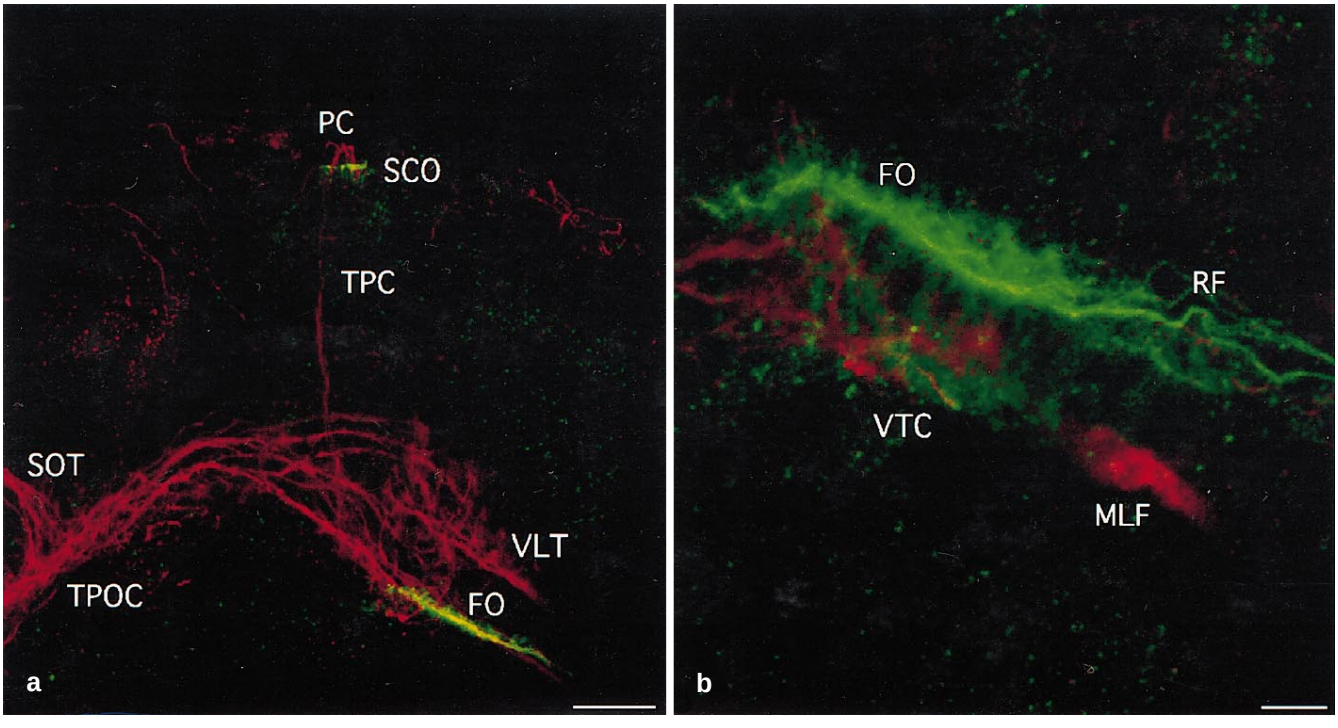
Fig. 3a, b Confocal laser scanned images of anti-RS fluorescence labelling of the floor plate in zebrafish and chick embryos. **a** Dorsal view of a whole mount zebrafish embryo at 15 h. The immunoreactive cells form a continuous band extending from the prosencephalon to the caudal end of the spinal cord. **b** Photomontage using Adope Photoshop of sagittal 100 µm sections of a whole chick embryo at stage 27. The whole floor plate including the caudalmost region of the spinal cord but also the SCO is labelled (*FP* floor plate, *SCO* subcommissural organ)





tioned with a vibratome (Microslicer DTK-3000W) at 250 µm thickness in the frontal or parasagittal planes.

Yolk material and the surface ectodermis were prepared from *Xenopus* embryos older than stage 25.

Zebrafish and *Xenopus* embryos were used as whole mounts for immunocytochemistry procedures.

Light microscopy immunocytochemistry

For all tissue samples the endogenous peroxidase was quenched with 0.3% hydrogen peroxidase containing 0.1% sodium azide. After pretreatment with a mixture of 1.5% powdered milk, 1% bovine serum albumin and 0.5% normal goat serum in TBS with 0.4% Triton X-100, to block nonspecific binding, we incubated with a rabbit polyclonal antiserum directed against RS (anti-RS, code K11, 1:1,000). This antiserum was prepared in our laboratory and tested for specifity in ELISA (Lichtenfeld et al. 1998). Next, the tissue samples were exposed to peroxidase-conjugated goat anti-rabbit IgG (Dakopatts, 1:500) at 4° C. Finally, the peroxidase was visualized using 3,3′ diaminobenzidine as chromogene. Between each of the steps, the tissues were rinsed with TBS, 0.4% Triton X-100. The incubation times for the antibodies ranged from 2 to 5 days dependent on the thickness of tissue samples.

Subsequent to the immunocytochemical labelling some vibratome sections and the whole mounts of zebrafish and *Xenopus* were embedded in paraffin (standard procedure) and 5–7 µm sections cuted on a Jung microtome (Biocut 2035), mounted on glass slides with egg albumin, and dried. Nuclei were counterstained with Mayer's hemalum. The slide preparations were viewed with a Leitz DMRD microscope equipped for differential interference contrast.

Fluorescence microscopy immunocytochemistry

Vibratome sections of chicken embryos and whole mount zebrafish embryos were used for immunofluorescence microscopy according to the protocol described above. For visualizing the anti-RS antiserum, indocarbocyanine (Cy3; Dianova 1:300)-conjugated goat anti-rabbit antibody was used as secondary antibody. For double-label experiments, a monoclonal anti-acetylated tubulin antibody (Sigma) and the anti-RS antiserum were used simultaneously. A mixture of appropriate Cy3- and dichlorotriazinylamino-fluoresceine (DTAF; Dianova 1:500)-conjugated antibodies was used as secondary antibodies. Finally, the specimens were embedded in Mowiol 40–88 (Aldrich) and viewed with a Leitz DMIRB inverted microscope equipped with a Leica TCS 4D confocal laser scanner. Photographs were mounted as composites using Adobe Photoshop.

The primary serum was replaced by rabbit normal serum as controls for the specifity of the anti-RS pattern.

Results

Schematic drawings of the brains of each species at the stage of earliest anti-RS immunoreactivity in the floor plate is shown in Figs. 1, 5, 7 and 9 as a guide for the histological sections. These medio-lateral views of the embryonic brains depict the gross morphology and key landmarks for orientation. Comparing the histological sections to the schemes should help to fix the rostral border of RS-immunoreactivity in the floor plate during their widest rostro-caudal extension.

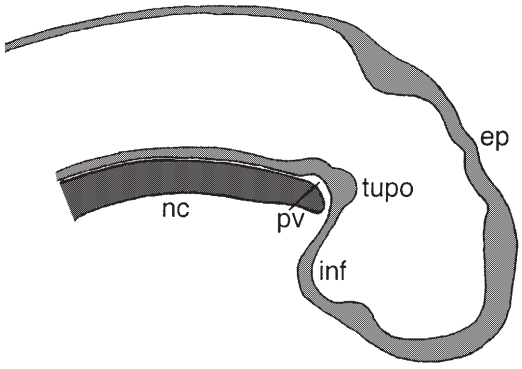


Fig. 5 Schematic drawing of a midsagittal brain section of stage 21 *Xenopus* embryo (compiled from data in von Kupffer 1906; Hausen and Riebesell 1991). The plica ventralis is well developed. The brain vesicle is enlarged and bulged in the infundibulum region (*ep* epiphysis, *inf* infundibulum, *pv* plica ventralis, *tupo* tuberculum posterior)

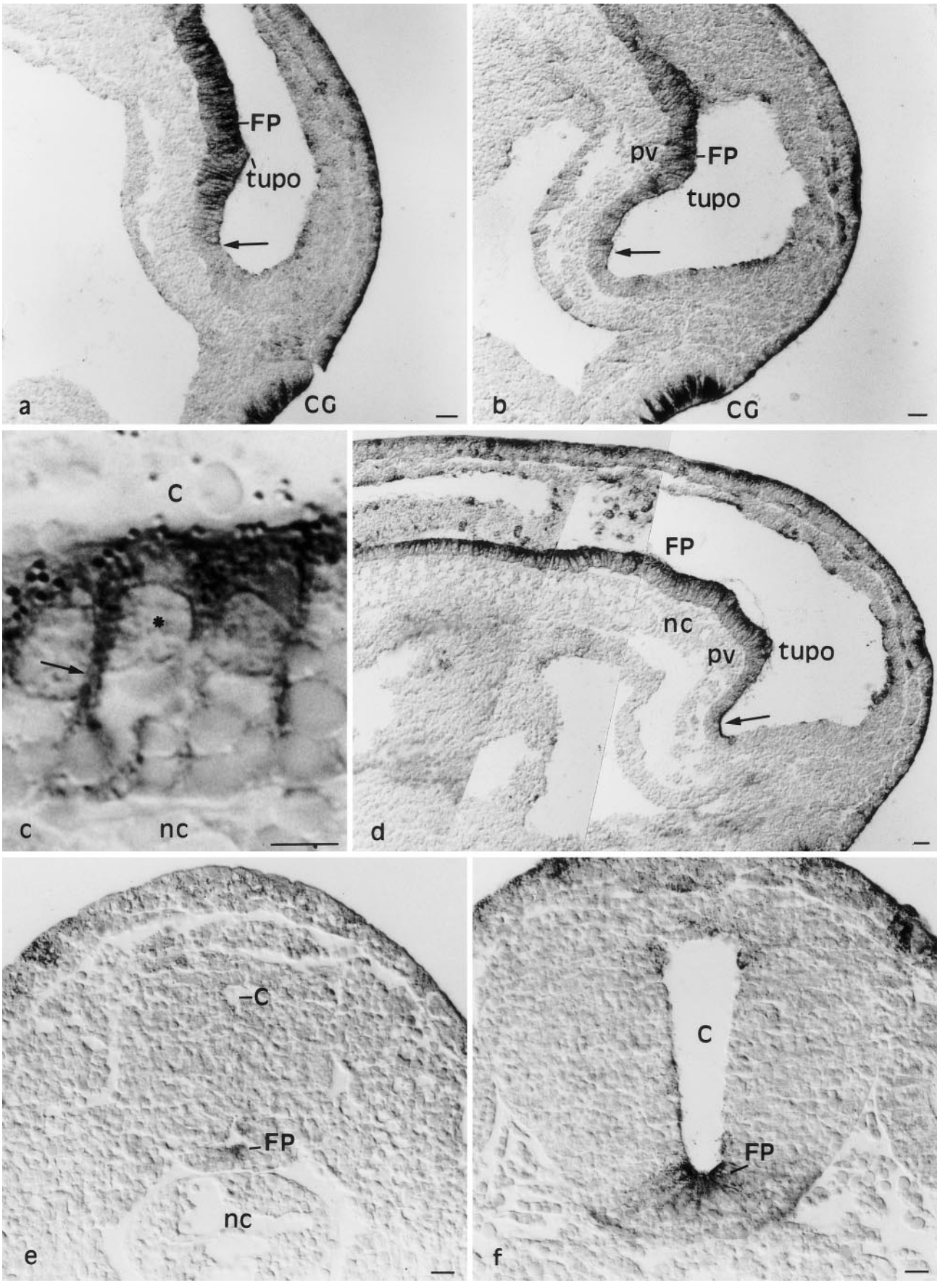
Zebrafish

Spatial distribution

Labelling with anti-RS antiserum in early stages (15–20 h) is found throughout the midline of the spinal cord as far as the prosencephalon (Fig. 1 a). The rostral floor plate cells displaying anti-RS reactivity are localized in a brain area in front of the notochord tip (Figs. 1, 2 a). The immunoreactive cells form a continuous band extending to the caudal end of the spinal cord. The spinal cord floor plate is only a single cell wide and possesses a distinctive triangular shape (Fig. 2 c). However, rostrally in the prospective mesencephalon, there is a ventro-lateral extension of floor plate immunolabelling (Fig. 2 b). The ubiquitous ventral anti-RS midline pattern disappears later, at first in the caudal diencephalon and in the rhombencephalon. After 48 h, immunopositive floor plate cells are restricted to the caudal two-thirds of the spinal cord and at the border between the mesencephalon and metencephalon (Fig. 2 d, e). These few rostral cells have bipolar aligned profiles and an apical process, which terminates on the central canal, and a basal process which projects towards the external limiting membrane (Fig. 2 e). The rostral immunoreactive floor plate cells thus resemble radial glia cells. At the base of these immunopositive pyramidal-shaped band of cells, immunonolabelling is also visible on transversal neurites crossing this area (Fig. 2 f).

Anti-RS immunoreactivity is also strongly present at 48 h in the SCO, that is localized in the dorsal di-mesencephalic boundary (Fig. 2 d, e). The SCO cells show the

Fig. 4a, b Double immunofluorescence staining with the anti-RS antiserum (*green*) and an anti-acetylated tubulin antibody (*red*). The 24 h zebrafish embryo is viewed from the lateral side using a confocal laser scanner. **a** The major axon tracts of the di- and mesencephalon and their correlation to RS-immunoreactive cells are shown. The RS expression in the SCO starts at the time of the formation of the posterior commissure. The FO is within an area where various axonal bundles meet. **b** Detail at higher magnification of the FO showing the radial character of the Reissner's fibre-forming cells with basal relation to the ventral tegmental commissure (*MLF* medial longitudinal fascicle, *PC* posterior commissure, *SOT* supraoptic tract, *TPC* tract of the posterior commissure, *TPOC* tract of the postoptic commissure, *VLT* ventral longitudinal tract, *VTC* ventral tegmental commissure). *Bars* **a** 30 µm, **b** 10 µm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same radial morphology as those cells in the ventral mes-metencephalic region (Fig. 2 e). RS is originally an exclusive secretory product of the floor plate. Later on, secretory material is also released by the SCO into the ventricle. It forms the Reissner's fibre that extends along the central canal and contacts remnants of the floor plate at the ventral mes-metencephalic region (Fig. 2 e). Anti-RS immunoreactivity is not observed in any neural structure outside the midline.

Temporal variations

Anti-RS immunoreactivity is first shown at 15 h (Figs. 1, 2a–c, 3a) in the midline of the neural plate just dorsal to the immunonegative notochord. In this stage of development anti-RS immunoreactivity is found throughout the widest rostro-caudal extension. Until stage 24 h the floor plate retracts rostrally. Simultaneously, a ventrolaterally extension of floor plate immunolabelling was detected in the mesencephalon. The floor plate cells in the region of mes-metencephalic boundary are immunolabelled most intensely at 48 h (Fig. 2 d, e). This expression is reduced after hatching and is still detectable in the early larvae, but is absent in the adult (data not shown).

While ventral anti-RS immunolabelling in the floor plate decreases during development, SCO cells in the midline of the caudal diencephalic roof plate are immunopositive at about 24 h (Fig. 4 a), the time when the outgrowth of the posterior commissure starts, and is still present in the adult.

Correlation of axon tracts to cells of RS-expression

We examined the relationship of developing tracts and commissures in the brain to cells that express RS. A simple scaffold of bilaterally symmetrical tracts and commissures were visualized using an antibody to acetylated tubulin. Immunodouble-labelling embryos at 24 h reveals that two of the commissures are located upon the endfeet of RS expressing cells. The ventral tegmental commissure are extended across the midline at the mesencephalic, strong RS-immunoreactive floor plate (Fig. 4 a, b). Furthermore dorsal, RS is expressed by the developing SCO at the time the posterior commissure forms (Fig. 4 a).

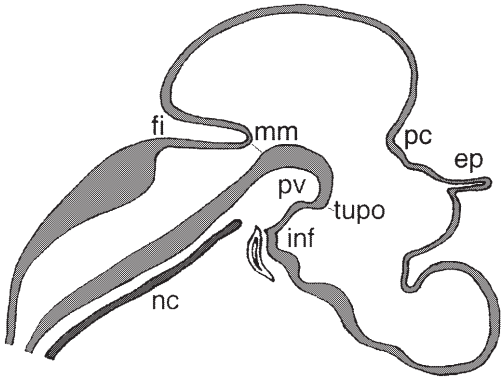


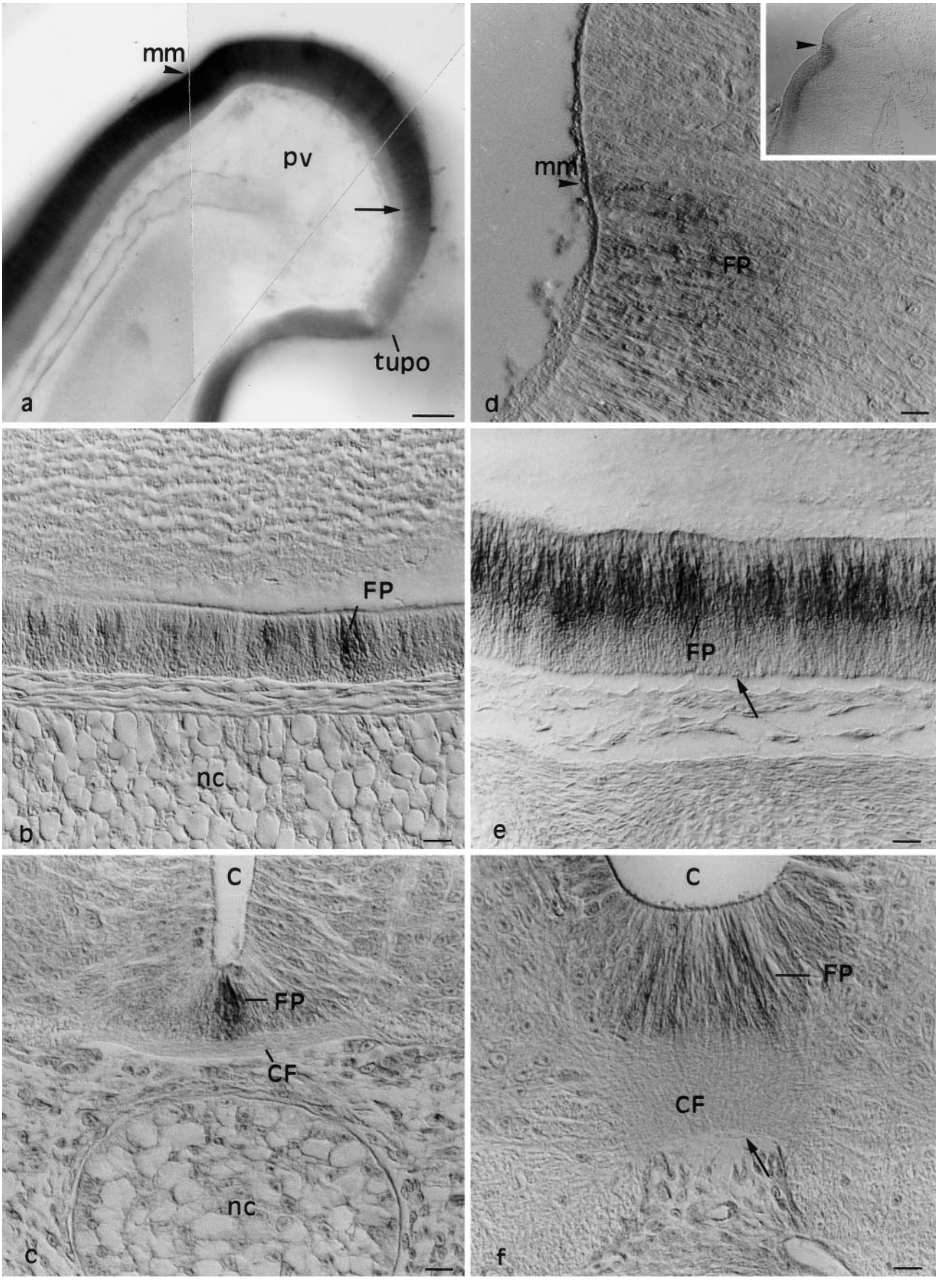
Fig. 7 Schematic drawing of a midsagittal section through the brain of a chick embryo in stage HH 27 (compiled from data in von Kupffer 1906; Henneberg 1937; Nieuwenhuys et al. 1998). The ventral di-mesencephalic border is localized just caudal to the tuberculum posterior. The mes-metencephalic border is situated after the curvature of the ventral flexure, just opposite to the fovea isthmi (*fi* fovea isthmi)

Xenopus

Spatial distribution

Anti-RS immunoreactive material is first detected in the ventral midline of the neural tube. Staining in the prosencephalon is found in an area of prechordal mesoderm (Figs. 5, 6 a). When the infundibulum is visible, the rostral border of the immunopositive reaction is characterized by the deepest point in the infundibular recess (Figs. 5, 6 a, b, d). While the lateral extension of the floor plate comprises only one or two cells in the spinal cord (Fig. 6 e), immunoreactive floor plate cells in the mesencephalon are present in the ventral third of the neural plate (Fig. 6 f). The floor plate cells show a characteristically alternating pattern that is a result of anti-RS reactive floor plate cells and unlabelled neural progenitors (Fig. 6 c). During development the maximal rostro-caudal immunoreactivity extends in the prospective diencephalon and throughout the mesencephalon, the rhombencephalon up into spinal cord. In the caudal trunk, however, stained floor plate cells are only scattered between non-immunoreactive cells. Finally, no significant cellular reaction is detectable in the tail region; only the extracellular immunoreactive material in central canal is visible where it forms the Reissner's fibre.

Fig. 6a–f Expression pattern of RS in the floor plate of *Xenopus* embryos. **a**, **b**, **d** Especially, the development in the prosencephalic region is illustrated at different times in sagittal paraffin sections. **a**, **b** The most rostral immunoreactive floor plate cells are localized in a prechordal region (*arrow*) at stage 20 (**a**). At stage 21 (**b**) the plica ventralis is more obvious. **c–f** Expression pattern of RS in stage 23. **d** Immunoreactivity in the floor plate is present in the area of the future infundibular recess (*arrow*) and more caudal as a continuous band of cells spanning the first two third of the spinal cord. **c** Detail at higher magnification of the spinal cord in **d** showing the bipolar character of the floor plate cells with an obviously apical process (*arrow*) enclosing unlabeled progenitor cells that have a large nucleus in a ventricular position (*asterisk*). The immunolabelling is more extensive in the apices. **e**, **f** Frontal sections demonstrating the different lateral floor plate extension. Whereas the lateral extension of the anti-RS immunoreactive floor plate consists of only one or a few cells in caudal regions (**e**), more rostrally, in the mesencephalon, there is a larger extension of floor plate-immunolabeling (**f**) (*C* primitive central canal/ventricular system, *CG* cement gland). *Bars* **a**, **b**, **d–f** 10 µm, **c** 5 µm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Temporal variations

The earliest expression of anti-RS immunoreactive material is detectable at the end of neurulation at about stage 20 (Fig. 6 a). However, this labelling is a transient pattern, which gradually becomes restricted rostrally and caudally during embryonic and larval periods. This restriction begins at the same time as the disolution of compact prechordal mesoderm about stage 23. At this stage the rostralmost immunoreaction extends to the floor of the infundibular recess (Figs. 5, 6 d). Until stage 43 the floor plate labelling retracts rostrally. Simultaneously, the caudal labelling decreases. After five days of development, the mesencephalic floor plate cells are strongly immunoreactive and show their original shape. At this stage the rhombencephalic floor plate is more developed, but shows only weak and diffuse immunoreactivity. Thus, the anti-RS immunoreactivity becomes restricted to the mes-metencephalic boundary (data not shown).

In the SCO anti-RS immunopositive cells first appear at stage 30 of embryonic development. These cells are the only source of RS secretion in the adult (data not shown).

Chick

Spatial distribution

At early embryonic stages the anti-RS immunolabelling of the floor plate extends throughout the mesencephalon, the rhombencephalon, and as far as to the end of the spinal cord (Fig. 1 b). The di-mesencephalic boundary represents the rostralmost appearance of RS expression in the floor plate (Figs. 7, 8 a). This rostral boundary retracts over the mesencephalon from stage 29 until stage 35. In older embryos the rostralmost RS expression in the floor plate is localized in the mes-metencephalic boundary (Fig. 8 d). The intensity of RS immunoreaction in this region is much higher than that of the floor plate more caudally. The anti-RS immunoreactive cells in the floor plate are radially orientated from their first appearance. Whereas the basal processes are connected with the external limiting membrane, they contact the ventricle surface apically (Fig. 8 b, c, e, f). In advanced developmental stages, the area between the central canal and the external limiting membrane is increased, resulting from additional commisural fibres. This distance is compensated by the extension of basal processes (compare Fig. 8 e, f). The immunolabelling is more extensive in the apices (Fig. 8 e, f). The embryonic CNS also shows differences in the lateral extension of immunolabelling. Up to stage 35 it is obvious that the immunopositive reaction has a larger lateral extension at the mes-metencephalic boundary than in the more caudal regions (data not shown).

Fig. 8 Expression pattern of RS in the central nervous system of chick embryos in stage HH27 (**a**–**c**) and HH 35 (**d**–**f**). **a** Midsagittal section of stage HH 27. The rostral RS-expression in the floor plate exceeds clearly the mes-metencephalic boundary (*arrowhead*) and starts in the di-mesencephalic area (*arrow*). **d** From stage HH 35 onwards the most rostral labeled floor plate cells are localized at the mes-metencephalic border (*arrowhead*) shown in an overview (*insert*) and in detail at greater magnification. **b**, **c**, **e**, **f** Comparision of immunoreactive floor plate cells in the spinal cord at different embryonic stages in sagittal (**b**, **e**) and frontal (**c**, **f**) sections. At stage HH 27 the cell bodies are localized near the external limiting membrane (**b**, **c**). Stage HH35 shows that the extensions of the basal processes compensate for the increasing distance (**e**, **f**) between the primitive central canal and the external limiting membrane (*arrow*). The radial orientation of immunoreactive floor plate cells becomes obviously in the frontal section (**c**, **f**). *Bars* **a** 200 µm, **b**–**f** 10 µm

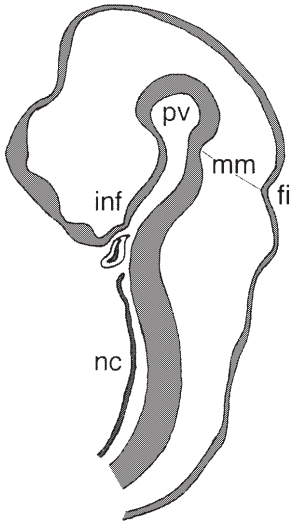


Fig. 9 Schematic drawing of a midsagittal brain section of rat embryo at E13 (compiled from data in McKanna et al. 1992). The ventral mes-metencephalic border is situated at the indentation opposite to the fovea isthmi (*fi* fovea isthmi)

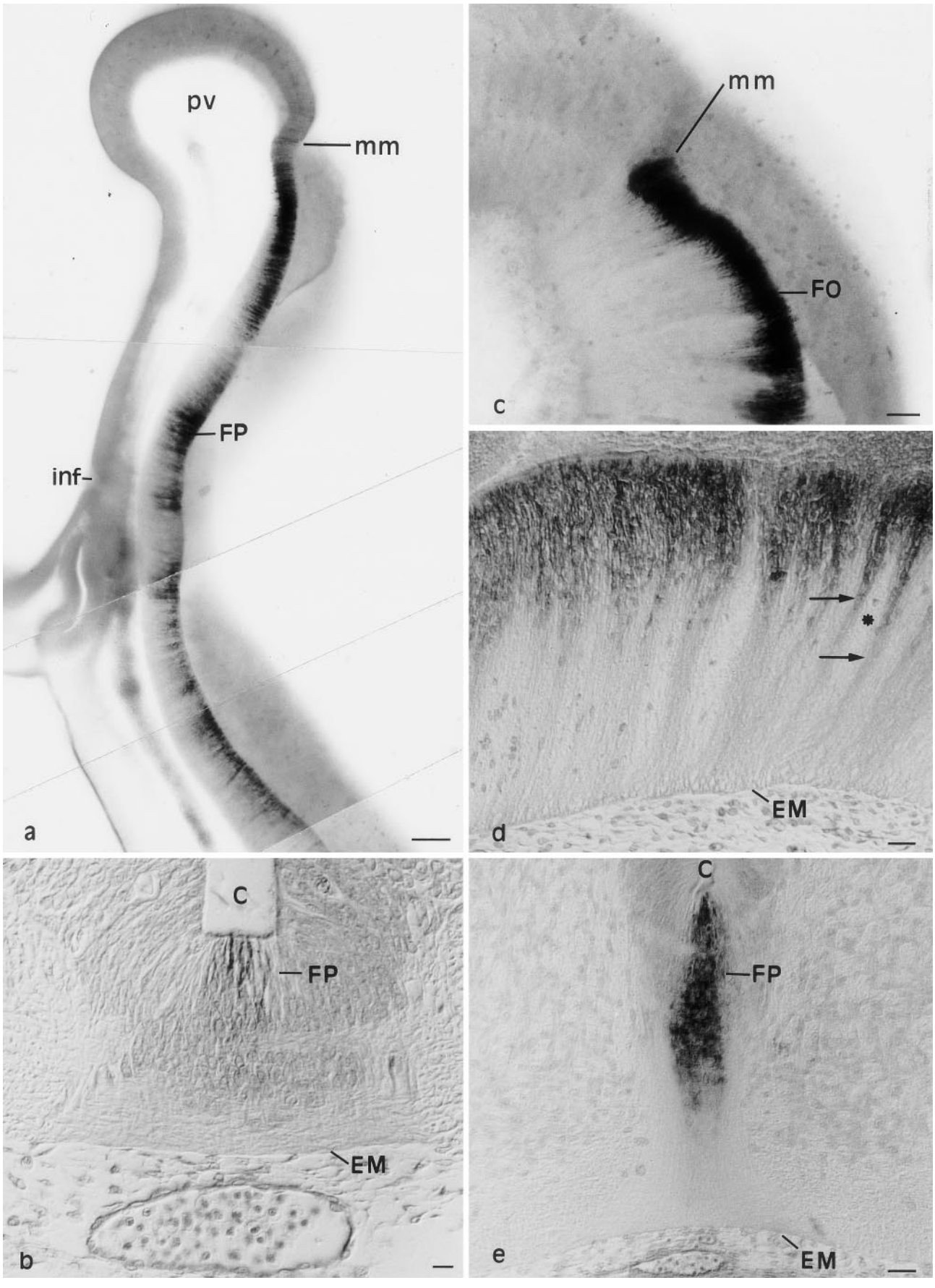
Temporal variations

Anti-RS immunolabelling is first detectable in the floor plate as well as in cells of the SCO at stage 18 (data not shown). From stage 35 until stage 41 the more caudally situated floor plate cells gradually decrease their RS expression while cells at the mes-metencephalic boundary express RS at an equally high level. Stage 43 reveals only a weak RS expression within the metencephalic floor plate, which disappears before stage 45. In the adult only the SCO immunolabelling remains (data not shown).

Rat

Spatial distribution

Floor plate cells of the rat begin to express anti-RS immunopositive material from about E13 (stage 84) onwards. The labelling starts immediately behind the mes-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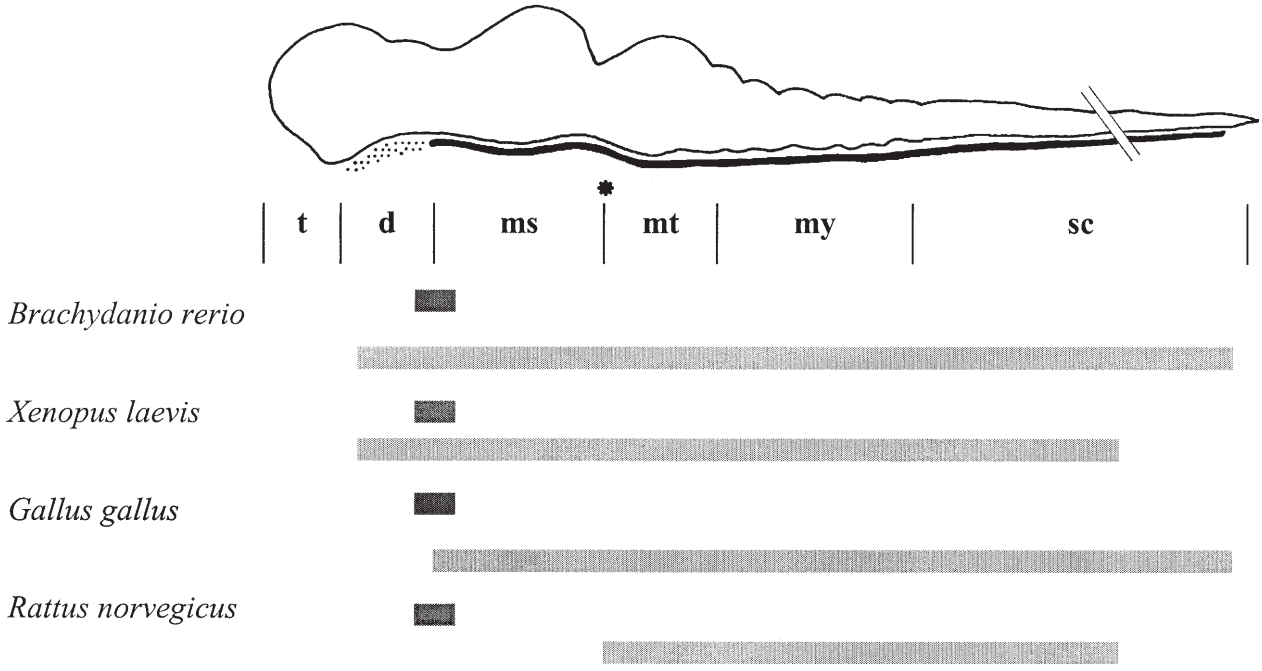

Fig. 11 Schematic organization of the early vertebrate neural tube and underlying axial mesoderm (*black* and *dotted* area). In addition to the secretory anti-RS reactive cells in the floor plate (*bright columns*) there is also an immunopositive glial complex in the roof plate at the border of the last diencephalic prosomere and mesencephalon (*dark columns*). The diagram compares the spatial RS expression between the four investigated species: zebrafish, *Xenopus*, chick and rat. Differences can be shown in the maximal rostro-caudal extension of anti-RS labelling in the floor plate of these four vertebrates. This labelling is a transient pattern that gradually becomes restricted rostrally as well as caudally during embryonic and larval periods to the mes-metencephalic boundary (isthmus region; *asterisk*), but is absent in the adults. Note that the floor plate extends to prechordal regions (*dotted* area) in zebrafish and *Xenopus*, but starts more caudally in chicken and rat. RS secretory activity of the SCO cells occurs later than in the floor plate, but is always present at the same dorsal position in all vertebrates. The SCO is also immunoreactive in the adult (*d* diencephalon, *ms* mesencephalon, *mt* metencephalon, *my* myelencephalon, *sc* spinal cord, *t* telencephalon)

Fig. 10a–e Expression pattern of RS in the floor plate of rat embryos shown in two characteristic developmental stages. **a** Mid-sagittal section of E13. The rostral limit of floor plate reacting with anti-RS was located in the isthmus region at the mes-metencephalic boundary. Caudally, it extends in the rhombencephalon and in the spinal cord. **b** Frontal section at E13 in a rhombencephalic region. **c**–**e** The highest level of anti-RS reactivity is observed at E16. **c** The floor plate in the isthmus region appears strongly reactive. **d** Higher magnification of the sagittal section (**c**) shows immunopositive cells forming a column-like pattern. This columns are formed by an alternating pattern of immunoreactive floor plate cells (*arrow*) and unlabeled neuronal tissue (*asterisk*). The RS is concentrated at the ventricular pole of the bipolar floor plate cells. **b**, **e** Frontal sections of E13 (**b**) and E16 (**e**) compare the distance between the central canal and the external limiting membrane in the rhombencephalon. The increasing radial extension is compensated by larger basal processes. There is a stronger floor plate labelling in E16. *Bars* **a** 200 µm, **b**, **c** 10 µm, **d**, **e** 20 µm

metencephalic boundary and extends caudally into the spinal cord (Figs. 9, 10 a, b). In comparison with staining in the spinal cord the immunoreactivity of the rhombencephalic region is more pronounced and here it is detected latest. No significant reaction is detectable more rostrally in the mesencephalon or diencephalon (Figs. 9, 10 a).

During development, the increasing radial extension of the tissue between the central canal and the external limiting membrane is compensated by longer basal processes of the floor plate cells (Fig. 10 d, e). The column-like association of anti-RS immunopositive cells (Fig. 10 c, d) is the result of increasing proliferation and/or migration of non-labelled cells in the ventral midline as well as of axonal crossing.

Temporal variations

Starting at E13, the highest level of anti-RS immunoreactivity in the floor plate is observed at E16 (stage 118; Fig. 10 c–e) before the staining decreases. In addition to this ventral pattern of RS expression, the first-labelled cells can be seen in the roof plate of the caudal diencephalon at E16 (stage 104), where its later forms the SCO. Whereas only diffuse immunoreactive material is labelled in the ventral midline after birth, these dorsal SCO cells retain their secretory activity (data not shown).

The RS expression pattern of the four investigated species in the stage of their widest rostro-caudal extension are summarized in Fig. 11, showing the differences in the spatial RS distribution.

Discussion

This study examines the temporal expression and spatial distribution of anti-RS immunoreactive material in the

central nervous systems of zebrafish, *Xenopus*, chicken and rat. We show that RS is expressed transiently in the developing floor plate of these representatives of the four major vertebrate classes.

Reissner's substance expression in the floor plate

Anti-RS immunolabeling is widely distributed in the ventral midline of the central nervous system. In chicken and zebrafish labelling is present throughout the whole floor plate, including the caudalmost part of the spinal cord. In the spinal cord of the zebrafish anti-RS immunoreactive cells form a neat median cell row that lies between the lumen of the neural tube dorsally and the notochord ventrally. They are identical to the floor plate cells described by Hatta (1992).

The floor plate of rat and *Xenopus* embryos lacks the strong RS expression in the caudal half of the spinal cord. This is in accord with Rodríguez et al. (1996) and Yulis et al. (1998), who also showed labelling with an anti-RS antiserum (code AFRU; Rodríguez et al. 1984). Various other molecules are also known to extend to different extents along the floor plate, and this varies also between species (Hamre et al. 1995). Both planar and vertical processes appear to have roles in region-specific patterning during differentiation of midline structures and neural tissue (Doniach 1993). We propose that floor plate migration during gastrulation could be inequal or that axial organizers have distinct properties remaining at different positions along the anterior-posterior axis in the four different vertebrates. This would explain the differences between the rostral and caudal expansion of anti-RS labelling in the investigated species.

Reissner's substance expression in the prosencephalon

Most apparent is the rostral localization of RS expression in regions of the prosencephalon. Following Kingsbury's conclusion (Kingsbury 1920) the floor plate is assumed to terminate at the isthmic fovea. Some authors agree with this floor plate extension up to the mes-metencephalic boundary because of the expression pattern of floor plate markers such as FP3, FP4 (Plazcek et al. 1993) and zn-5 (Hatta et al. 1991). Studies with anti-RS antisera code AFRU (Rodgríguez et al. 1996) and code K11 (our results) in embryonic rats also reveal this rostral floor plate boundary.

However, anti-RS labeling is detectable also more rostrally in the chicken mesencephalon as well as in the prospective diencephalon in the early stages of the zebrafish (15 h) and *Xenopus* (stage 20). The distribution of RS to these rostral midline regions was found with anatomical characteristics within the early brain. The expansion of the floor plate in prechordal regions is in accord with the model of Shimamura et al. (1995). They postulate the more rostral extension of the floor region extending beyond the anterior end of the notochord. It was shown by acetylcholinesterase staining of chicken that the floor plate ends at the caudal mammillary body (Puelles et al. 1987), which is localized in a prechordal region. Annexin IV expression in murine embryos also defines a floor plate region that extends from caudally in the spinal cord all the way rostralwards to the diencephalon (Hamre et al. 1996). Furthermore, the zebrafish mutations *cyclops* (Hatta et al. 1991; Brand et al. 1996), *one eyed pinhead* and *bozozok* (Schier et al. 1996) that affect the floor plate, have also been found to result in defects in the formation of the prechordal plate. At early stages, these mutant embryos do not express *shh/vhh-1*, which is a marker of notochord and floor plate (Roelink et al. 1994), and like NK2.2 (Barth and Wilson 1995) of the ventral prosencephalon. From this genetic study and also from transplantation experiments (Hatta et al. 1994), the prechordal ventral midline seems to be equivalent to the notochordal floor plate, which strongly suggests the continuity of floor plate properties into the ventral diencephalon (Hatta et al. 1994). The early rostral RS expression pattern in the zebrafish, *Xenopus* and the chicken also provides further arguments for this suggestion.

Reissner's substance expression in the mes-metencephalic region

A conspicuous anti-RS pattern appears in the mes-metencephalic region in the four investigated vertebrate species in later embryonic stages. This region corresponds to the FO described by Olsson (1956) in embryonic anamniote brains. Whereas the anti-RS immunoreaction disappears in the floor plate cells in the caudal metencephalon and myelencephalon as well as in the spinal cord and rostrally in the diencephalon, cells in the mes-metencephalic region show a lateral extension and strongly express RS. Especially in zebrafish and *Xenopus* the RS expression in this small area is very strong and can be observed after hatching during the larval period. Until now no other floor plate molecule has been described with these special restriction in the mes-metencephalic region as RS.

However, in contrast to Yulis et al. (1998), who showed a temporally earlier RS-expression in the mes-metencephalic region than in the other floor plate, the RS-immunoreactivity appeared simultaneously in the floor plate of all investigated species of our study. In the early stages a clear distinction between the floor plate cells along the body axis could not be made since they constitute a homogeneous cell population that form a continuous cell stripe. Thus, in our opinion the cell shape and the secretion of the FO in the mes-metencephalic region need not result from the specialization of these cells. On the contrary, these features could indicate that the floor plate cells in this region maintain their original status throughout a greater part of embryonic and larval development.

The mes-metencephalic boundary appears to be a central source of control, acting as an organizer in early developmental stages (Marin and Puelles 1994, Rétaux and Harris 1996). It is defined by the overlapping expression

of *Pax*-family genes (*Pax2*/*Pax-b*, *Pax5*, *Pax8*), *Wnt-1* and *Engrailed*. Furthermore, *FGF8*, that codes a secreted factor with midbrain-inducing and polarizing effects, is also expressed in this region (for review, see Ang 1996; Joyner 1996; Rétaux and Harris 1996). The gradual restriction of RS expression to the mes-metencephalic boundary could also reflect further complex differentiation processes in this region in later developmental stages.

The RS expression in the floor plate is a conserved molecular pattern in the chordate neural tube. RS secreting cells already form a neuronal landmark in cephalochordates (*Branchiostoma lanceolatum*) between the brain vesicle which appears to be homologous to the vertebrate diencephalon (Lacalli et al. 1994) and the more caudally located neuronal regions. Therefore, the infundibular organ in *Branchiostoma* (Olsson and Wingstrand 1954; Sterba et al. 1983; Olsson et al. 1994) and the FO in vertebrates are homologous structures, whereas the active secretory cells of the SCO form a new vertebrate landmark dorsally. It is an exciting problem to understand the molecular control of this, obviously original, embryonic pattern of RS in the floor plate and further on in the roof plate. It deals with the question of the lineage of this secretory radial glia.

Putative role of Reissner's substance within the floor plate

Solubilized proteins from bovine Reissner's fibre are able to interfere with the aggregation of neuronal cells and induce the outgrowth of processes in in vitro systems (Gobron et al. 1996; Monnerie et al. 1997). Therefore, these authors postulated a morphogenetic activity of the SCO/Reissner's fibre complex on cell populations in the developing CNS and on neuritic outgrowth. Although the compounds expressed in the floor plate and SCO share similar epitopes they are not absolutely identical (Rodriguez et al. 1996). We nevertheless presume that these secretions subserve similar functions. It has been suggested that Netrin-1a, the zebrafish homologue to chicken Netrin-1 (Serafini et al. 1994), which is expressed in a spatiotemporal correlation to extending tracts and commissures, is involved in guidance of growth cones (Lauderdale et al. 1997). Our results in the zebrafish demonstrate that RS expressed in a temporal-spatial pattern that suggests that it could also be involved in axonal decussation. This is supported by our observation that RS was localized in basal parts and endfeets of FO cells but also appeared to be present extracellularly in the area of commissural axons. Furthermore, as is shown both in the SCO and the FO these cells start their strong RS-expression temporally consistent with the formation of the posterior commissure (dorsal to the SCO) and the ventral tegmental commissure (ventral to the FO) at about 24 h of embryonic development (Chitnis and Kuwada 1990). Thus, RS could exert its effect as a secretion of the growing commissural fibres. Future in vitro assays may help to clarify the roles of RS in morphogenetic processes in the floor plate during vertebrate development.

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