SURFACE MARKERS OF REGIONALIZATION IN THE VERTEBRATE NERVOUS SYSTEM

Thesis by

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Dedication

I dedicate this thesis to my wonderful parents. Aai and Baba, this is for you, with love, for everything you did for me since those trying days of monsoons, mud and "me ha dagad chickhlaat budawoon budawoon...."

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ABSTRACT

In order to identify new molecules that might play a role in regional specification of the nervous system, we generated and characterized monoclonal antibodies (mAbs) that have positionally-restricted labeling patterns.

The FORSE-1 mAb was generated using a strategy designed to produce mAbs against neuronal cell surface antigens that might be regulated by regionally-restricted transcription factors in the developing central nervous system (CNS). FORSE-1 staining is enriched in the forebrain as compared to the rest of the CNS until E18. Between E11.5-E13.5, only certain areas of the forebrain are labeled. There is also a dorsoventrally-restricted region of labeling in the hindbrain and spinal cord. The mAb labels a large proteoglycan-like cell-surface antigen (>200 kD). The labeling pattern of FORSE-1 is conserved in various mammals and in chick.

To determine whether the FORSE-1 labeling pattern is similar to that of known transcription factors, the expression of BF-1 and Dlx-2 was compared with FORSE-1. There is a striking overlap between BF-1 and FORSE-1 in the telencephalon. In contrast, FORSE-1 and Dlx-2 have very different patterns of expression in the forebrain, suggesting that regulation by Dlx-2 alone cannot explain the distribution of FORSE-1. They do, however, share some sharp boundaries in the diencephalon. In addition, FORSE-1 identifies some previously unknown boundaries in the developing forebrain. Thus, FORSE-1 is a new cell surface marker that can be used to subdivide the embryonic forebrain into regions smaller than previously described, providing further complexity necessary for developmental patterning.

I also studied the expression of the cell surface protein CD9 in the developing and adult rat nervous system. CD9 is implicated in intercellular signaling and cell adhesion in the hematopoetic system. In the nervous system, CD9 may perform similar functions in early sympathetic ganglia, chromaffin cells, and motor neurons, all of which express the protein. The presence of CD9 on the surfaces of Schwann cells and axons at the appropriate time may allow the protein to participate in the cellular interactions involved in myelination.

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INTRODUCTION

A central issue in development is how complex, specialized structures are formed from a relatively simple early embryo. The vertebrate neural tube, an ectodermal derivative, gives rise to the entire central nervous system (CNS) and the neural crest. The latter gives rise to most of the peripheral nervous system except for the derivatives of the placodes. The anteroposterior and dorsoventral axes of the embryo are specified before neurulation. Key features of the neural tube develop according to these axes: the forebrain forms at the anteriormost end of the neural tube and the neural crest migrates from the dorsal margin of the tube. The signals that mediate neural induction as well as axis specification originate from the underlying mesoderm, and these signals are both planar and radial (Spemann, 1938; Sater et al., 1993). Following axis specification, further patterning is required so that the forebrain, midbrain, hindbrain and spinal cord are differentiated. Moreover, each of these areas are further subdivided into the various structures of the mature nervous system. In addition, cues are laid down to guide migrating cells and growing axons to their appropriate locations. Finally, the system must allow for relative changes in size and distance of a neural area with respect to its target during development, modification by activity-dependent mechanisms and, in some cases, recovery upon injury.

Many of the signals involved in these processes must be location-specific, and the molecules involved must therefore be spatially and temporally restricted in the developing CNS. In order to identify new molecules that have positional distributions appropriate for playing a role in some of these processes, we generated monoclonal antibodies (mAbs) using the cyclophosphamide immunosuppression method. My thesis is based on studies of two mAbs that recognize antigens with positionally-regulated epitopes in the developing nervous system. This Introduction reviews the literature pertaining to patterning of the nervous system and candidate molecules that may participate in this process from the earliest stages of development.

Early patterning in the vertebrate embryo: mesodermal induction

Based on work done primarily in amphibian embryos, the basic body plan is thought to be generated by inductive interactions between the animal

and vegetal regions of the embryo, the future ectoderm and endoderm, respectively. This is thought to be the earliest inductive event in the embryo. The equatorial zone of the blastula lying between these two regions forms the mesoderm (Nieuwkoop, 1969). Mesoderm-inducing activity can be assayed *in vitro*; explants of ectodermal or endodermal regions alone do not give rise to mesoderm, but endoderm can induce ectoderm to form mesoderm

A second inductive event occurs within the band of mesodermal cells at the equator that is regionalized such that dorsal mesoderm is present at the site of the organizer and ventral mesoderm is at the opposite side, on the equator. Dorsal mesoderm exerts a dorsalizing influence on the rest of the mesoderm. This effect diminishes with distance. Dorsal mesoderm gives rise to the entire craniocaudal extent of the notochord and part of the somite tissue, including muscle cells. Ventral mesoderm never contributes to the notochord; in isolation, it gives rise to little, if any somite tissue but forms largely mesothelium, mesenchyme and blood islands, the ventral mesodermal derivatives. Under the influence of the dorsal mesoderm of the organizer, however, ventral mesoderm can be dorsalized, in culture and *in vivo*, to the extent that it contributes to somites but not to notochord. Ventral mesoderm does not have a complementary ventralizing effect on dorsal mesoderm (Smith and Slack, 1983). Thus, there is a difference in the inductive capacity of the dorsal, organizer mesoderm and the ventral mesoderm, observed from the earliest stages of mesoderm induction. The basis of this special ability of the organizer remains to be understood, but a likely determinant is the dorsoventral polarity in the egg that is initiated with respect to the point of entry of the sperm and the axis of the first cleavage (Black and Gerhart, 1985), such that dorsal structures normally develop from the region opposite to the entry of the sperm (Nieuwkoop, 1969, 1977).

The dorsoventral differences in mesoderm arise from differences in the inductive ability of vegetal cells. On the organizer side of the blastula, dorsal vegetal cells specifically induce dorsal mesoderm and similarly on the opposite side, ventral vegetal cells induce ventral mesoderm. The inductive activities involved in these phenomena differ qualitatively: quantitative changes in the amount of inducing dorsal vegetal tissue will generate altered amounts of notochord-type mesoderm, but not ventral type mesoderm (Dale and Slack, 1987)

A variety of substances have been tested for their ability to induce mesodermal fate in ectodermal explants, significant amongst them being conditioned medium from a xenopus XTC cell line, XTC-CM (Smith, 1987), and members of the fibroblast growth factor (FGF), TGF-β and *Wnt* growth factor families. Kimelman and Kirchner (1987) showed that while basic FGF (bFGF) can induce muscle, a dorsal mesodermal derivative, it primarily induces ventral derivatives. The action of bFGF is potentiated by TGFβ1, a cytokine that does not have any effect by itself. Rosa et al. (1988) showed that TGFβ2 has dorsal mesoderm-inducing activity similar to XTC-CM. In addition, XTC-CM contains TGFβ-like activity in other assays that is partially blocked by antibodies against TGFβ2 but not TGFβ1. A candidate for an endogenous mesoderm inducing factor is the gene *Vg1*, a member of the TGF-β family whose transcript, of maternal origin, is localized in the vegetal cells after fertilization (Weeks and Melton, 1987). Other TGF-β family members, the activins, are potent mesoderm inducers, as are growth factors of the *Wnt* family (Sokol and Melton, 1992). The actions of these molecules are discussed in a later section.

Evidence for an *in vivo* role for activin was provided by Hemmati-Brivanlou and Melton (1992) in experiments involving injection of the message for a truncated, non-functional activin receptor. This treatment is thought to interfere with endogenous receptor function due to the formation of inactive receptor heterodimers, or due to competition with the endogenous receptor. The result with such truncated receptor injections is inhibition of mesoderm induction in the embryo and in explants, upon treatment with activin, but not with bFGF. Mesodermal induction is assayed by the upregulation of markers *Xwnt*, *goosecoid*, *X-bra*, and *Xhox-4*, which are not detectable in uninjected embryos treated with activin. These effects are reversed by coinjection of functional activin receptor mRNA. Surprisingly, the neurectoderm-specific marker NCAM is detected at high levels in truncated receptor-injected explants, demonstrating that neural-specific gene induction is not dependent on mesoderm formation. This result also suggests that activin must be inhibited for neural induction.

More recently identified molecules that may participate in these processes are other members of the TGF-β family, DVR-4, and *noggin*. DVR-4 can override the dorsalising effects of activin, giving rise to ventral type mesoderm (Jones et al., 1992), while *noggin* is a secreted factor expressed in the

organizer region, and can mimic the inductive properties of the organizer (Smith and Harland, 1992)

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Anteroposterior patterning in mesoderm and induction of neurectoderm

Steinbeisser et al. (1993) demonstrated that injection of *goosecoid* or activin mRNA into embryos produces a partial secondary axis consisting of structures caudal to the ear. Injection of *Xwnt-8* results in a complete secondary axis. Thus *goosecoid*, activin, and *Xwnt-8* are all capable of inducing dorsal-type mesoderm, but their actions differ with respect to the extent of the anteroposterior axis induced. It is significant that the rostral limits of *goosecoid* and activin-induced secondary axis structures coincide with the expression boundary of the rostral-most *Hox* genes. This raises the possibility that activin and *goosecoid* might be involved in initiating *Hox* gene expression.

Xhox-3 is an early mesodermal marker expressed in a graded manner with highest levels at the posterior end and is thought to be required for normal posterior development . Overexpression of *Xhox-3* interferes with differentiation of anterior structures, the region where the gene is normally expressed at its lowest levels. Blocking its function by injecting antibodies causes posterior (trunk and tail) abnormalities (Ruiz i Altaba and Melton, 1989; Ruiz i Altaba et al., 1991). bFGF induces high, posterior-like levels of *Xhox-3* in explanted animal caps, while XTC-MIF (Xenopus XTC cell line mesoderm inducing factor) induces lower, anterior-like levels of *Xhox-3*. When these induced mesodermal tissues are tested for their ability to induce specific secondary neural/epidermal structures, the explants treated with bFGF induce spinal cord and dorsal fin, giving a partial secondary axis in the resulting two-tailed embryo. Explants treated with XTC-MIF, however, induce brain and eyes in the partial secondary axis, resulting in two headed embryos (Ruiz i Altaba and Melton, 1989).

Planar versus radial signals

Experiments performed with "Keller" explants (Keller and Danilchik, 1988) show that the morphogentic movements associated with neurulation, neural induction, anteroposterior patterning and position-specific neural gene induction can be directed by planar signals alone (Doniach et al., 1992; Keller et al., 1992; Sater et al., 1993). In these explants, the involuting dorsal

mesoderm, which is responsible for neural induction, is not in vertical contact with the ectoderm, but rather connected to it at the posterior end. The anterior ends of the ectoderm and mesoderm point in opposite directions. Inductive signals can therefore only travel through the plane of the tissues to reach the ectoderm. This configuration permits not only neural induction, assayed by the upregulation of NCAM, but also position-specific expression of other molecules. In Keller explants, *En-2* is expressed in a location similar to its normal region of expression at the midbrain-hindbrain junction, *Krox-20* is seen in two bands just posterior to *En-2*, similar to its expression in rhombomere 3 and 5, and *Xlhbox 1* and *6* are detected in the spinal region. These genes are not induced in explants of dorsal ectoderm cultured without contact with dorsal mesoderm, showing that mesoderm is necessary for anteroposterior patterning in ectoderm. Furthermore, planar contact with dorsal mesoderm is sufficient to induce similar expression patterns of these three genes in ventral ectoderm, even though this region normally does not express these markers. (Doniach et al., 1992).

A particularly remarkable feature of planar induction is the distance at which it can be effective; neuronal differentiation is induced even at the distal tips of the explants, furthermost from the ectoderm-mesoderm junction. Planar explants do not, however, appear to induce anterior neural structures (other than rudimentary retinal tissue). In contrast, explants that permit only vertical but not planar signals between mesoderm and ectoderm induce less robust differentiation of neurons, but form well-developed anterior neural structures (Sater at al., 1993), suggesting that both planar and vertical signals act together to pattern the neural tube *in vivo*.

Dorsoventral patterning in the neural tube

From the earliest stages, the notochord is a powerful regulator of dorsoventral specification in the neural tube. The mediolateral axis of the neural plate prior to closure corresponds to the future dorsoventral axis of the neural tube. Thus the medial region of the neural plate is the location of the future floorplate, and progressively lateral regions of the neural plate form increasingly dorsal parts of the neural tube. Neural crest migration occurs from the dorsal-most folds of the neural tube, beginning just before neural tube closure.

Dodd, Jessell and co-workers have shown that the notochord is necessary and sufficient for floorplate induction. Notochord ablation results in neural tubes without floorplate, while transplantation of an additional notochord causes an extra floorplate in the region of the neural tube closest to the transplant. In these experiments, the presence of the floorplate is confirmed by the detection of the markers FP1 and FP2 (Yamada et al., 1991). In addition, motor neuron differentiation is regulated by these manipulations. In the absence of notochord and floorplate, motor neurons, identified by the expression of Islet-1 (a LIM homeodomain protein), fail to differentiate. While signals for floorplate induction are contact-mediated, motor neuron differentiation is induced by diffusable cues. Both types of signals originate initially from the notochord and later from floorplate cells themselves (Yamada et al., 1993). Different regions of the neural plate do not remain equally responsive to inductive signals, however. The competence of lateral neural plate cells to respond to floorplate-inducing signals from the floorplate declines with age. The absence of floorplate in the forebrain appears to be due to the lack of competence of rostral neural tube cells to respond to inductive signals, as well as to a lack of floorplate-inducing activity in the prechordal mesoderm that underlies the rostral CNS (Placzek et al., 1993).

The notochord influences gene expression in cells along the entire dorsoventral extent of the neural tube. Normally restricted to the alar plate, the expression domains of *dorsalin-1* and *Pax-3* expand ventrally upon notochord removal, and are compressed upon transplantation of an additional, ectopic notochord (Basler et al., 1993; Goulding et al., 1993). Furthermore, analysis of mouse mutants that lack all or parts of their notochord reveals altered expression of *Pax-1* and *Pax-3* in the somites. Similar defects are observed in mutants where the notochord forms but degenerates, suggesting that the notochord is important for both induction and maintenance of these genes (Dietrich et al., 1993).

Just as the notochord plays a role in the induction of ventral cell types in the neural tube, *dorsalin-1* is implicated in promoting the differentiation of dorsal derivatives such as the neural crest. *dorsalin-1* is a TGF-β family member expressed in the dorsal part of the neural tube after closure. It is also suggested to dorsoventrally limit the induction of ventral cell types such as motor neurons by the notochord and floorplate (Basler et al., 1993).

The notochord appears not to be the sole initiator of dorsoventral patterning in the neural tube, however. Examination of notochordless tadpoles and Keller explants that lack an underlying notochord reveals some aspects of dorsoventral differentiation to be induced by the cells of the notoplate (which lie at the ventral midline of the neural tube and normally give rise to floorplate in amphibians). In these studies, differentiation of Rohon-beard neurons, primary (but not secondary) motor neurons, as well as dorsoventrally restricted, bilateral expression of *Xash* (Xenopus *achaete-scute* homolog) is observed in the absence of an underlying notochord, presumably due to signals from the notoplate (Clarke et al., 1991; Ruiz i Altaba et al., 1992; Zimmerman et al., 1993).

Dorsoventrally-regulated molecules in the developing spinal cord (and evidence for their function)

A number of putative transcription factors and secreted molecules are expressed in dorsoventrally-restricted patterns in the developing spinal cord. Most of these have homologs or related family members in *Drosophila*, such as members of the *HoxB* family, *Pax3, 6,* and *7, HNF-3β/pintallavis, Dorsalin-1, Wnt-1* and *3,* and *shh* (*sonic hedgehog*). Of these, *shh* is a putative secreted molecule with a striking distribution that suggests it might be a floorplate-inducing factor. Homologs of *hh* have been implicated as patterning molecules in many systems. In *Drosophila, hh* is thought to participate in a positive feedback loop involved in specifying segment polarity. *hh,* presumably induced by *en* in the posterior half of each segment, signals the upregulation of *wg* and *dpp* in the anterior half; the latter are in turn necessary for *en* expression in the posterior half (Tabata et al., 1992). *hh* also participates in *Drosophila* wing patterning (Basler and Struhl, 1994).

In vertebrates, there is strong evidence that *shh* is a retinoic-acid induced morphogen or intermediary signaling molecule in the chick limb bud (Riddle et al., 1993). In the notochord, *shh* continues to be expressed after notochord-inducing activity has ended, but the temporal pattern of expression of *shh* in the floorplate suggests that it might be a floorplate-secreted inducing factor, acting on lateral floorplate cells as well as motor neurons (Echelard et al., 1993). In support of this is the evidence that COS cells expressing *vhh* (vertebrate homolog of *hh*) can induce floorplate and motorneurons in neural plate explants (Roelink et al., 1994). In addition,

transgenic mice that misexpress *shh* behind a *Wnt-1* enhancer upregulate the floorplate marker *HNF-3β* in ectopic locations in the hindbrain. In these animals, ectopic *shh* is not sufficient to induce floorplate in dorsal spinal cord. This may be due to the relatively late onset of transgene expression in this region (Echelard et al., 1993).

The role of *dorsalin-1* in specifying dorsal characteristics in the neural tube was discussed above. *Pax3, 6* and *7* are transcription factors containing a paired domain as well as a homeodomain. *Pax3* and *7* are expressed in the alar plate of the spinal cord, and *Pax3* is expressed in the roof plate as well (Goulding et al., 1991; Jostes et al., 1991). *Pax6* is present in the basal plate (Walther and Gruss, 1991). Homozygous mutations in *Pax3* (known as *splotch* in mice and Waardenberg's syndrome in humans) cause severe defects of neural tube closure and defects in pigmentation, the latter presumably due to defects in the neural crest (Epstein et al., 1991; Tassabehji et al.,1992; 1993). Mutations in *Pax6* have defects in the lens and nasal placodes (Hill et al., 1991).

Other dorsoventrally regulated molecules include the *Wnt* genes that are present in a restricted dorsal region of the spinal cord midbrain and forebrain (Roelink and Nusse, 1991), *Dbx* , expressed in bilateral stripes at the midline (Lu et al., 1992), and some genes of the *Hox* family that are expressed in temporally dynamic, dorsoventrally restricted patterns thought to correlate with the appearance of specific cell groups along that axis (Graham et al., 1991).

Patterning in the hindbrain: Segmentation and Hox genes

Segmentation is an elegant and economical means of establishing a body plan. Briefly, it involves setting up an iterated pattern of regulatory genes capable of directing the formation of repeated structures in each segment, after which each segment can be modified for local specializations. There is overwhelming evidence that invertebrate systems such as *Drosophila* are patterned in this manner, through the interactions of gap, pair-rule, segment polarity and segment identity genes (Ingham and Martinas-Arias, 1992, and references therein). These genes encode DNA-binding proteins that function as transcription factors. Segment identity genes are members of the *antennapedia* and *bithorax* complex (ANT-C and BX-C), and share closely related versions of a homeobox sequence encoding a 61 amino acid DNA-

binding homeodomain. Each of these transcription factors can activate a cascade of downstream events where they are expressed. Mis-expression of segment identity genes causes homeotic transformations in the ectopic regions of expression. The ANT-C and BX-C are also called collectively, the homeotic cluster (HOM-C) (McGinnis and Krumlauf, 1992).

Many *Drosophila* transcription factors are evolutionarily conserved. In particular, the HOM-C appears to have been expanded by duplications such that mammals have 4 homologous clusters called *HoxA, B, C* and *D* (Duboule and Dolle, 1989; Graham et al., 1989). Most of the members of the HOM-C are represented in each cluster, with several new members added during the course of evolution. These genes are expressed in striking patterns in the mammalian CNS suggesting a segmental basis for its patterning. Corresponding members in each cluster are called paralogs, and have very similar expression patterns, with some exceptions (Hunt et al., 1991). Within a cluster, each member has a sharp rostral limit of expression in the CNS, neural crest, paraxial mesoderm or ectoderm that coincides with a different axial level. There is a remarkable order in the arrangement of the genes in each cluster along the chromosome, which correlates with their rostral boundaries of expression such that the genes at the 3' end have the rostral-most limit of expression, with few exceptions. (McGinnis and Krumlauf, 1992). The patterns of expression of these genes is spatially and temporally complex (Hunt et al., 1991; Graham et al., 1991).

In the hindbrain, various *Hox* genes have rostral limits of expression that coincide with different rhombomere boundaries. Thus rhombomeres can be uniquely identified by a distinct combination of *Hox* gene expression (Wilkinson et al., 1989). Once formed, rhombomere boundaries appear to be barriers to cell mixing between adjacent rhombomeres (Fraser, 1990). This provides a means by which cells in a given rhombomere, once specified with a positional identity in terms of their pattern of gene expression, can be physically segregated from cells of a different identity. These groups of cells can then initiate segment-specific developmental programs. Neural crest cells arising from particular cranial rhombomeres appear to retain their positional specification and generate discrete peripheral structures accordingly (Wilkinson, 1993).

If *Hox* genes are essential for segmental identity, then interfering with their function should disrupt cell fate. Such alterations have been reported in

mice lacking functional *HoxA1, A3,* or *B4* genes. *Hox A1* knockout mice have disruptions in the hindbrain with greatly reduced rhombomeres 4 and 5 (r4 and r5), defects in neurogenic crest derivatives, as well as altered wiring of cranial nerves (Chisaka et al., 1992; Lufkin et al., 1991; Carpenter et al., 1993; Dolle et al., 1993). In *Hox A3* knockout mice the defects are restricted to cranial crest derivatives (Chisaka and Capecchi, 1991). Mice lacking functional *Hox B4* have homeotic transformations of cranial vertebrae, but apparently normal neural structures (Ramirez-Solis et al., 1993). It is significant that the mutant phenotypes do not show defects in all areas of expression of the normal *Hox* gene. Presumably, paralogs from the other clusters with similar expression patterns can make up for the absence of a given gene.

Regulation of *Hox* genes

Data about Hox gene regulation *in vivo* comes from work done using transgenic mice carrying constructs of a reporter, lac Z, fused to a putative *Hox* regulatory sequence. The finding that the enhancer sequences of *HoxB2* contain three putative Krox-20 binding sites prompted the generation of a transgenic mouse line carrying *HoxB2* regulatory sequences (Sham et al., 1993). Expression of this construct closely resembles the pattern of endogenous *Krox-20,* which is specific to r3 and r5. Further transgenic analysis using constructs with mutations in the Krox-20 binding sites of the *HoxB2* enhancer shows that these sites are required for expression of the reporter in r3 and r5. In addition, ectopically expressed *Krox-20* causes ectopic expression of the lac Z construct, showing that *Krox-20* is sufficient for *HoxB2* expression. The normal expression domain of *HoxB2* extends caudally from r3. This implies that different parts of the expression pattern of a given *Hox* gene may be regulated by different molecules (Sham et al., 1993).

A role for Retinoic acid

Retinoic acid is implicated an endogenous regulator of *Hox* gene expression. Embryonal carcinoma (EC) cell lines are useful model systems in which this phenomenon has been extensively studied. Derived from murine or human tumors, EC cells can be induced to express certain neuronal characteristics such as neurofilament proteins by agents like BUdR or retinoic acid. Only retinoic acid-induced differentiation causes the upregulation of a

large number of *Hox* gene family members. These genes are not detectable in the uninduced cells (Mavilio et al., 1988). Moreover, *Hox* genes are induced in the order in which they are located on the chromosome, with the 3' genes induced at low concentrations of retinoic acid (10nM), and progressively more 5' genes requiring high concentrations (10μM), while the most 5' members of the complex are not induced at all. In addition, the 3' genes have a faster time course for stable induction than the 5' genes (Simeone et al., 1990). This quantitative regulation suggests an *in vivo* mechanism by which a gradient of retinoic acid could generate the spatial pattern of *Hox* gene expression.

Embryos exposed to retinoic acid lack well-differentiated anterior structures. In mouse, *Xenopus* and zebrafish, retinoic acid exposure causes anteroposterior transformations in the CNS. In *Xenopus*, concentrations of retinoic acid similar to those found *in vivo* cause microcephaly associated with a dramatic reduction of the forebrain and midbrain, as well as a lack of eyes and nasal pits; ear formation and posterior CNS development is normal (Durston et al., 1989). Similar treatments reveal a concentration-dependent change in the expression patterns of *Xhox3* and serotonin, consistent with the formation of posterior cell types in the forebrain (Ruiz i Altaba and Jessell, 1991). In mouse embryos, retinoic acid treatment alters hindbrain segment identity, transforming specific segments into posterior counterparts. This effect is well demonstrated in transgenic mice which carry lacZ reporters driven by *HoxB1, B2* or *Krox-20*, regulatory elements. In all of these mice, r2 and r3 appear to be transformed into r4 and r5, respectively. This conclusion is supported by duplication of the r4 and r5-like expression patterns of these genes in such mice, once in the normal r4 and r5, and again in r2 and r3. There is also a change in the migration and outgrowth patterns of the neural crest and motor neurons in r2 and r3, which resemble those of r4 and r5 in the transgenic mice (Marshall et al., 1992).

Support for a role for retinoic acid during normal development *in vivo* comes from measurements of endogenous retinoic acid in amphibians; these levels are similar to those found in the chick limb bud, where retinoic acid is implicated in the patterning of the anteroposterior axis (Durston et al., 1989). Furthermore, retinoic acid receptor (RAR) and retinoid X receptor (RXR) isoforms, as well as cellular binding proteins for retinol and retinoic acid are present in a variety of embryonic tissues in different patterns, consistent with a complex embryonic response to exogenous or endogenous retinoic acid

(Denker et al., 1990; Gustafson et al., 1993; Maden et al., 1990; Ruberte et al., 1993; Vaessen et al., 1990). Most importantly, null mutant mice lacking RARγ display homeotic transformations of the rostral axial skeleton, demonstrating that this retinoic acid receptor is required for patterning in this region (Lohnes et al., 1993). Mice lacking RARα1 appear normal, however, suggesting overlapping functions and redundancy in the RARs (Li et al., 1993).

Specification of the Midbrain

The cerebellum, and the superior (tectum) and inferior colliculi are formed rostral to the hindbrain. Most of the data regarding the specification of these structures involves homologs of two *Drosophila* segment polarity genes, *wingless* and *engrailed*. The former is a putative secreted signaling molecule, and the latter a homeobox gene. In vertebrates, there is evidence that wingless homologs, called *Wnt* genes, as well as *En* genes are involved in midbrain specification. There are at least ten genes in the mouse *Wnt* family, and two in the *En* family.

Early expression of *Wnt-1* is restricted to the presumptive midbrain, similar to that of *En-1*, and also that of the later expressed *En-2*. *Wnt-1* gradually becomes restricted to a narrow ring just anterior to the midbrain-hindbrain junction. Transplants of this region induce ectopic *En* expression in chick, suggesting that *Wnt-1* may upregulate *En* expression in surrounding tissue (Bally-Cuif et al., 1992). Interestingly, this result is observed when the transplant is placed in pretectal or dorsal thalamic locations but not in the ventral thalamus or telencephalon, indicating a difference in responsiveness in the recipient regions to inductive signals (Martinez et al., 1991). In normal chick tecta, *En* expression is found in a caudorostral gradient at embryonic day 2. Ectopic tecta transplanted or induced anterior to the mesen-diencephalic border show a reverse *En* gradient, with both normal and ectopic tecta expressing low levels of *En* nearest the border. The closer the ectopic tectum is to this border, the weaker the expression of *En*. These results suggest that the mesen-diencephalic border contains *En*-repressing agents (Itasaki et al., 1991). One functional consequence of an altered *En* gradient is that the polarity of the retinotectal projection to the ectopic tectum matches the rostrocaudal orientation of the *En* gradient (Itasaki and Nakamura, 1992). At embryonic day 3, the tectum can no longer regulate its gradient of *En*

expression, and rotated tectal primordia retain their original gradient even after transplantation to a location that causes reversal at earlier stages (Itasaki et al., 1991). Retinotectal projections display corresponding polarities, matching the *En* gradient in both cases (Itasaki and Nakamura, 1992).

Mice homozygous for mutated *Wnt-1* have virtually no midbrain or cerebellum, while the rest of the animal appears normal (McMahon and Bradley, 1990). In contrast, mice that do not express *En*-2 display only subtle alterations in cerebellar foliation, suggesting functional redundancy between *En-1* and *En-2* in the midbrain (Joyner et al., 1991).

Regionalization of the forebrain

The forebrain is the only prechordal part of the CNS, meaning that the notochord terminates caudal to the forebrain, as do the somites, both of which play critical roles in patterning the rest of the CNS. Prechordal mesoderm underlying the forebrain, however, is thought to perform similar functions with respect to the forebrain. The forebrain is a very specialized, complex structure that cannot be obviously reduced to a set of iterated segments, since different parts of the forebrain have very different structures, patterns of axon outgrowth and cellular organization. On the other hand, there is morphological, cellular, and molecular evidence that the forebrain is divisible into separate areas (Herrick, 1910; Puelles, 1987; Simeone et al., 1992; Bulfone et al., 1993; Figdor and Stern, 1993; Fishell et al., 1993; Price, 1993). There are, however, several different interpretations regarding the arrangement of these units, whether these are true "segmental" areas, and even regarding which methods are best used to identify boundaries (Puelles, 1987; Bulfone et al., 1993; Fraser, 1993; Krumlauf, 1993; Puelles and Rubenstein, 1993). A central question is whether forebrain patterning is an extension of that in the rest of the CNS, or completely different from it. Does the forebrain develop as a set of "neuromeres,' a strategy that has precedence in the patterning of the hindbrain?

The telencephalon gives rise to neocortex, paleocortex and archicortex, which are formed from different regions of the paired telencephalic vesicles, as well as the basal ganglia, which arise from the basal telencephalon. The boundary between the vesicles and the basal telencephalon is also a barrier to cell migration (Fishell et al., 1993). The existence of this boundary is supported by differential expression of many genes. For example, genes of the

Dlx group are expressed in the basal telencephalon but not the telencephalic vesicles, while *BF-1* and *Otx1* show the opposite distribution (Puelles and Rubenstein, 1993). Within the telencephalic vesicles, some markers distinguish between prospective neocortex and archicortex (*BF-1, Otx-1;* FORSE-1; Puelles and Rubenstein, 1993; Tole and Patterson, 1994). None of the markers, however, display differential distributions within prospective neocortex.

A controversial issue is how the telencephalon is connected to the rest of the CNS (Puelles, 1987 and references therein). Figdor and Stern (1993) view the entire telencephalon as the rostral-most division of the CNS, while Bulfone et al. (1993) model it as a dorsal extension of the hypothalamus, the rostral-most part of the CNS being part hypothalamic and part telencephalic. At present, it is difficult to decide between these models.

The diencephalon is somewhat easier to subdivide, as there are morphological features such as identifiable sulci, ridges, zones of reduced cell density and axon tracts that separate various regions. There is still no consensus, however, on how many divisions exist. The classical view of Herrick (1910) identifies four regions called the epithalamus, dorsal thalamus, ventral thalamus, and hypothalamus. More recent models have proposed either two divisions (D2 and D1; Figdor and Stern, 1993), or five divisions (prosomeres p2-p6; Bulfone et al., 1993), of the same diencephalic areas. Herrick's epithalamus and dorsal thalamus are contained within p2 of Bulfone et al., and within D2 of Figdor and Stern. Herrick's ventral thalamus corresponds to p3 of Bulfone et al., and together with the hypothalamus, corresponds to D1 of Figdor and Stern . Herrick's hypothalamus is further divided into p4-p6 (as is the telencephalon, viewed as a dorsal extension of the hypothalamus) in the model of Bulfone et al. In addition, Bulfone et al. propose additional boundaries perpendicular to those mentioned above, so that in their model the diencephalon is patterned in a grid-like manner (see schematics in Puelles and Rubenstein, 1993). It is important to note that Herrick used morphological sulci as his dividing boundaries, while Figdor and Stern tested these boundaries for cellular mixing across them, and retained those that served as barriers to migrating cells. Bulfone et al. use molecular differences to distinguish between areas, and observe that in some cases, molecular boundaries do not correspond exactly to sulci, but to nearby

ridges, and hence they question the appropriateness of using sulci as valid boundaries.

It is clear that the complexity of molecular patterns in the developing diencephalon exceeds the data on cellular mixing at this point. Many of the molecules described as regionally-restricted within the developing forebrain are transcription factors belonging to the homeobox family (*Otx1,2; Emx1,2; Dbx; Gbx; Dlx1,2,3,4; Nkx 2.1, 2.2)* or the *HNF-3/forkhead* family (*BF-1*) (Rubenstein and Puelles, 1994). Additional restricted distributions in early CNS development have been reported for the cytoplasmic antigen PC3.1 (Arimatsu et al., 1992), *Wnt* family secreted factors (Salinas and Nusse, 1992), and cell-surface antigen FORSE-1 (Stainier at al, 1991; Tole et al., 1994; Tole and Patterson, 1994).

The molecular data is promising in that it points to very early differences in gene expression in the proliferative neuroepithelium. These data suggest that the differences in transcription can provide the basis for the implementation of area-specific developmental programs. Support for this hypothesis awaits data from perturbation experiments.

Functional domains in the brain: cortical prespecification versus innervation-dependent identity

The cortex is divided into distinct, functionally specialized areas, which differ in their intra-cortical and subcortical connections as well as in cytoarchitectonic features. These regions are thought to be specified by a combination of genetic and epigenetic events. In the prespecification hypothesis, the neuroepithelium contains a fate map, the "protomap," which can be propagated to the cortical plate by neurons that migrate in a manner that maintains the positional information of the map (Rakic, 1988). In the epigenetic hypothesis, the cortical plate is specified by afferents (O'Leary, 1989). There is evidence for both the above hypotheses, in different regions of the cortex.

The best example of cortical prespecification is the early commitment of neurons to limbic cortex. LAMP (Limbic-system associated membrane protein), a 64-68 kD protein, is expressed specifically in migrating and postmitotic cortical plate neurons of limbic cortical areas. Transplantation studies show that neurons in prospective limbic areas have an early plastic period, after which they are committed to expressing LAMP even upon

transplantation to LAMP-negative locations (Barbe and Levitt, 1991). Such ectopic LAMP-positive neurons receive limbic thalamic projections in their new location, whereas transplants performed before they are committed to expressing LAMP do not receive such inputs (Barbe and Levitt, 1992; Levitt et al., 1993).

Within the neocortex, there is evidence that subplate neurons play a role in the innervation of cortical areas. Prior to invading the cortical plate, thalamic afferents accumulate and "wait" in the region of the subplate. Ablation of visual subplate neurons at the onset of the waiting period causes axons from the lateral geniculate nucleus to grow past their target cortical area in a anomalous route. Similar results were seen for innervation of auditory cortex by afferents from the medial geniculate nucleus upon ablation of auditory subplate (Ghosh and Shatz, 1993).

There are some examples of innervation-defined specializations within neocortical areas, such as ocular dominance columns in layer IV of visual cortex, and vibrissae-associated "barrels" in somatosensory cortex. These patterns are thought to be generated by activity-dependent competition between innervating axons, and can be modified by weakening, ablating, or blocking activity in some of the inputs, which causes these inputs to lose cortical territory to the stronger inputs (Hubel et al., 1977; Chapman et al., 1986; Reiter and Stryker, 1988). In addition, neocortical areas appear to be plastic to the extent of being able to accept totally abnormal inputs: visual cortex can form "barrels" when transplanted in the location of somatosensory cortex (Schlaggar and O'Leary, 1991); auditory cortex can process misrouted visual inputs (Sur et al., 1988) and can even show some stimulus-preference characteristics of normal visual cortical neurons (Roe et al., 1992).

Such studies show that neocortex is remarkably plastic, and support the hypothesis that thalamic innervation plays a significant role in specifying the identity of uncommitted neocortical areas. On the other hand, evidence suggestive of intrinsic differences within the prospective neocortex comes from the work of Arimatsu et al. (1992), who showed that E13 rat dorsomedial neuroepithelium can be distinguished from lateral neuroepithelium by differential expression of the PC3.1 antigen. The first evidence of intrinsic, early differences between functionally distinct neocortical areas comes from the analysis of a transgenic mouse line, H-2Z, which contains a construct of regulatory elements from a major histocompatibility complex class I gene

linked to lac Z. In these mice, the blue X-gal product is found specifically in layer IV of somatosensory cortex in a striking pattern that corresponds to specialized formations like barrels, and also in somatosensory areas that represent other body regions. The ability to express the transgene is retained by somatosensory tissue upon transplantation to other, non-expressing areas, even when the transplantation is performed at ages prior to thalamic innervation of the transplanted tissue. Other, non-expressing cortical explants do not upregulate the transgene upon transplantation into somatosensory cortex (Cohen-Tannoudji et al., 1994). These studies provide the first molecular evidence of regionalization within neocortical areas.

To summarize, there is evidence supporting prespecification of different types of cortex. Within neocortex, while most of the evidence supports thalamic innervation-dependent specification of areal identity, some early intrinsic differences between areas are suggestive of a role for prespecification of the neocortex as well.

Future directions

In this introduction, I have reviewed the patterning of the vertebrate nervous system from the earliest stages. Much is known about the complexity of regionalization at different developmental time points. However, while some of the molecules likely to be involved are beginning to be identified, the mechanisms by which regional specification occurs have yet to be unraveled. How are complex patterns of putative regional identity genes generated from simple early patterning cues? How does expression of a specific regulatory gene lead to formation of unique structural features, and what are the intermediaries involved? For example, while there are many transcription factors whose expression patterns distinguish between presumptive cortex and basal telencephalic derivatives, how is the appropriate area programmed to generate a layered cortical plate? Elucidating these mechanisms is critical to further our understanding of how the brain is built.

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Chapter 1

FORSE-1: A Positionally-Regulated Epitope in the Developing Rat Central Nervous System

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Abstract

We designed a protocol to identify cell surface molecules expressed in restricted spatial patterns in the developing central nervous system (CNS) that might be regulated by regionally-restricted transcription factors. The immunogen was a membrane fraction from NT2/D1 embryocarcinoma cells that were induced to differentiate into neurons and up-regulate Hox gene expression in response to retinoic acid. One monoclonal antibody (mAb), FORSE-1, specifically labels the rostral rat CNS from the earliest stages. Staining is observed in the rostral but not caudal neural folds of the embryo prior to neural tube closure. Staining is enriched in the forebrain as compared to the rest of the central nervous system (CNS), until E18. Between E11.5-E13.5, only certain areas of the telencephalon and diencephalon are labeled. Later, up to E17.5, FORSE-1 labeling is specifically restricted to the telencephalon, where a correlation with mitotic activity is apparent; the ventricular zone labels with FORSE-1, while the cortical plate is negative. The staining of the neuroepithelium is intensified by acetone fixation, which also reveals, between E11.5 and E13.5, a dorsoventrally-restricted, FORSE-1-positive region of the spinal cord. After E18, the entire CNS is labeled, through adulthood. The mAb labels the surfaces of dissociated, living cells. Other, non-CNS areas of FORSE-1 labeling are nasal and otic placodes, nasal epithelium, nasal glands, and early (E9.5-10.5) endoderm. mAb FORSE-1 recognizes an epitope present on both a high molecular weight (>200 kD) proteoglycan from embryonic and early postnatal brain, and on a 80 kD doublet that is restricted to the CNS in the adult.

These findings suggest the FORSE-1 antigen as a candidate cell surface molecule for mediating regional specification from the earliest stages of CNS development.

Keywords: forebrain, cyclophosphamide immunosuppression, dorsoventral, position, proteoglycan, NT2/D1, rostrocaudal

Introduction

The development of the vertebrate central nervous system (CNS) is a complex process during which the neural tube gives rise to a variety of different neural structures. A critical issue is how these differences are generated. Transcription factors with positionally restricted patterns could specify regional identity and their actions could be expanded upon by other, downstream target genes. Of these downstream effectors, those that are on the cell surface would be capable of mediating intercellular interactions and axon guidance (Wilson et al., 1993). In the hindbrain, for example, the positions of the transient folds of the rhombomeres are correlated with the rostral limits of expression of several HoxB genes (Bogarad et al., 1989; Frohman et al., 1990; Graham et al., 1988; Hogan et al., 1988; Hunt et al., 1991; Krumlauf et al., 1987; Schughart et al., 1988; Vogels et al., 1990). The spatial overlap of these genes suggested a role in defining axial position and cell identity, and disruption of Hox gene expression leads to alterations in cell migration and fate (Chisaka and Cappechi 1991; Chisaka et al., 1992; Lufkin et al., 1991). Limited information is available concerning candidate downstream molecules. Hox 2.4 and 2.5 differentially regulate, *in vitro*, a construct containing the promotor of NCAM (Jones et al., 1992), and NCAM is enriched at rhombomere boundaries. Antigens bearing the HNK-1 epitope are also specifically present in odd-numbered rhombomeres in the hindbrain (Kuratani, 1991). These and other surface/extracellular molecules could play a role in giving each rhombomere a unique identity.

Regional specification of the developing forebrain, in contrast, is complicated by the fact that different areas have very diverse architectonic characteristics. In the mammalian forebrain, the olfactory bulb consists of 3-layered paleocortex; in the telencephalon, the medial walls give rise to 1-layered archicortex, the lateral walls give rise to 6-layered neocortex, and the basal telencephalon forms the striatum; the diencephalon gives rise to thalamic structures. Thus, these various regions may not represent simple modifications of iterated segmental domains.

While the Hox genes are not expressed rostral to the hindbrain, homeobox-containing and other classes of transcription factors have recently been found to overlap in interesting patterns in the developing forebrain. BF-1, Dlx-1, Dlx-2 , and TTF-1 are expressed in regions of the olfactory bulb and telencephalon (Tao and Lai, 1992; Price et al., 1991; Porteus et al., 1991; Robinson et al., 1991; Lazzaro et al., 1990); Dlx-1, Nkx2.2, and TTF-1 demarcate overlapping regions in the diencephalon (Price et al., 1992); Otx-1, Otx-2, Emx-1, Emx-2 are expressed in nested domains extending

from the forebrain to the hindbrain (Simeone et al., 1992); MASH-1 is expressed in a subpopulation of neural precursors in spatially restricted domains in the CNS (Lo et al., 1991). These various patterns are suggestive of a role in defining regional identity, and some of their boundaries are consistent with proposed rostrocaudal and dorsoventral domains in the forebrain (Bulfone et al, 1993). However, little is known about the downstream targets of these genes. It seems likely that critical intercellular interactions in the developing forebrain will involve secreted, cell surface and extracellular matrix molecules.

In order to identify cell surface molecules that are expressed in restricted distributions during CNS development and that may be responsive to position-specifying transcription factors, we generated monoclonal antibodies (mAbs) against the membrane fraction of retinoic acid-induced, human embryocarcinoma (NT2D1) cells. In response to retinoic acid, these cells express neuronal differentiation markers as well as certain Hox genes (Andrews, 1984, Mavilio et al., 1988, Simeone at al, 1990). We reasoned that neuronal differentiation, coupled with specific Hox gene expression, could result in the production of downstream, cell surface antigens with interesting positional distributions. We used cyclophosphamide immunosuppression (Matthew and Patterson, 1983; Matthew and Sandrock, 1987; Ou et al., 1991) against the membranes of uninduced NT2D1 cells to bias the immune response in favor of novel membrane antigens that are induced by retinoic acid treatment.

One mAb generated from this fusion, FORSE-1 (forebrain-surface-embryonic), labels the embryonic rat forebrain very specifically. Within the forebrain, FORSE-1 has a restricted staining pattern that demarcates subregions of the telencephalon and diencephalon that have not been previously described. In this paper, we describe the immunohistochemical labeling pattern of FORSE-1 during development and the biochemical characterization of the antigens recognized by the mAb.

MATERIALS AND METHODS

Cell Culture

Clone D1 of the NTERA-2 (referred to as NT2/D1) human EC cells were obtained from P. W. Andrews (The Wistar Institute of Anatomy and Biology). NT2/D1 cells were maintained at high density in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum . Cultures to be

exposed to retinoic acid (RA) were established by plating a trypsinized single cell suspension of undifferentiated NT2/D1 cells at a density of 10^6 cells per 75 cm^2 tissue culture flask (Andrews, et al., 1984 and Andrews, 1984). RA (10 mM solution in dimethyl sulfoxide, all-trans; Sigma chemicals) was added to a final concentration of 10^{-5}M. The medium was changed with fresh medium containing RA every 48 hr. Differentiation was monitored by morphological changes in the cultures, and by the proportion of cells positive for the glycolipid surface marker, A2B5 (Andrews et al., 1990). The RA-induced immunogen (see below) was generated from cells that had been exposed to 10^{-5}M RA for two weeks.

Preparation of the immunogen

Undifferentiated and RA-induced NT2/D1 cells were harvested by rolling 3mm diameter glass beads (Andrews, personal communication) over the surface of confluent cell layers, in a few ml of medium. The intact cells were then centrifuged at 1000 rpm for 10 min. in an IEC HN-SII tabletop centrifuge. The cell pellets were then resuspended in homogenization buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 2mM EGTA) containing five protease inhibitors: 1mM phenylmethylsulfonylfluoride, 20µg/ml aprotinin, 20µg/ml turkey egg white trypsin inhibitor, 2 mM benzamidine, and 5 mM N-ethylmaleimide, and homogenized with a Polytron (Brinkman Instruments Co.). The homogenates were centrifuged at 1000 rpm, as described above, to remove nuclei and cell debris. The resulting supernatants were then centrifuged at 100,000 x g for 1hr in a Beckman 70 Ti rotor. The pellets were resuspended in homogenization buffer and washed twice by centrifugation at 100,000 x g for 1 hr and subsequent resuspension. The final pellets were resuspended in homogenization buffer. This material, which will be referred to as the membrane/cytoskeletal fraction was either used immediately or frozen at -80°C until further use. All procedures were carried out at 4°C. Protein was determined by the method of Lowry et al. (1951) or by the BCA protein assay (Pierce Chemical Co.).

Immunization and mAb production

Balb/c mice were injected with either undifferentiated NT2/D1 membrane/cytoskeletal fractions (RA (-))or RA-induced NT2/D1 membrane/cytoskeletal fractions (RA(+)) according to the schedule described below. The myeloma cells used for the fusion were HL1-653 (Taggart and Samloff, 1983).

Day 1: 300 μg RA(-) plus 0.2 ml RIBI adjuvant (RIBI Immunochem)
plus cyclophosphamide (100 mg/kg body weight) (i.p.)
Day 2: Cyclophosphamide only (i.p.), same dose.
Day 3: Cyclophosphamide only (i.p.), same dose.
Day 8: Serum screening
Day 14: 300 μg RA(+) plus 0.2 ml RIBI adjuvant (i.p.)
Day 22: Serum screening
Day 23: Boost #2 mouse with100 μg RA(+) (intrasplenic injection)
Day 26: Fuse #2 mouse. 1.65×10^8 spenocytes were collected and fused with 6.2×10^7 HL1-653 myeloma cells (Kohler and Milstein, 1975). Cells were plated at 2.2×10^5 cells/well.

Serum screening results (days 8 and 22): Sera samples were tested against the RA (-) and/or RA(+) membrane/cytoskeletal preparations on dot blots.

Day 8 screen	RA(-) titer	RA(+) titer
Control	1:500	
Exp #1	<1:50	
Exp #2	<1:50	
Day 22 screen		
Control	1:5000	1:5000
Exp #1	1:100	1:100
Exp #2	1:250	1:500

The control mouse received the antigen injection without cyclophosphamide.
Exp#1, #2: experimental mice received the antigen injection plus cyclophosphamide.
The Exp #2 mouse was selected for fusion because it showed a difference in titer against the RA(+) versus the RA(-) preparations.

Immunohistochemistry

Pregnant female Sprague Dawley rats were obtained from Simonsen laboratories, Gilroy, CA. The morning after the day of mating is designated as day 0.5. Whole embryos, embryonic, and adult tissues were directly frozen without fixation in O.C.T. (Tissue-tek) , on dry ice. Sections (10 μm) were cut using a Bright

cryostat (Hacker Instruments, Inc.), and placed on gelatin-coated slides. After air drying, the sections were either stained immediately or stored at -80°C. Acetone fixation, when performed, was carried out using acetone for 10 minutes, 0°C, prior to the staining procedure given below.

The sections were preadsorbed with 2% goat serum in PBS, and incubated in primary antibody (hybridoma supernatant) for 1 hour at room temperature (RT), washed twice with PBS, and then incubated in the secondary antibody that was either FITC-conjugated (FITC-goat anti-mouse IgM, Tago Inc., used at 1:100 in PBS) or biotinylated (Vector Mouse IgM ABC-peroxidase kit). In the latter case, the Vector ABC procedure was followed without blocking endogenous peroxidase, using a negative control (no primary antibody) to assess background staining.

For BrdU labeling, cultures were stained live for FORSE-1, similar to the procedure for sections, using FITC-labeled secondary antibody, and then, after fixation, were stained for BrdU. The fixation consisted of 70% ethanol, 30 minutes, at -20°, followed by PBS washes, and then 2N HCl, 10 minutes, at room temperature. After PBS washes, and treatment with 0.1M $Na_2B_4O_7.10H_2O$ for 10 minutes at room temperature, the staining procedure for BrdU was begun, similar to that used for sections. Anti BrdU antibody (DAKO #M744) was used at 1:20, and Texas red labeled goat-anti mouse IgG (Southern Biotech) was used as the secondary antibody. FITC and Texas red-labeled slides were mounted in glycerol containing n-propyl gallate (8mg/ml final concentration, dissolved in 0.1M Tris-HCl pH9). Peroxidase-labeled slides were mounted in Aquamount (BDH limited, Poole, England). Slides were observed and photographed using a Zeiss ICM 405 microscope. Kodak Tri-X Pan 400 film was used for fluorescence pictures, and Kodak Plus-X Pan 125 or Kodak Gold 100 film for bright field pictures.

BrdU incorporation

E16 telencephalic lobes were dissected in Ca^{++}/Mg^{++} free Hanks-A solution, and incubated in 1 mg/ml collagenase (Worthington Biochemical Corp.) in Hanks-A for 1 hour at 37°C. The enzyme was then blocked by the addition of serum. Cells were washed free of collagenase by pelleting at 1000rpm in a table top centrifuge and resuspending in culture medium (repeated twice). Undissociated clumps were broken up by gentle trituration using a flame-polished Pasteur pipette that had been preadsorbed with serum. Cells were plated on polylysine-laminin-coated tissue culture dishes (Corning, 35 mm). L15 CO2 medium (Hawrot and Patterson,

1979) supplemented with 10% FBS was used as the tissue culture medium. BrdU was added to a final concentration of 10mM. Cells were cultured for 16 hours.

Preparation of tissue fractions

Tissues from various age rat embryos, postnatal and adult rats were dissected and immediately frozen on dry ice. The postnatal and adult rats were killed by asphyxiation with carbon dioxide. Upon thawing, tissues were homogenized by hand or by using a Polytron (Brinkman Instruments Co.) in homogenization buffer. The homogenates were centrifuged at 1000 rpm for 10 min., to remove nuclei and cell debris. The supernatants were centrifuged at 100,000 x g for 1 hr. The supernatants resulting from this centrifugation were either used immediately or frozen at -80°C, and will be referred to as the soluble fractions. Pellets were resuspended in homogenization buffer and washed twice by centrifugation at 100,000 x g for 1 hr. The final pellets were resuspended in homogenization buffer. These preparations, which will be referred to as the membrane/cytoskeletal fractions, were either used immediately or frozen at -80°C. In some cases, the membrane/cytoskeletal fractions were also solubilized in non-ionic detergent. This was done by first recentrifuging these fractions at 100,000 x g for 1 hr. The pellets were then thoroughly resuspended by homogenization in homogenization buffer containing 1% Triton X-100 or NP40, and centrifuged again at 100,000 x g for 1 hr. The resulting supernatants contain the non-ionic detergent-solubilized proteins.

Enzymatic Digestions

Soluble fractions of embryonic, early postnatal, and adult CNS tissues solubilized with 1% NP40 were directly digested with the following glycosidases: chondroitinase ABC (2.0 U/ml, from proteus vulgaris; Sigma), hyaluronidase (500 U/ml, from leeches; Sigma), heparinase I (100 U/ml, from flavobacterium heparinum; Sigma), keratanase (2.5 U/ml, from pseudomonas species; Sigma), Endoglycosidase H (0.5 U/ml, recombinant E. Coli; Boehringer Mannheim), Neuraminidase (0.2 U/ml, from vibrio cholerae; Boehringer Mannheim), N-glycosidase F (4.0 U/ml, from flavobacterium meningosepticum; Boehringer Mannheim). In each case, approximately 100 µg of protein in the homogenization buffer described above (including protease inhibitors) was digested for 12-16 hr at 37° C. As controls, identical protein samples were treated in exactly the same way, but did not include enzyme. At the end of the incubation period all samples were diluted 1:1 with 2X SDS-PAGE reducing sample buffer (20% glycerol, 4% SDS, 0.13

M Tris, 2% β-mercaptoethanol, and 0.01 bromophenol blue (pH 6.8), to terminate the digests.

Electrophoresis and Immunoblotting

One-dimensional SDS-PAGE was performed as described by Laemmli (1970) in 1.5-mm-thick, 7% polyacrylamide slab gels. All samples were diluted 1:1 with 2X nonreducing or reducing sample buffer (see above) prior to loading. Nonreducing sample buffer is identical to reducing sample buffer, but does not contain b-mercaptoethanol. Immunoblots were performed as described by Kaprielian and Patterson (1993), with the modifications listed below. First, proteins present within the resolving as well as the stacking portions of the gels were routinely transferred to nitrocellulose. Primary antibody incubations were for 5 hr at room temperature or 12-16 hr at 4°C, with either mAb FORSE-1 or mAb 52G9 (undiluted hybridoma supernatant). The nitrocellulose filters were incubated with goat anti-mouse IgM affinity-purified antisera conjugated to peroxidase (Chemicon, Int.), and diluted 1:200, to detect primary antibody binding.

RESULTS

mAb production and screening

We used the cyclosphosphamide immunosuppression method to bias the immune response towards membrane antigens on retinoic acid-induced NT2D1 embryocarcinoma cells. Hybridoma supernatants were screened on frozen, sagittal sections of whole E14.5 and E18.5 rat embryos, postfixed with 1% paraformaldehyde. This protocol reflects a compromise between the need to preserve the integrity of the neural tissue through fixation and a desire to preserve the proteins in their native state for later identification and isolation (Patterson, 1992).

Of 960 hybridomas generated, approximately 640 supernatants contained mAbs and were screened on sections. Approximately half of these did not detectably label the CNS, and most of the other half labeled the entire CNS of one or both ages, with no positional differences in intensity. mAb FORSE-1 was selected for further study because it intensely labels the E14.5 forebrain, while the rest of the CNS appears negative.

FORSE-1 labeling in the CNS: Forebrain, midbrain, hindbrain, and spinal cord

In rat embryos, FORSE-1 binding is detected as early as E9.5, labeling the rostral but not the caudal neural folds (Fig. 1). In addition, the dorsal surface of the entire embryonic ectoderm, and the endoderm labels with FORSE-1. At E10.5, the procencephalon, but not the remainder of the CNS, labels with FORSE-1 (not shown). The pharyngeal arches, Rathke's pouch , and gut label as well (not shown).

From E11.5 to E13.5, telencephalic and diencephalic staining is clear (Fig. 2D). As summarized in a reconstruction of serial sections, restricted areas of these structures are FORSE-1-negative (Fig. 2B). The entire neuroepithelium is proliferative at these stages, and hence has no distinguishable layers. The medial walls of most of the telencephalic bulbs are negative, while the rest of the telencephalon is positive, with the basal telencephalon (the ventral region of the telencephalic bulbs) being most intensely labeled. In the diencephalon, there are distinct FORSE-1-positive areas separated by FORSE-1-negative areas (Fig. 2D). The midbrain and hindbrain are sometimes weakly labeled at these stages, while the cord is consistently negative. Weak midbrain-hindbrain labeling is described under the later section on acetone fixation.

At E15, labeling is further restricted such that FORSE-1 binding in the telencephalon is maintained, with the basal telencephalon being most intensely labeled, while the diencephalon is FORSE-1-negative (Fig. 3). This contrasts with the pattern at earlier stages where both the telencephalon and the diencephalon are strongly labeled. The lateral and dorsal telencephalon displays label throughout the neuroepithelium. The cortical plate, where postmitotic neurons accumulate, is not yet detectably formed. The basal telencephalon shows the most intense FORSE-1 label, but this label is in the ventricular and adjacent regions, while the outer region containing fibers and postmitotic cells is negative. The rest of the CNS is negative, except for labeling along the midline of the hindbrain (Fig. 3).

At E17.5, the rostrocaudal restriction of FORSE-1 labeling is similar to that at E15 in that only the telencephalon labels while the rest of the CNS is negative. Within the telencephalon, FORSE-1 now labels only the ventricular zone of dividing cells, while the cortical plate, where postmitotic neurons accumulate, is negative (Fig. 4). That FORSE-1 labels cells in S-phase is confirmed by culturing dissociated E16 telencephalon cells in the presence of BrdU for 16 hours and double-labeling for FORSE-1 (Fig. 5). FORSE-1 clearly labels BrdU-positive cells. Since FORSE-1 also labels BrdU-negative cells, it is likely that the FORSE-1 antigen

continues to be expressed for some time on postmitotic cells. FORSE-1 labeling is seen on live, intact cells, demonstrating that the antigen is on the cell surface.

The olfactory bulb is FORSE-1-positive in the glomerular layer from E17.5 through postnatal life. The staining appears to be around the glomeruli, where the periglomerular cells, mitral cells, and interstitial tufted cells are located, and not within the glomeruli, where olfactory sensory axons make their synapses (Fig. 6 A-D). By P10, this glomerular layer labeling is much weaker. In the adult, the entire bulb is very weakly stained (not shown).

After E18.5, the FORSE-1 labeling pattern changes to one where many neurons in the CNS (except for the olfactory bulb, described above) are positive (Fig. 7). This pattern remains into maturity. In the adult, the entire CNS has weak, patchy FORSE-1 labeling in the grey matter (not shown).

FORSE-1 labeling in sensory structures

The nasal pits are FORSE-1-positive from E11.5 through E14.5. At E15.5, the nasal epithelium, now thrown into folds, is FORSE-1-positive and continues to be so until birth, with binding mainly on the surface of the epithelium, lining the cavity, and to a lesser degree within the sensory epithelium itself (Fig. 6G,H). Other nasal tissues, the lateral nasal, septal, and vomeronasal glands, are FORSE-1-positive from E17.5 through P10 (Fig. 6E, F). The vomeronasal organ itself is negative at all ages (not shown).

The retina is FORSE-1-positive upon acetone fixation, from E11.5-E15, (not shown), but subsequently becomes negative until birth, when the inner nuclear layer of the retina is FORSE-1-positive. This staining continues in the adult (not shown).

The otic placodes are FORSE-1-positive from E10.5 onwards (E12.5, Fig. 2D). From E15 to E17.5, the cochlear ducts stain with FORSE-1, primarily on the inner surface (Fig. 3). At birth, no labeling of otic structures is detected (not shown).

Effect of Acetone Fixation on FORSE-1 labeling

The FORSE-1 epitope is sensitive to paraformaldehyde, ethanol, and methanol fixation, therefore all of the data described above is from fresh frozen sections, either unfixed or postfixed in 1% paraformaldehyde. Postfixation in acetone (10 minutes, 0°C or -20°C) remarkably intensifies the staining in areas that are FORSE-1 positive, while background staining (no 1° antibody or irrelevant IgM 1° antibody) remains very light (Figs. 3 and 8). The ability of acetone to increase the

sensitivity of staining by the FORSE-1 antibody was tested by using various dilutions of FORSE-1 supernatant on acetone-fixed sections, described below. In addition, we determined, using dissociated cells, that the subcellular localization of the staining with and without acetone fixation was similar, i.e. acetone-fixed, dissociated E15 telencephalic cells appeared to show surface label, similar to the labeling of dissociated, live, unfixed cells (not shown).

We observed no change in the overall pattern of FORSE-1 staining after acetone fixation at any age except from E11.5 to E13.5. At E15, acetone fixation results in an intensification of telencephalic staining, as well as minor areas of diencephalic staining (Fig. 3). From E11.5-E13.5, however, acetone fixation enables us to visualize FORSE-1 staining in a restricted part of the dorsal half of the entire CNS, from midbrain to the caudal-most spinal cord (Fig. 8). The dorsal-most area of the neural tube was consistently negative, as was the entire ventral half, resulting in a dorsoventrally-restricted band of FORSE-1 staining . When the FORSE-1 mAb is diluted, however, the forebrain staining remains while, with increasing dilution, the spinal cord, and then the midbrain and hindbrain staining becomes undetectable, even after acetone fixation (Fig. 8). This dilution result suggests a gradient of FORSE-1 antigen expression from high in the forebrain to low in the spinal cord. Such a gradient is supported by immunoblots of subdivisions of the CNS at E12 (Fig. 10; see following section). Acetone may modify the FORSE-1 epitope resulting in better binding of the mAb, or allow the mAb improved access to the epitope.

Identification of the FORSE-1 antigens

To identify the FORSE-1 antigen, forebrains from E14.5 rat embryos were homogenized in low salt, and soluble and membrane fractions were separated by centrifugation. When the proteins in these two fractions were subjected to immunoblot analysis, FORSE-1 is found to bind a high molecular weight region, in both soluble and membrane-bound forebrain fractions, which does not migrate out of the stacking portion of the gel (Fig. 9A). There is no obvious staining of any proteins present within these two fractions when FORSE-1 is omitted from the incubation, and a variety of other antibodies generated from the same fusion do not label the high molecular weight region labeled by FORSE-1 (not shown). The heterogeneous and very large size of the FORSE-1-positive band suggest that the antigen is proteoglycan-like (Herndon and Lander, 1990; Fryer et al., 1992; Maeda et al., 1992).

The nature of the FORSE-1 antigen in older animals was determined by performing immunoblots using protein fractions isolated from newborn (P0) forebrains and subregions of the adult CNS. While FORSE-1 binds similar high molecular weight regions in the soluble and membrane-bound fractions of the P0 forebrain, this labeling is completely absent in any of the adult brain fractions (Fig. 9A). FORSE-1 does, however, specifically label a doublet centered at about 80 kD in the membrane-bound, but not the soluble (not shown) fractions, derived from the adult cerebrum and spinal cord, and a similar doublet that migrates slightly faster than 80 kD in the membrane fraction derived from the adult cerebellum (Fig. 9A). The adult antigens are likely to be integral membrane proteins as they are fully extracted from the membrane fractions by Triton X-100 and NP40 (not shown). These bands are not labeled when FORSE-1 is omitted from the incubation, nor are they stained by a variety of other antibodies generated in the same fusion (not shown). Consistent with our immunohistochemical results, there is no obvious binding to soluble or membrane-bound fractions derived from the following adult tissues: dorsal root ganglia, sciatic nerve, kidney, liver, and adrenal medulla (not shown). We also performed immunoblots using 52G9, a previously characterized mAb that labels the forebrain of rat embryos (Stainier et al., 1991). Surprisingly, we find these immunoblots (Fig. 9B) are essentially identical to those generated with FORSE-1(Fig. 9A). Moreover, in the immunoblot analyses of the rostrocaudal distribution of the embryonic/P0 antigen, and the carbohydrate content of both the embryonic/P0 and adult antigens using FORSE-1 described below, identical results were obtained with 52G9 (not shown).

A stringent test of whether the high molecular weight, FORSE-1-immunoreactive species is, in fact, the antigen visualized immunohistochemically in the embryo, is to analyze its rostrocaudal distribution. To do this we generated soluble and membrane-bound fractions from E12.5 and E15 forebrain, midbrain and hindbrain, spinal cord, and body (minus spinal cord). Equal amounts of protein from each fraction were subjected to an immunoblot analysis using FORSE-1. At both these ages, the biochemically-detected rostrocaudal difference in the distribution of the FORSE-1 epitope parallels the immunohistochemical results (Fig. 10A). In addition, FORSE-1 detects similar levels of the embryonic antigen in protein fractions derived from P0 forebrain, cerebellum, and spinal cord (Fig. 10B), consistent with the lack of an immunohistochemical rostrocaudal gradient at this age. These data taken together indicate that the embryonic FORSE-1 epitope most likely resides on a high molecular weight, proteoglycan-like antigen.

The proteoglycan-like characteristics of the embryonic/P0 antigen prompted us to determine whether the FORSE-1 epitope is composed of amino acids or carbohydrate. To do this we digested soluble fractions of P0 rat forebrain with the following glycosidases: chondroitinase ABC, hyaluronidase, heparitinase, keratinase, endoglycosidase-H, neuraminidase, or N-glycosidase F, and performed an immunoblot analysis using mAb FORSE-1. Chondroitinase ABC digestion yields a more tightly resolved, faster migrating (although still within the stacking gel) FORSE-1-immunoreactive species (Fig. 11A). None of the other glycosidases affected the mobility of the antigen or the binding of mAb FORSE-1(not shown). This result suggests that the embryonic/P0 antigen is a proteoglycan that contains chondroitin sulfate.

The migration (highly resolved bands) and non-ionic detergent extractability of the adult antigens are not proteoglycan-like, but are rather suggestive of a membrane glycoprotein. To test this we digested membrane fractions of adult rat cerebellum (solubilized with 1% NP40) with the same glycosidases used to characterize the embryonic antigen, and performed an immunoblot analysis with FORSE-1. While N-glycosidase F completely abolishes the binding of FORSE-1, none of the other glycosidases affect the mobility of the adult cerebellar antigen or the binding of FORSE-1 (Fig. 11B; not shown). Identical results were obtained using adult cerebrum and spinal cord membranes (not shown). In all cases, FORSE-1 binding was eliminated whether the solubilized membranes were digested prior to SDS-PAGE or whether the nitrocellulose transfers of the resolved proteins were incubated with N-glycosidase F prior to the addition of the mAb (not shown). These data suggest that the adult antigens are glycoproteins that contain N-linked high mannose, most likely in the form of hybrid and complex chains, and that the FORSE-1 epitope present on these antigens is composed of carbohydrate.

Comparison with mAb 52G9

We compared the immunohistochemical labeling pattern of FORSE-1 with that of 52G9 (Stainier et al., 1991). At all ages, consistent with the results of the immunoblot analysis (see above), the binding patterns of the two mAbs are identical, including the restricted labeling of the CNS (Fig. 3), nasal epithelia, and otic structures. Taken together, the immunohistochemical and immunoblot data indicate that FORSE-1 and 52G9 recognize epitopes on the same embryonic and adult antigens.

DISCUSSION

Developmental expression of the FORSE-1 epitope in the CNS

From the earliest stages, prior to closure of the neural tube, the FORSE-1 mAb exhibits a positionally-restricted pattern of binding in the CNS. This suggests that the FORSE-1 antigens could play a role in patterning the neural tube. To understand the spatial regulation of the FORSE-1 antigens, the important features of the staining pattern are discussed separately below, and the data is summarized in Table I.

Proliferative neuroepithelium staining (E9.5- E17.5). That cells of dividing neuroepithelium specifically label with FORSE-1 is well demonstrated at E17.5 (Fig. 4) where staining is seen in cells of the ventricular zone but not the cortical plate.

Regional specificity in the E11.5-E13.5 forebrain. The forebrain contains some FORSE-1-positive and some FORSE-1-negative areas. From E11.5 to E13.5, the basal telencephalon and the lateral walls of the telencephalic bulbs label with FORSE-1 while the medial walls do not, even upon acetone fixation, and the boundaries between the positive and negative regions are sharp. In the diencephalon, FORSE-1 displays a unique labeling pattern which we have compared in detail to several transcription factors (Tole and Patterson, 1994). The FORSE-1 labeling pattern is not identical to any of the known transcription factors in the early forebrain, and serves to further subdivide the developing forebrain.

Dorsoventral restriction (midbrain-hindbrain-cord; E11.5-E13.5. Dorsoventral restriction within the forebrain is discussed above). Acetone fixation reveals a dorsoventrally restricted region of FORSE-1 labeling along the entire rostrocaudal extent of the CNS, from E11.5 to E 13.5. This FORSE-1-positive region lies in between a small dorsal-most area that is FORSE-1-negative, and most of the ventral half of the CNS. Transcription factors Pax3 and 7 have similar dorsoventrally restricted distributions; Pax6, in contrast, appears to have a complementary distribution to that of FORSE-1 in the spinal cord. Members of the HoxB family all display spatially and temporally regulated patterns along the dorsoventral axis and show a striking dorsal restriction in the spinal cord between E12.5-E14.5 in the mouse (Graham et al., 1991). Graham et al. suggest that the dynamic pattern of expression of these genes could reflect their role in conferring rostrocaudal identity to different classes of neurons as they are born in different locations along the dorsoventral axis. Dbx-1 is expressed in a narrow, dorsoventrally restricted stripe in the embryonic spinal cord (Lu et al., 1992), and MASH-1 is transiently expressed in a dorsal region of the ventricular zone in the

embryonic spinal cord (Lo et al., 1991). All these expression patterns reveal molecular differences in cells based on their dorsoventral position.

Widespread labeling of mature neurons (E18-Adult). From late embryonic life through adulthood, the FORSE-1 pattern appears to no longer be subject to the spatial regulation discussed above. Instead, neurons throughout the CNS label with FORSE-1, with local variations in intensity of labeling. This is consistent with the possibility that the FORSE-1 >200 kD antigen has different roles in early versus late embryonic life. The rostrocaudal regulation discussed above and the expression pattern from late embryonic life to adulthood together are reminiscent of the biphasic expression pattern of NCAM in the developing mouse CNS (Bally-Cuif et al., 1993). NCAM is first expressed in a rostrocaudal, position-dependent pattern, and then is expressed by almost all postmitotic neurons. It is significant that throughout embryonic and early postnatal life, the FORSE-1 antigen identified in the CNS is a high molecular weight proteoglycan (discussed further below), indicating that the change in expression pattern from a restricted to a widespread one does not appear to be accompanied by a change in the identity of the antigen. In the adult, however, FORSE-1 binds an 80kD doublet.

Secretion-like staining. The mAb FORSE-1 labeling of the E10.5 luminal face of the nasal epithelium, cochlear duct, optic cup, and the midline of the neural tube, the E9.5 dorsal surface of the ectoderm, the E9.5/E10.5 endoderm/gut, and the E10.5 pharyngeal arches appears to be greatly enriched on the surface of the cells adjacent to a lumen or enclosed space, and is reduced or undetectable deeper in the epithelium itself, or along any other, non-luminal cell surfaces. This pattern is particularly well illustrated in the nasal epithelium, where it appears as if the mucus layer is the main site of staining, with modest staining within the epithelium itself. This finding, and the observation that the lateral nasal, septal, and vomeronasal glands label intensely, suggests that the FORSE-1 antigen may be a component of nasal secretion. Newborn lateral nasal glands also have a high molecular weight (>200kD) antigen that binds FORSE-1 (not shown). A number of carbohydrate-containing surface molecules have been detected in the developing chick olfactory placode, using antibodies against N-CAM, keratan sulphate, heparan sulphate proteoglycan, and a panel of lectins (Croucher and Tickle, 1989). These reagents were found to bind the early nasal placode and at later stages, the olfactory epithelium, much like FORSE-1. N-CAM has also been found in the developing otic placode (Richardson et al., 1987), paralleling FORSE-1 staining. In the other areas where FORSE-1 displays a secretion-like staining pattern, the antigen may

have a role in lining the lumen, as part of the extracellular matrix. Similar observations have been reported for the staining pattern of mAbs against CDA-3C2 (carbohydrate differentiation antigen-3C2; Prouty and Levitt, 1993). This antigen is also a high molecular weight proteoglycan, and labels only the apical borders of, for example, early neurectoderm, endoderm, as well as nasal and otic placodes. It is interesting that probes to many of the transcription factors discussed above also label the nasal epithelium, otic, and optic placodes. Perhaps some of these molecules, including the FORSE-1 antigen, play a role in defining the topology (outside versus inside surfaces) of the ectoderm and endoderm. All the surfaces listed above that are labeled by FORSE-1 start out as exterior surfaces, and no surface that faces the interior of the animal is labeled.

Other features of FORSE-1 labeling. In the developing olfactory bulb, FORSE-1 staining is detected around the glomeruli, where periglomerular cells and interstitial tufted cells, which are among the output cells of the olfactory bulb, are located. Periglomerular cells are generated primarily after birth, but interstitial tufted cells are generated in the same time period as when FORSE-1 labels the glomerular layer (E15 -birth; Bayer, 1983). It is significant that here, too, FORSE-1 labels cells as they are newly generated, and not afterward, until late in postnatal life, when the entire CNS labels with FORSE-1. Adult, anosmic olfactory bulbs do not label more intensely around the glomeruli than normal olfactory bulbs (not shown), suggesting that innervation does not directly regulate the FORSE-1 antigen.

FORSE-1 antigens

FORSE-1 recognizes an epitope on apparently distinct bands on immunoblots, and these are regulated with different developmental time courses. From E12.5 through early postnatal life, FORSE-1 binds a high molecular weight antigen in soluble and membrane-bound forebrain fractions that does not migrate out of the stacking gel even when subjected to SDS-PAGE under reducing conditions. A number of properties of this antigen indicate that it is likely to be a proteoglycan (Zaremba et al., 1989). The estimated molecular weight (>200 kD) of the FORSE-1 immunoreactive species, its broad, diffuse migration pattern, are consistent with this antigen being a proteoglycan. The most compelling evidence on this point is provided by enzymatic digestion. That chondroitinase ABC digestion of soluble and membrane-bound forebrain fractions results in a tighter FORSE-1-positive band that migrates faster on SDS-PAGE, strongly suggests that

this antigen is a proteoglycan that contains chondroitin sulfate. The presence of the FORSE-1 immunoreactive proteoglycan in soluble as well as membrane-bound forebrain fractions is consistent with other studies that describe a similar subcellular distribution of proteoglycans (Zaremba et al., 1989; Oohira et al., 1988; Herndon and Lander, 1990; Maeda, et al., 1992).

A number of recent studies have described the expression of developmentally-regulated proteoglycans in the mammalian CNS (Herndon and Lander, 1990; Streit et al., 1990; Cole and McCabe, 1991;Maeda et al., 1992; Fryer et al., 1992), but none have reported a proteoglycan epitope whose expression is positionally- and developmentally-regulated as in the present case. That the biochemistry accurately reflects the distribution of the antigen detected immunohistochemically is especially clear at E12.5 and E15, where consistent with the immunohistochemistry, immunoblot analysis clearly shows a forebrain-enriched distribution of the proteoglycan bearing the FORSE-1 epitope. During early postnatal development, in contrast, FORSE-1 labels sections of rostral and caudal CNS equally, and immunoblot analysis detects no positional difference in the expression of the proteoglycan. These results strongly suggest that we have correctly identified the antigen bearing the FORSE-1 epitope.

In immunoblots of membrane-bound fractions derived from the adult brain and spinal cord, FORSE-1 binds a doublet of proteins centered at about 80 kD. These FORSE-1 antigens appear to be integral membrane glycoproteins and not proteoglycans, because they are completely solubilized by non-ionic detergents, their mobility is not affected by enzymes such as chondroitinase ABC, and N-glycosidase F treatment removes the FORSE-1 epitope. The latter result suggests that these antigens contain N-linked high mannose oligosaccharides, most likely in the form of hybrid and complex chains, and further indicates that the epitope recognized by FORSE-1 is composed of carbohydrate. This suggests that the FORSE-1 epitope on the embryonic antigen also contains N-linked high mannose oligosaccharides. However, N-glycosidase F treatment does not remove the epitope on the embryonic antigen, perhaps due to the enzymes' inability to access the appropriate site. This epitope is CNS-specific as it is not present in the PNS or in a variety of non-neuronal tissues. In addition, the size of the doublet, at least in the adult, is region-specific. The forebrain and spinal cord express a doublet centered at about 80 kD, while the molecular weight of the doublet in the cerebellum is slightly smaller. This size disparity could be due to the presence of additional carbohydrate on the forebrain/spinal cord proteins. It is also possible that the core proteins in

each doublet are distinct. Several such region-specific carbohydrate-bearing antigens have been shown to be expressed during the early development of Drosophila and grasshopper (Snow et al., 1987), rat (Dodd et al., 1988) and chick (Canning and Stern, 1988; Roberts et al., 1991).

Comparison with mAb 52G9

As described in the Results section, we find that mAb FORSE-1 and mAb 52G9 have identical labeling patterns in our hands. Some of our findings, like the staining of the telencephalon, olfactory bulb, nasal pits, otic placodes, and Rathke's pouch, agree with those of Stainier et al. (1991). Our other results, particularly the biochemical characterization of the antigen, the pattern of labeling in the forebrain, the transient, dorsoventrally-restricted labeling of the entire CNS, and the presence of staining at E18 and beyond, are very different from those previously reported for mAb 52G9. We do not, at any age, observe a single, restricted region of labeling in the diencephalon as was reported for mAb 52G9. Rather, this restricted region is part of the pattern we demonstrate in the diencephalon from E11.5 to E13.5 (Fig. 2D), which is clearly visualized in horizontal sections. Stainier et al. used sagittal sections, which do not permit visualization of each of the regions in a single section. Moreover, despite many variations in our methods, and replication of the methods of Stanier et al., neither mAb yields the 55kD band on immunoblots of E15 forebrain that was reported by them.

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FIGURE LEGENDS

Figure 1: FORSE-1 labeling in the E9.5 rat embryo. Sections of E9.5 rat embryos stained with FORSE-1 show label in the rostral neural folds, (arrows) while the primitive streak, (ps) at the caudal end of the embryo is unlabeled (A). In addition, the dorsal surface of the entire embryonic and extraembryonic ectoderm (small arrowheads), and the endoderm (large arrowheads) labels with FORSE-1. This labeling is also present in a more caudal section (B), where the neuroepithelium itself does not label (arrows point to neural folds). Scale bar is 200μm.

Figure 2: FORSE-1 labels the E12.5 CNS. (A-C), a reconstruction of the labeling pattern of FORSE-1 in the E12 rat embryo. (A), Schematic of a lateral view of an E12 embryo showing the CNS, with colouring showing the staining pattern of FORSE-1 in the telencephalon (red) and diencephalon (blue). This pattern was reconstructed from serial sections cut as in (B). (B), a cutaway view of B, showing the plane of sectioning, and a representative section after staining with FORSE-1. (C), serial sections of the head of an E12.5 embryo cut as shown in C, displayed in order from upper left to lower right, stained with FORSE-1, show labeling of the nasal pits (np), telencephalon (t) and diencephalon (d), but not the midbrain (mb) and hindbrain (hb). Within the telencephalon, a restricted region of the medial walls remains unlabeled (arrows), and the diencephalon shows three FORSE-1-positive regions (arrowheads) separated by FORSE-1-negative regions. (o) is the otic placode that shows FORSE-1 staining. The developing eye, (e), does not label with FORSE-1. Scale bar is 1mm.

Figure 3: FORSE-1 labels E15 rat telencephalon. Rows (A), (B), (C), and (D) each show serial sections of an E15 rat embryo, through the brain, cut as shown in (E), stained with cresyl violet, FORSE-1 (unfixed sections), FORSE-1 (acetone fixed sections), and 52G9. (A) and (B) show the telencephalon and diencephalon (d). In the telencephalon, FORSE-1 staining is present throughout the neuroepithelium, except for the outer region of the basal telencephalon (bt). The diencephalon does not label with FORSE-1 at this age. The cochlear ducts (cd) also label with FORSE-1. Acetone fixation enhances but does not alter this labeling pattern, except that a greater area of the basal telencephalon shows label, and minor areas of labeling appear along the ventricular zone of the diencephalon. 52G9 shows a labeling pattern very similar to FORSE-1, where the telencephalon, but not the diencephalon, and the cochlear ducts are stained. (C) shows the telencephalon,

diencephalon and part of the hindbrain, (hb), and (D) shows the midbrain, (mb) and hindbrain. FORSE-1 does not label the diencephalon, midbrain, or hindbrain, except for label along the midline of the hindbrain. The spinal cord does not appear in these sections since the head of the embryo was frozen separate from the body in preparation for sectioning. Scale bar is 1 mm.

Figure 4: FORSE-1 labels E17.5 telencephalon. A, B are serial, transverse sections of an E17.5 rat brain, stained with cresyl violet (A), and FORSE-1 (B), showing the lateral wall of the telencephalic bulb, where FORSE-1 labels the ventricular zone (vz) but not the cortical plate (cp). Scale bar is 250 μm.

Figure 5: FORSE-1 labels BrdU-incorporating cells in culture. (A) is a culture of dissociated E16 telencephalon, stained live with FORSE-1. (B) is the same field visualizing BrdU incorporation, and (C) is a phase contrast photograph. FORSE-1 labels the surfaces of BrdU-positive cells (arrows), and also a few cells that have not incorporated BrdU (small arrowheads), which are presumably newly postmitotic cells, while some, presumably mature cells, remain unlabeled by FORSE-1 (large arrows). Scale bar is 10 μm.

Figure 6: FORSE-1 labels embryonic rat olfactory bulb and nasal cavity. (A, B), (C, D), (E, F), and (G, H) are serial transverse sections of an E21 rat embryo, stained with FORSE-1 (A, C, E, G) and methyl green (B, D, F, H). (A) shows FORSE-1 label in the glomerular layer of the olfactory bulb (ob) and in the olfactory epithelium (oe), while other areas of the olfactory bulb, the nasal cavity, and the retina (r), visualized in (B), are unlabeled. (C) is a higher magnification of the olfactory bulb, showing that the FORSE-1 label is in the glomerular layer of the olfactory bulb (gl), around the glomeruli, where the periglomerular cells and interstitial tufted cells are located, and not within the glomeruli where olfactory axons make their synapses. Other areas of the olfactory bulb visualized in (D), are unlabeled. (E) shows the FORSE-1 label in the lateral nasal gland (lng) and in the olfactory epithelium (oe). (G, H) are higher magnifications of sections similar to (E, F), showing that the FORSE-1 label is in the mucus lining of the nasal epithelium, while there is reduced label within the epithelium itself. Scale bars are 0.5 mm in (A, B) and (E, F), 100 μm in (C, D), and 10 μm in (G, H).

Figure 7: FORSE-1 labels the E21 CNS. Sections of E21 brain (A, C) and spinal cord (B, D), labeled with FORSE-1 (A, B) show non-uniform labeling in all regions of the CNS. A negative control, (C, D), using no primary antibody, reveals little background staining in the CNS, while the bones of vertebral column and ribs present around the spinal cord label equally in both (B) and (D). Scale bars are 250 μm.

Figure 8: Effect of acetone fixation on FORSE-1 staining. Sections of E12.5 embryos through the forebrain (fb) and hindbrain (hb), (A, C, E, G), and spinal cord (sc), (B, D, F, H), were labeled with cresyl violet (A, B), FORSE-1, no fixation (C, D), diluted FORSE-1, acetone fixation (E, F), and FORSE-1, acetone fixation (G, H). On unfixed sections, (C, D), FORSE-1 labels only the forebrain , and not the hindbrain or spinal cord. Acetone fixation (G, H) reveals label in the dorsal part of the midbrain and spinal cord, as well as an intensified staining of the forebrain. However, if acetone-fixed sections are labeled with diluted FORSE-1 (E, F), the staining is essentially identical to (C, D), where only the forebrain is labeled. Scale bars are 1mm in the top row, and 0.5 mm in the bottom row.

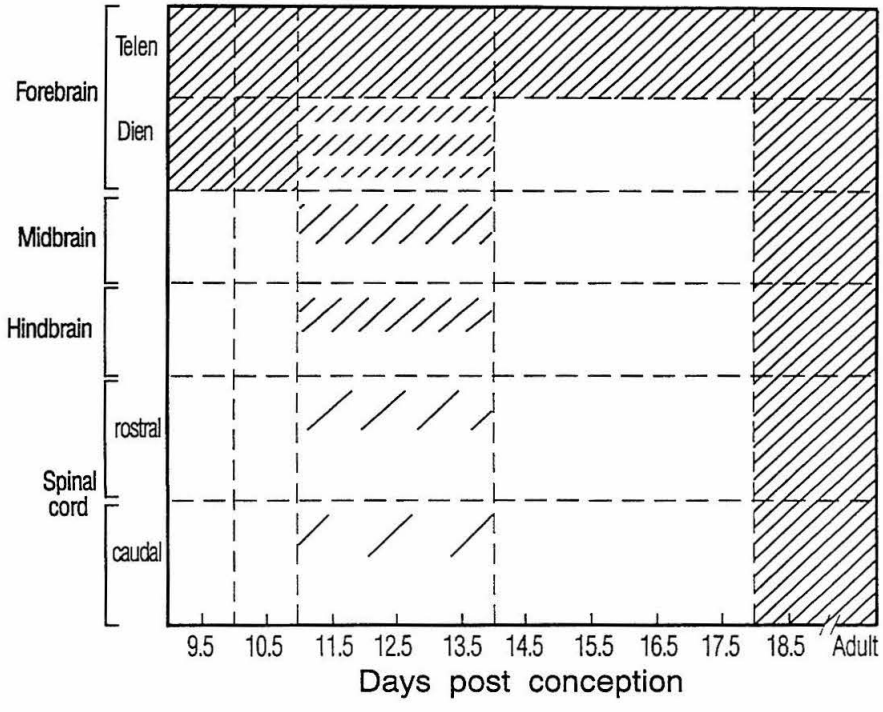
Figure 9: FORSE-1 and 52G9 both recognize a proteoglycan in the embryonic and newborn rat forebrain, and regionally expressed 80 kD doublets in the adult brain. Soluble (s) and membrane/cytoskeletal (p) protein preparations from E15 and P0 rat forebrains, as well as membrane/cytoskeletal fractions (p) of adult rat cerebrum (cbr), cerebellum (cbl), and spinal cord (sp. c.), were subjected to SDS-PAGE and immunoblot analysis. (A) shows the binding of mAb FORSE-1 to a proteoglycan in the embryonic and P0 forebrain, and to 80 kD doublets in the adult rat crb, cbl, and sp.c.. (B) shows very similar binding of mAb 52G9 to essentially identical nitrocellulose transfers. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse IgM secondary antibody.

Figure 10: The FORSE-1 epitope on the proteoglycan is enriched in the rostral CNS early in embryonic development. Soluble (s) and membrane/cytoskeletal (p) protein fractions generated from various rostrocaudal regions of the E12.5 and E15, as well as the P0 CNS were subjected to SDS-PAGE and immunoblot analysis. In (A), the top panel shows the binding of FORSE-1 to the proteoglycan in the E12.5 protein fractions, while the bottom panel shows the binding to the E15 fractions. Both immunoblots show that the expression of the FORSE-1 epitope on the

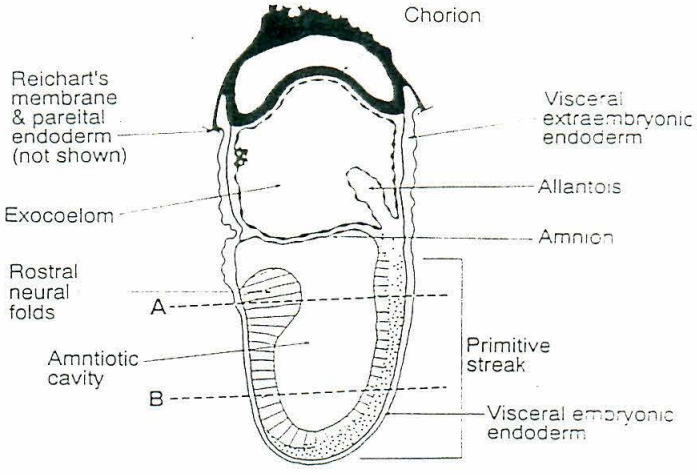
proteoglycan is enriched in the forebrain. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse IgM secondary antibody. Panel (B) shows the binding of FORSE-1 to fractions of P0 rat forebrain. Similar levels of the proteoglycan are detected in soluble as well as membrane/cytoskeletal fractions.

Figure 11. The FORSE-1 epitope is composed of carbohydrate. (A) Equal amounts of soluble protein derived from the P0 rat forebrain were digested with chondroitinase ABC as described in the materials and methods. These digests, along with an undigested sample containing the same amount of protein, were subjected to SDS-PAGE and immunoblot analysis. FORSE-1 binds the proteoglycan in the control, undigested sample(-), and to a more resolved, faster migrating species in the sample digested with chondroitinase ABC (CH.). (B), a membrane/cytoskeletal fraction derived from the adult rat cerebellum, solubilized with 1% NP40, was digested with N-glycosidase F (N-Gly) as described in the materials and methods. This digest and an undigested sample of the same protein fraction were subjcted to SDS-PAGE and immunoblot analysis. N-glycosidase F completely eliminates the binding of mAb FORSE-1 to the 80 kD doublet. The upper arrow indicates the border between the stacking and running gels for both A and B. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse IgM secondary antibody.

Table I : FORSE-1 labeling in the developing CNS



Legend: Hatches represent FORSE-1 staining, with greater densities reflecting higher labeling intensities. In the E11-E14 diencephalon, the 3 stripes of hatching represent the regional restriction described in Figure 2. In the E11-E14 midbrain, hindbrain, and spinal cord, the restricted region of hatching represents the staining observed in transverse sections, visualised after acetone fixation, as described in Figure 8.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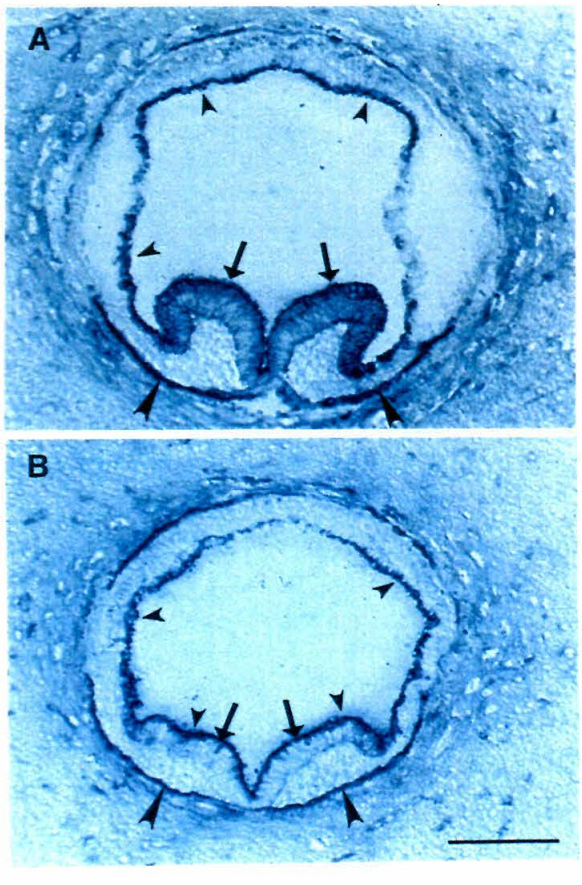
Allantois
Exocoelom
Amnion
Rostral neural folds
A
Amntiotic cavity
Primitive streak
B
Visceral embryonic endoderm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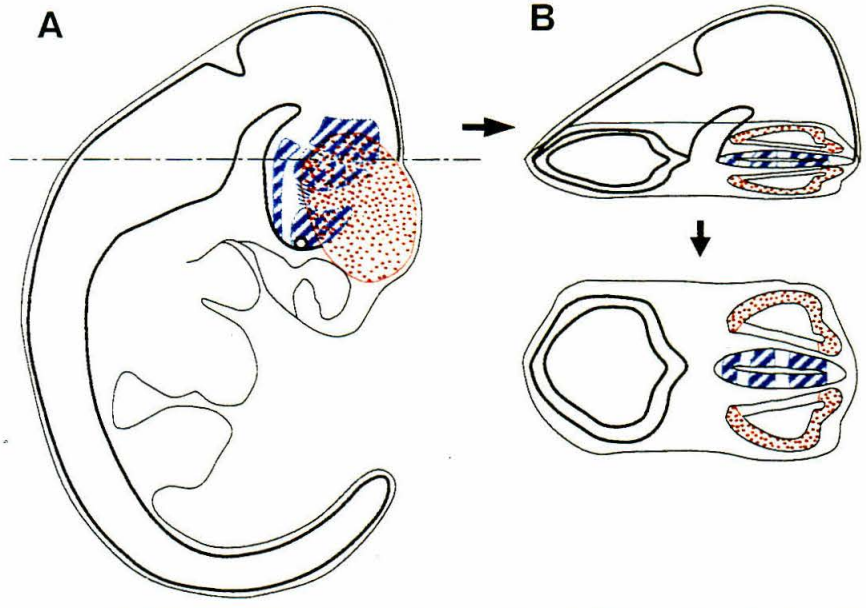
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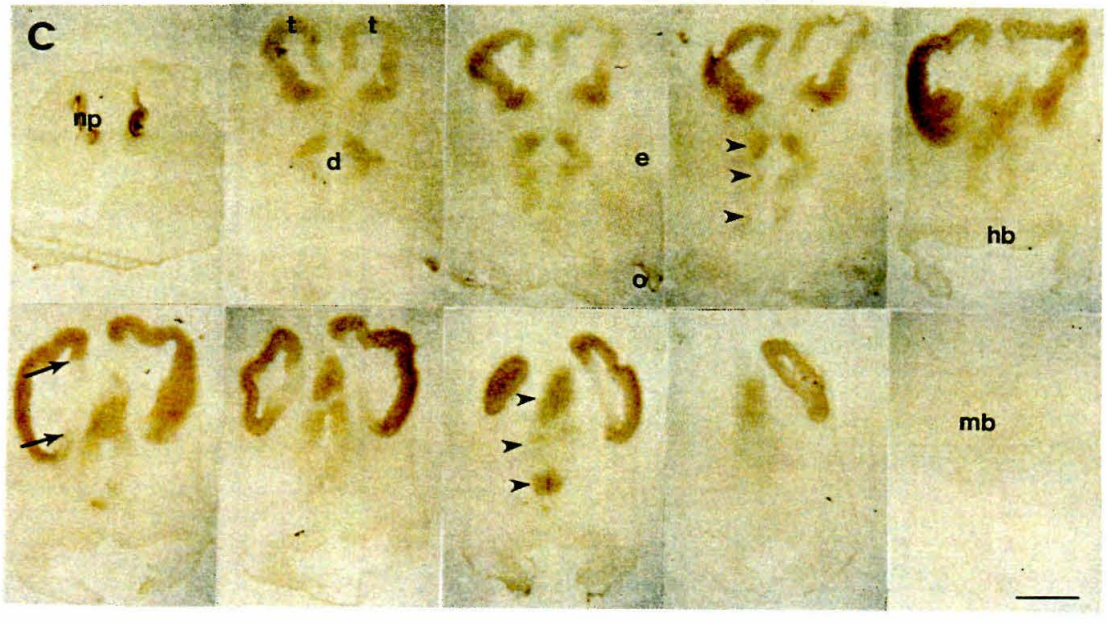
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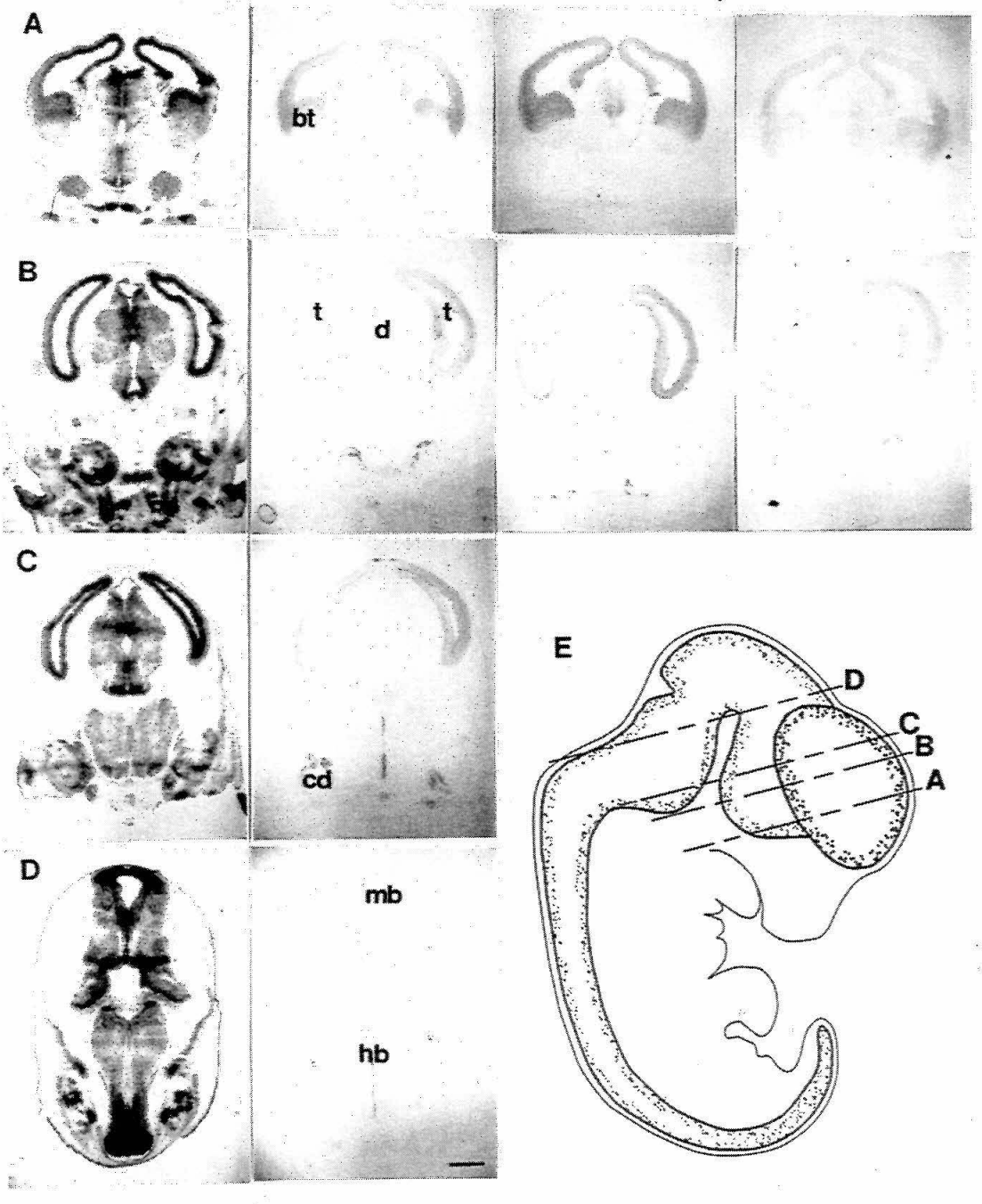
Cresyl violet
FORSE-1(unfixed)
FORSE-1(acetone)
52G9
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bt
t
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cd
mb
hb
D
C
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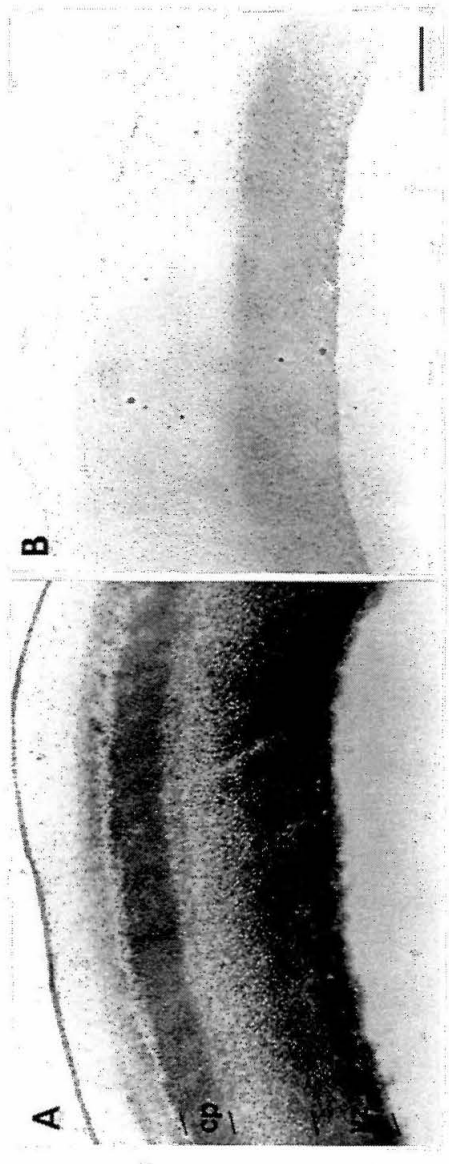
A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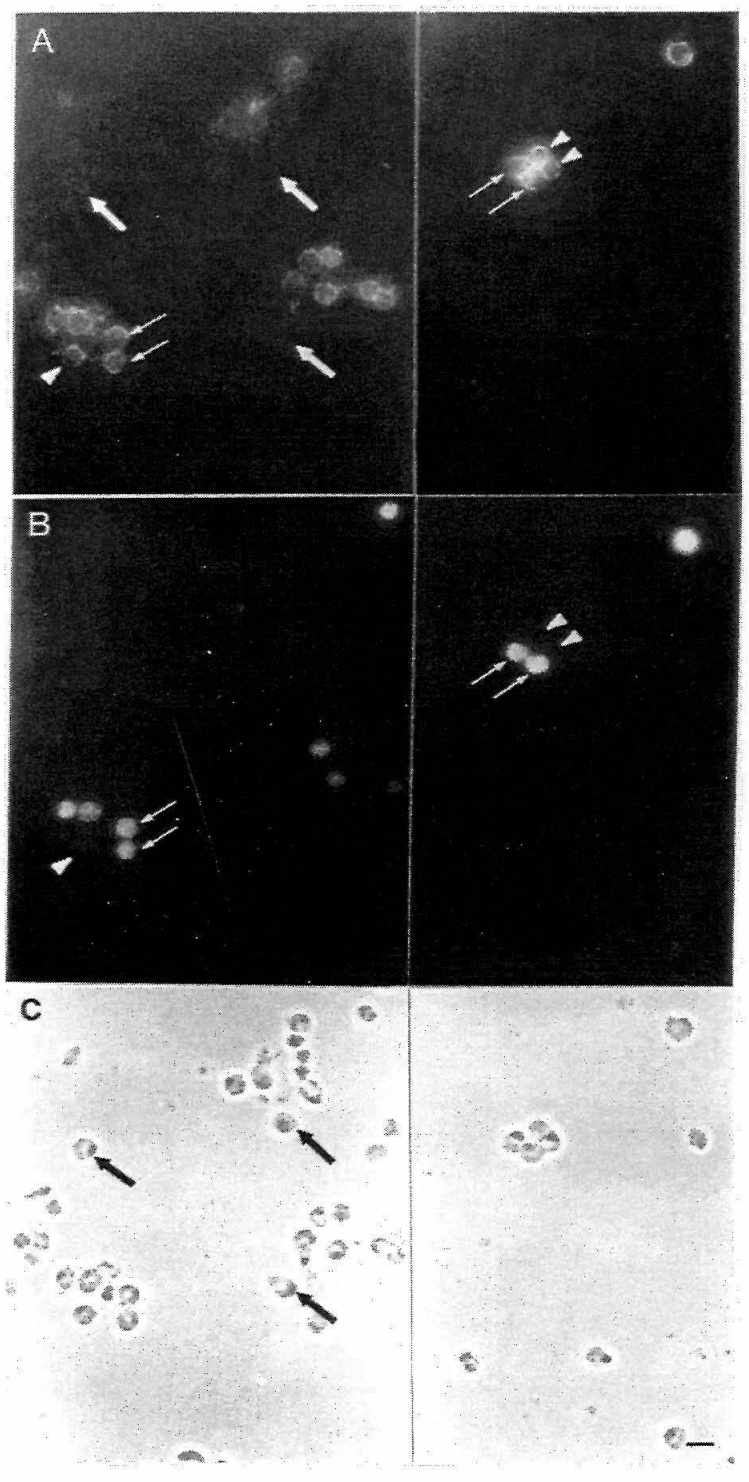
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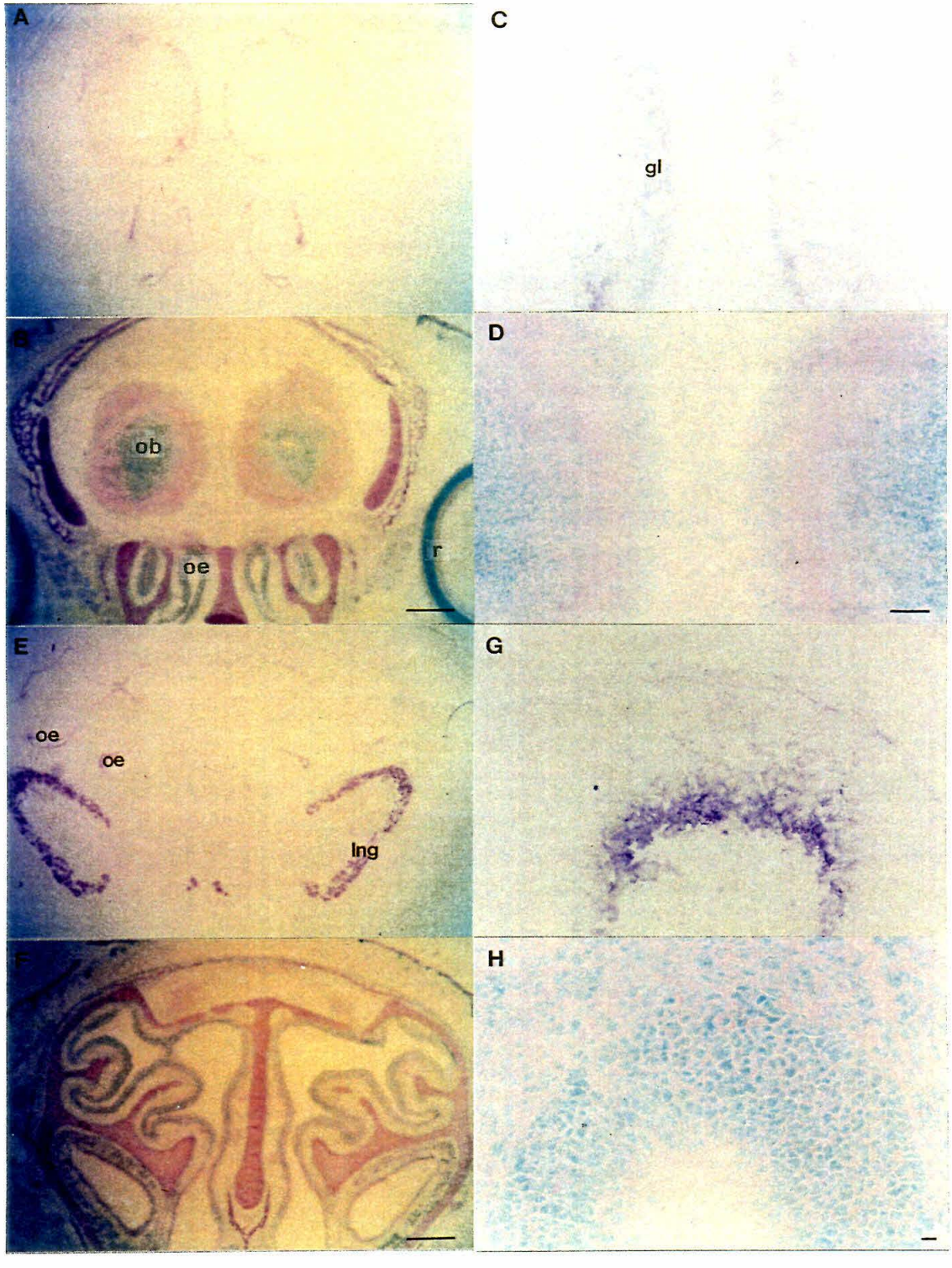
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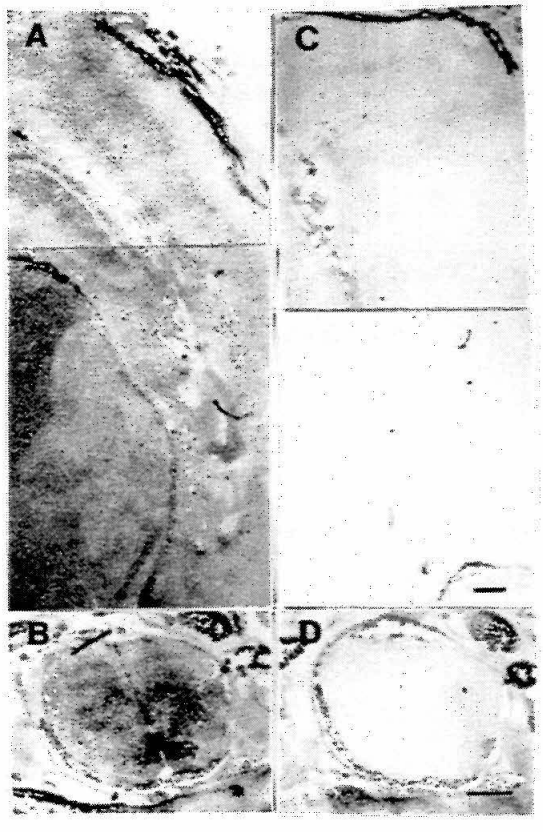
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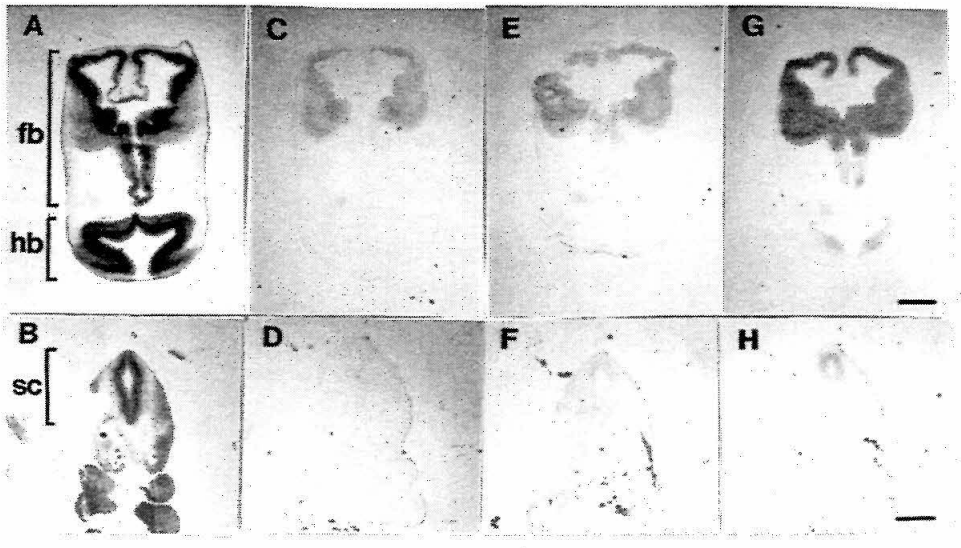
A
fb
hb
C
E
G
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sc
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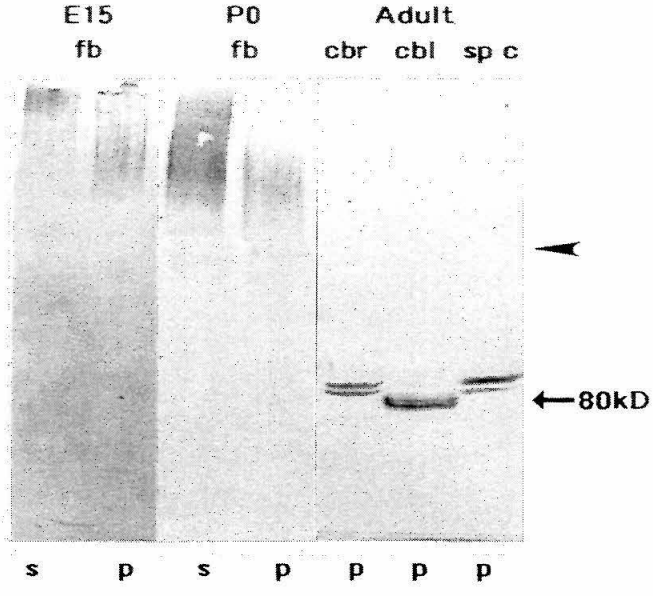
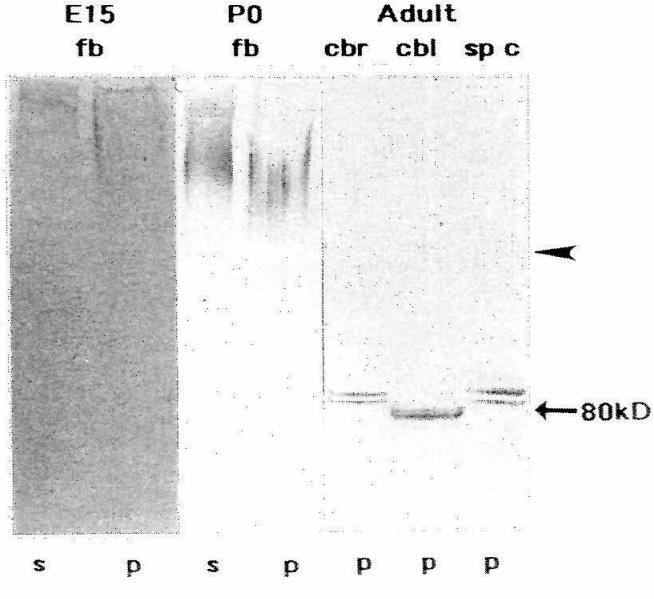
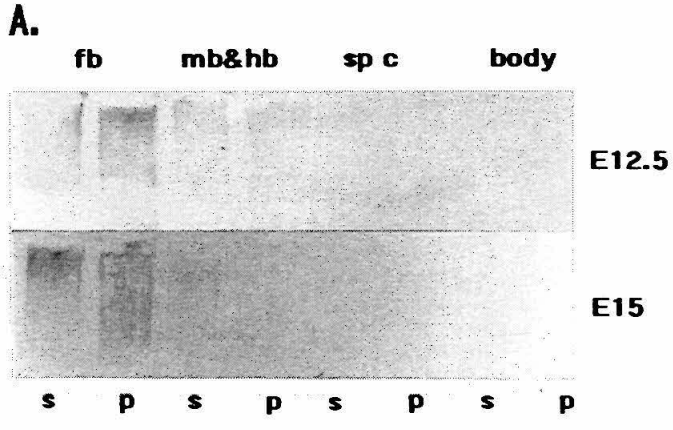
F
H

A. FORSE-1



B. 52G9




A.
fb
mb&hb
sp c
body
E12.5
E15
s
p
s
p
s
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s
p
B.
fb
cbl
sp c
P0
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p
s
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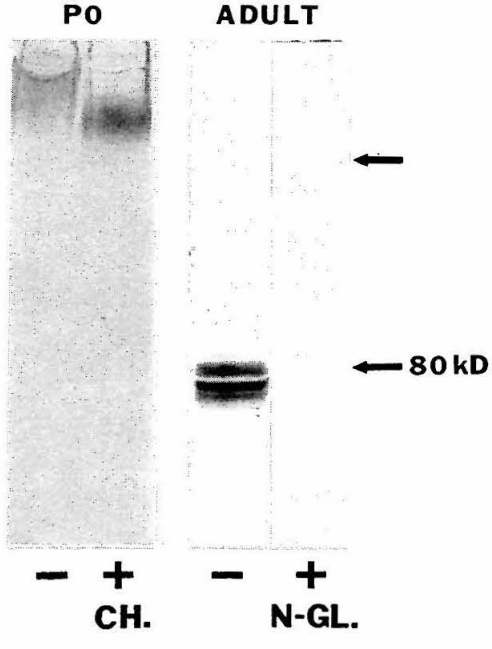
s
p


P0
ADULT
80kD
−
+
CH.
−
+
N-GL.

Chapter 2

Regionalization of the developing forebrain: A comparison of FORSE-1, Dlx-2 and BF-1

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ABSTRACT

The FORSE-1 monoclonal antibody (mAb) was generated using a strategy designed to produce mAbs against neuronal cell surface antigens that might be regulated by regionally-restricted transcription factors in the developing CNS. To determine whether FORSE-1 has a labeling pattern similar to that of known transcription factors, the expression of BF-1 and Dlx-2 was examined by *in situ* hybridization on sections serial to those labeled with FORSE-1. We find a striking overlap between BF-1 and FORSE-1 in the telencephalon; both are expressed in the lateral but not the medial walls of the telencephalon, and the boundaries of expression are apparently identical. FORSE-1 is detected prior to BF-1 expression in the neural tube, however. FORSE-1 and Dlx-2 have very different patterns of expression in the forebrain, suggesting that regulation by Dlx-2 cannot by itself explain the distribution of FORSE-1. However, they share some sharp boundaries in the diencephalon. In addition, FORSE-1 identifies some previously unknown boundaries in the developing forebrain. These results indicate that a new, cell surface marker can be used to subdivide the embryonic telencephalon and diencephalon into regions smaller than previously described, providing necessary complexity to the developmental patterning in the forebrain.

Keywords: regionalization, forebrain, neuromeres, prosomeres, diencephalon, thalamus, telencephalon

Introduction

The forebrain is structurally and functionally the most complex region of the mammalian central nervous system (CNS). How the different regions of the embryonic forebrain give rise to various adult structures is not well understood. Moreover, it is important to determine if patterning in the forebrain is an extension of that in the midbrain, hindbrain, and spinal cord, with similar features. Part of this analysis involves searching for transverse and longitudinal domains in the forebrain that are delineated before, or coincident with, overt cellular differentiation. Recent data concerning molecular differences (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Price, 1993) and cell mixing boundaries (Figdor and Stern, 1992) between various forebrain regions have forced a reevaluation of classical views.

Herrick (1910) proposed that various regions of the diencephalon are organized as longitudinal columns, the epithalamus, dorsal thalamus, ventral thalamus, and hypothalamus, separated by ventricular sulci. In this model, the longitudinal axis of the diencephalon cannot be an extension of the axis of the spinal cord and hindbrain, but is separate and nearly perpendicular to it. The expression patterns of several transcription factors and other molecules present in restricted regions of the diencephalon (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Price, 1993) do not fit the regional boundaries on Herrick's model, however. Instead, these data fit the "neuromeric theory" (Puelles, 1987; Bulfone et al., 1993), which proposes that the longitudinal axis of the diencephalon is continuous with that of the spinal cord and hindbrain, and curves with the flexures of the neural tube. Furthermore, various diencephalic regions can be organized as transverse domains about this longitudinal axis. FORSE-1, a cell surface, proteoglycan-like antigen exhibits regional specificity in the developing diencephalon (Tole et al., 1994), and may therefore contribute to this analysis. FORSE-1 labeling also exhibits regional specificity in the telencephalon (Tole et al., 1994). Bulfone et al. (1993) interpret the telencephalon to be a dorsal extension of the rostral-most diencephalic regions, rather than an independent, rostral-most structure in the CNS. In the neuromeric theory, the telencephalon is subdivided into transverse domains that are dorsal extensions of the diencephalic domains.

Since FORSE-1 is a cell surface molecule, it may be regulated by a regionally restricted transcription factor with an identical pattern of

expression. On the other hand, several transcription factors, with expression patterns only partially overlapping that of FORSE-1, may act as combinatorial regulators of FORSE-1. We have compared the FORSE-1, BF-1, and Dlx-2 patterns in this context.

Materials and Methods

For *in situ* hybridization, all reagents and glassware were maintained RNAase free. The probes were prepared using the Ambion MEGAscript kit, and digoxigenen-11-UTP (Boehringer Mannheim), following the manufacturers' instructions. The probes were stored in hybridization buffer, at a concentration of 0.5-1µg/ml at -20°C. Hybridization buffer for Dlx-2 consisted of 50% formamide, 5 X SSC, 10mM β-mercaptoethanol (BME), 2X Denhardts, and 250µg/ml tRNA (modified from Bulfone et al., 1993). Hybridization buffer for BF-1 consisted of 50% formamide, 0.3M NaCl, 20mM Tris pH 8.5 mM EDTA, 10mM sodium phosphate buffer, pH 8, 1X Denhardts, 10mM BME, and 500µg/ml tRNA (modified from Tao and Lai, 1991).

Fresh frozen embryos were sectioned at 15µm, and placed on Superfrost/Plus slides (Fisher Scientific). They were dried overnight at room temperature (RT), and either stored at -80°C or processed immediately. All subsequent treatments, including hybridization, were carried out in slide mailers (Baxter). Sections were fixed in freshly prepared 4% paraformaldehyde for 20 minutes at RT, followed by three washes in PBS. Dehydration was carried out through grades (30%, 50%, 70%, 80%, 95%, and 100%) of ethanol. Sections were then air dried, and treated with 1µg/ml proteinase K in buffer containing 100 mM Tris-HCl, pH 8, and 50 mM EDTA, for 30 minutes at 37°C. This was followed by fixation once more in 4% paraformaldehyde for 20 minutes at RT, a PBS rinse, and acetylation, which consisted of rinsing with 0.1M TEA, draining well, and incubating in a solution of freshly dispersed acetic anhydride in 0.1 M tetraethyl ammonium (TEA) at a concentration of 0.25%, for 10 minutes at RT. Sections were then rinsed in water, air dried, and either stored at 4°C for a few days or used immediately.

Prehybridization consisted of a 1 hour incubation in hybridization buffer, at the same temperature used for hybridization. Hybridization was performed for 16 hours at 48°C for Dlx-2 and at 58°C for BF-1. At the end of this period, the probe was saved for reuse, and sections were washed twice in

2X SSC with 10 mM BME for 30 minutes at RT, followed by RNAase treatment for 40 minutes at RT using a solution of 20µg/ml RNAase A plus 1-10 units/ml RNAase T1 in 2X SSC with 1 mM EDTA. This was followed by two 30 minute washes at 56°C for Dlx-2 and 60°C for BF-1, in a solution of 2X SSC, 50% formamide, 50 mM EDTA and 10 mM BME. Sections were then rinsed in 0.2 X SSC at RT, washed twice in 10 mM Tris- HCl, pH 7.5 and 150 mM NaCl, and blocked in 20% heat-inactivated sheep serum in PBS containing 0.1% Tween-20 (PBS-Tw).

Overnight incubation followed in a solution containing anti-digoxigenen Fab fragments, conjugated to alkaline phosphatase (Boehringer Mannheim). These fragments were preabsorbed to reduce background labeling, as follows: Embryos of the same age and species that was being used for *in situ* hybridization were dissected out, cut into small pieces, fixed in 4% paraformaldehyde for 1 hour at RT, washed in PBS, and stored at 20°C in methanol. The samples were then rehydrated through grades of methanol up to 50% methanol, then incubated in 25% methanol, 75% PBS-Tw, and finally in 100% PBS-Tw. The pieces were centrifuged to obtain a loose pellet, which was minced to obtain a coarse homogenate, and resuspended in twice the volume of anti-digoxigenen Fab fragments at an initial concentration of 1:200, in PBS-Tw with 1% heat-inactivated sheep serum. This incubation was performed for 3 hours with gentle end-over-end mixing. The tissue fragments were then centrifuged down, and the antibody-containing supernatant removed and diluted with 20% sheep serum in PBS-Tw to give a final antibody concentration of 1:1000. This antibody solution was stored at 4°C, and re-used several times.

Incubation of sections in the above antibody solution was performed overnight, at 4°C, with gentle rocking. Sections were then washed 3 times for 30 minutes each at RT, in PBS-Tw containing 2 mg/ml BSA. This was followed by a 10 minute incubation, at RT, in developing buffer, which contained 100 mM Tris pH9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, and freshly added levamisole at a concentration of 1.2 mg/ml. The color reaction was carried out by incubation in developing buffer with 4.5 µl/ml NBT (from a 75 mg/ml stock in 70% di-methyl formamide) and 3.5 µl/ml BCIP (from a 50 mg/ml stock, in 100% di-methyl formamide). The incubation was performed for 10-40 hours, and terminated when the color reaction had developed satisfactorily, by fixing in 0.1M MOPS, 2mM EGTA, 1mM $MgSO_4$,

and 3.7% formaldehyde for 1 hour at RT. Finally, sections were washed in PBS, dehydrated through grades of ethanol, and mounted in Permount.

Immunohistochemistry and photography was performed as in Tole et al., 1994.

Results

We compared the patterns of FORSE-1 labeling with BF-1 *in situ* hybridization on serial sections of E12.5 rat embryos. The angle of sectioning is indicated in the schematic diagram in Fig. 1. FORSE-1 and BF-1 show a striking overlap in the telencephalon, with apparently identical boundaries such that the lateral, dorsal, and ventral regions of the telencephalic bulbs are positive for both molecules, while a restricted region of the medial walls (arrows) does not show the expression of either molecule. FORSE-1 also labels some parts of the diencephalon, while BF-1 expression is restricted to the telencephalon. We examined E9.5 rat embryos to determine if BF-1 is expressed as early as FORSE-1, prior to neural tube closure. We do not detect BF-1 transcripts in the embryo at this stage, while the rostral neural folds label intensely with FORSE-1 (Fig. 2).

To compare the FORSE-1 pattern with the expression pattern of Dlx-2, we used sagittal (Fig. 3 and 4) and transverse (Fig. 5) sections of embryonic day E13.5 rat embryos. Alternate sections were labeled with FORSE-1 or processed for Dlx-2 *in situ* hybridization. Next to each serial pair is a line drawing that illustrates the boundaries revealed by that pair. Our data on the expression pattern of Dlx-2 in rat is consistent with earlier characterizations in mouse (Porteus et al., 1991, Price et al., 1991, Robinson et al., 1991, Bulfone et al., 1993).

While their overall patterns of expression are very different, Dlx-2 and FORSE-1 both respect the zona limitans intrathalamica (zli), which separates the dorsal and the ventral thalamus (DT, VT). Dlx-2 is expressed in the ventral thalamus (Fig. 3A, B, C, E; arrow points to the zli), while FORSE-1 labels both the dorsal and ventral thalamus, but does not label the zli in between (Fig. 3G, H, I, K; large arrow points to the zli). High magnification views of similar sections are shown in Fig. 4 (see legend). There are cells present in the zli that do not label with FORSE-1 (Fig. 4F). The zli is the future site of the mammilothalamic tract. The characteristic FORSE-1 gap at the zli is detected before the tract forms. FORSE-1 also respects 2 boundaries

rostral to the zli in the basal plate of the diencephalon (Fig. 3H, K, medium and small arrows). Basal plate labeling begins rostral to the ventral thalamus (neuromere p3 of Bulfone et al., 1993), in the mammillary area (MA), which lies in neuromere p4 of Bulfone et al., 1993. FORSE-1 labeling thus precisely delineates the p3/p4 boundary (Fig. 3H, K, medium arrow), in that labeling is observed rostral to the boundary in the MA of the basal plate of the diencephalon, and caudal to it in the ventral thalamus, in the alar plate of the diencephalon. Further rostrally, a distinct gap in the FORSE-1-positive region of the basal plate delineates the p4/p5 boundary (Fig. 3H, K, small arrow). The p3/p4 boundary and the p4/p5 boundary lie within the D1 region of Figdor and Stern (1992), which comprises diencephalic areas rostral to the zli.

FORSE-1 also labels the midbrain (Mb, Fig. 3G, H, K), hindbrain (Hb, Fig. 3G-L) and spinal cord (arrowheads, Fig. 3I-L), in a dorsoventrally restricted manner (Fig. 3E-H, arrowheads). FORSE-1 and Dlx-2 share a common, unlabeled zone in the supraoptic-paraventricular region (SPV) as well as the adjacent eminentia thalami (EMT) (Fig. 3D, J).

In the telencephalon, the medial ganglionic eminence (MGE) is positive for both Dlx-2 and FORSE-1 (Fig. 3D, J, F, L; Fig.4C, G). FORSE-1 is absent in a sub region if the MGE, however (arrow, Fig. 3J, L; asterix, Fig. 4G). FORSE-1 labels the lateral but not the medial wall of the telencephalon (Fig. 3G-L), and the FORSE-1-negative region of the medial wall is seen in Fig. 3Eand K (high magnification pictures in Fig 4D, H). Other FORSE-1 positive regions are the retina (Fig. 3G), otic placode (Fig. 3G,H, K, L), and the nasal pits (Fig. 3I-L).

Transverse sections through an E13.5 rat embryo, as shown in the schematic in Fig. 1, illustrate that the dorsal-most epithalamus (ET) and ventral most region in the diencephalon do not label with FORSE-1 (Fig. 5C). In the basal plate, there is a FORSE-1-positive region (Fig. 5C), which is restricted so that it does not extend as far as the alar-basal boundary; the latter is marked by the ventral boundary of Dlx-2 (Fig. 5A, dashed line). The ventral thalamus, labeled by Dlx-2, also expresses FORSE-1, although because section 5C is more caudal than 5A, the region of FORSE-1 labeling in the ventral thalamus is smaller than the Dlx-2-expressing region. Sagittal sections confirm that FORSE-1 does indeed label the entire Dlx-2-positive ventral thalamus (Fig. 3) . Further dorsally, there is a distinct gap in FORSE-1 staining at the zli (arrow, Fig. 5A, C), above which the FORSE-1-positive dorsal

thalamus is seen (Fig. 5C), the identity of which is confirmed by examination of the sagittal sections in Fig. 3. Sections 5B and 5D are more rostral than those in 5A and 5C. Dlx-2 is not expressed in the walls of the telencephalic vesicles, but is found in the lateral and medial ganglionic eminences (LGE and MGE, respectively), and in the contiguous anterior preoptic area (POA). Further ventrally, Dlx-2 is not present in the posterior preoptic area (POP), but is detected again in the suprachiasmatic area (SCH, Fig. 5B). FORSE-1 labels the lateral and dorsal, but not the medial telencephalic walls, as well as the lateral and medial ganglionic eminences, with a negative region within the latter. Unlike Dlx-2, FORSE-1 labels both the POA as well as the POP (Fig. 5D).

A composite schematic diagram of our FORSE-1 data, combined with Dlx-2 expression is shown in Figure 6A. The Dlx-2 portion of the schematic is taken from Bulfone et al. (1993). Figure 6B shows a schematic of the FORSE-1 labeling pattern combined with that of BF-1.

Discussion

Bulfone et al. (1993) and Puelles and Rubenstein (1993) have proposed a model of the developing forebrain in which it is divided into multiple transverse and longitudinal domains revealed by molecular markers. The attractive feature of such a developmental plan is that different areas of the map are identified by a unique combination of genes expressed there, much like combinations of Hox gene expression can be used to individuate the rhombomeres in the hindbrain (Krumlauf, 1993; McGinnis and Krumlauf, 1992; Wilkinson et al., 1989). Identification of new molecules with expression patterns in the forebrain will allow all areas of the embryonic brain to be delineated in this fashion. Since the forebrain is composed of so many different structures, a developmental map of considerable complexity will be required to pattern it. The labeling pattern of FORSE-1 fits well with several previously characterized boundaries, but also subdivides some existing domains, thereby making a finer grained map. Examples of boundaries of FORSE-1 expression that coincide precisely with previously identified boundaries are the zli, which is the p2/p3 boundary of Bulfone et al. (1993), and the D1/D2 of Figdor and Stern (1993). The zli separates the FORSE-1-positive dorsal thalamus from the FORSE-1-positive ventral thalamus. There are also two transverse boundaries of FORSE-1 staining in the basal plate of the diencephalon that coincide precisely with the p3/p4 and the p4/p5

boundaries of Bulfone et al. (1993), both of which lie within the D1 region of Figdor and Stern (1993). Some of the boundaries revealed by FORSE-1 and other regionally-restricted molecules, e.g. the zli in the diencephalon, and the LGE-cortical boundary in the telencephalon, have been shown to be boundaries of cell migration (Figdor and Stern, 1992; Fishell et al., 1993).

New boundaries delineated by FORSE-1 expression are in the medial ganglionic eminence (Fig. 3D,J, F, L; Fig. 4C, G) and the longitudinal boundaries in the basal plate (Figs. 3H-K). It will be important to determine if these boundaries coincide with cell lineage restriction or serve as barriers to cell migration (Fraser, 1993).

We have suggested that FORSE-1 is a candidate for a downstream target of regionally-restricted transcription factors, possibly mediating their actions in patterning the forebrain (Tole et al., 1994) . In the telencephalon, the FORSE-1 pattern is very similar to that of BF-1. However, unlike other members of the HNF-3/forkhead family that are expressed in regions of the early neural fold-stage embryo, we find BF-1 is not detectable at this stage. Similarly, the earliest stage that Dlx-2 expression has been reported is after the neural tube has closed (Bulfone et al 1993b). Thus, at these early stages, FORSE-1 must be regulated by other transcription factors. BF-1 is temporally and spatially in a position to regulate a portion of the expression of a FORSE-1 pattern, after neural tube closure. On the other hand, it is also possible that FORSE-1 is one of the cell surface mediators that regulate regionally-restricted transcription factors. Thus, FORSE-1 could modulate, for example, BF-1 in the telencephalon, Dlx-2 in some parts of its telencephalic and diencephalic expression domains, and even Pax-3 and 7 in the dorsal spinal cord.

Since the FORSE-1 antigen is on the cell surface, and likely to be a large proteoglycan, its function may lie in its influence on the nature of the extracellular environment in regions where it is expressed. Proteoglycans can bind growth factors and cell adhesion molecules and binding is required for optimal function of molecules such as bFGF (Yayon et al., 1991; Rapraeger et al., 1991) and NCAM (Reyes et al., 1990). Some proteoglycans absorb large amounts of water, thereby providing a hydrated environment. The chondroitin sulfate and keratan sulfate chains of aggrecan are thought to contribute to the hydration of cartilage. In addition, the long glycosaminoglycan chains on proteoglycans have been suggested to play a role in modulating cell aggregation through steric exclusion, simply by taking up

the space or fluid volume available to surrounding cells, causing them to pack closer (Morris, 1993).

In a number of instances, FORSE-1 labeling is present in two adjacent areas, but absent from the boundary between them. This could be an effective means of enabling cells from neighboring regions, or incoming and outgoing axons, to sense that they are crossing from one area to another. In one case, a FORSE-1-negative boundary, the zli, has been shown to be a border that dye-injected cells do not cross (Figdor and Stern, 1993); the dorsal and ventral thalami, which the zli separates, are both FORSE-1-positive. Figdor and Stern (1993) also report that the zli, but not the dorsal and ventral thalamus, is enriched in NCAM; in these structures, NCAM labeling is precisely complementary to that of FORSE-1, suggesting that FORSE-1 and NCAM might play opposite roles here. The zli marks the path of a future diencephalic axon tract, the mamillothalamic tract (Figdor and Stern, 1993; Fraser, 1993). FORSE-1 labeling is also absent from the site of the stria medullaris tract, at the dorsal margin of the ventral thalamus (Coggeshall, 1964; Figdor and Stern, 1993); this is the only FORSE-1-negative region in an otherwise continuous, transverse zone of FORSE-1 labeling, that extends from the ventral thalamus into the telencephalon.

The absence of FORSE-1 expression in the zli and in the stria medullaris tract is consistent with the idea that proteoglycans provide barriers to axon growth. For example, the roof plate of the spinal cord and tectum expresses a keratan sulfate during a period when axons grow along, but not across the plate (Snow et al., 1990). Interestingly, the roof plate does not label with FORSE-1, while an adjacent dorso-ventrally restricted region does. Proteoglycans have been suggested to play a role in the formation of olfactory bulb glomeruli by confining ingrowing axons (Gonzalez et al., 1993), and perturbation studies have provided evidence for the influence of chondroitin sulfate proteoglycans upon the direction of retinal ganglion cell axon outgrowth (Brittis et al., 1992). In addition, β-amyloid peptide has been shown to induce cortical glial cells to deposit chondroitin sulfate-containing proteoglycan, which blocks the ability of the peptide to support adhesion and outgrowth of cortical neurons *in vitro* (Canning et al., 1993). Finally, the presence of chondroitin sulfate proteoglycans in adult but not neonatal glial scar tissue, correlates with the inability of adult scar tissue to support neurite outgrowth (McKeon et al., 1991). The presence of chondroitin sulfate

proteoglycans after P2/3, has also been suggested to serve as a barrier to regenerating sensory axons in the dorsal root entry zone (Pindzola et al., 1993).

Together, FORSE-1, Dlx-2, BF-1 and other regionally-expressed genes serve to distinguish different areas of the developing forebrain at a molecular level. Some of these molecules, particularly those expressed early in neural tube formation, may specify the generation of different areas. Any of these molecules could also be responsible for subsequent expression of regional characteristics that ensure restriction of cell mixing, regulation of cell migration, or axon guidance. FORSE-1, a cell-surface molecule expressed from the earliest stages of neural tube formation, is in a position to play a role in such phenomena.

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Figure Legends:

Figure 1: FORSE-1 and BF-1 expression in the E13.5 CNS. (A, B) and (C, D), serial sections of the head of an E13.5 embryo cut as shown in the accompanying schematic, hybridized with the antisense probe to BF-1 (A, B), or labeled with FORSE-1 (C, D). The angle of sectioning was tilted, so that the two halves of the sections pass through different levels in the forebrain, illustrated by the two planes of sectioning. BF-1 and FORSE-1 are detected in the basal, lateral, and dorsal telencephalon, while the medial walls remain unlabeled. The boundaries between the labeled and unlabeled regions in the telencephalon are marked by arrowheads, and appear to be very similar for BF-1 and FORSE-1. FORSE-1 also labels some areas in the diencephalon (d), while BF-1 remains restricted to the telencephalon. Scale bar is 1mm.

Figure 2: FORSE-1 and BF-1 expression in the E9.5 rat embryo. Serial sections of E9.5 rat embryos stained with FORSE-1 (A) or hybridized with the antisense probe to BF-1 (B) show label FORSE-1 in the rostral neural folds, (arrowheads), while BF-1 is undetectable in the embryo at this stage. Scale bar is 200μm.

Figure 3: FORSE-1 and Dlx-2 expression in the E13.5 CNS. (A-L), serial pairs of sagittal sections of an E13.5 embryo, hybridized with the antisense probe to Dlx-2 (A-F), or labeled with FORSE-1 (G-L). FORSE-1 labels the dorsal and ventral thalamus, but not the zli in between (zli, long arrow, G, H, I, and K). FORSE-1 also labels the mammillary area which lies in p4, in the basal plate of the diencephalon. p4 is between the medium and small arrows, which mark the p3/p4 and the p4/p5 boundaries, respectively, in H and K. FORSE-1 is also detected in p5 in the basal plate of the diencephalon, so the p4/p5 boundary is seen as a distinct gap in FORSE-1 labeling (small arrow, H and K). FORSE-1 is absent from the stria medullaris tract, seen at the dorsal margin of the ventral thalamus (arrowhead, K). Dlx-2 is present in the ventral thalamus (A, B, C, E) with a sharp boundary at the zli (arrow, A, B, C, E). Both Dlx-2 and FORSE-1 are absent from the SPV and EMT (open arrow, D and J). In the medial ganglionic eminence, FORSE-1 expression is more restricted than Dlx-2, such that there is a region that is negative for FORSE-1 within the MGE (arrow, D, J, F, L). FORSE-1 labels a dorsoventrally-restricted region of the midbrain (G, H, K), hindbrain (G-L), and spinal cord (small arrowhead, I-L). In addition, FORSE-1 labeling is detected in the nasal pits, otic placode, and retina (G-

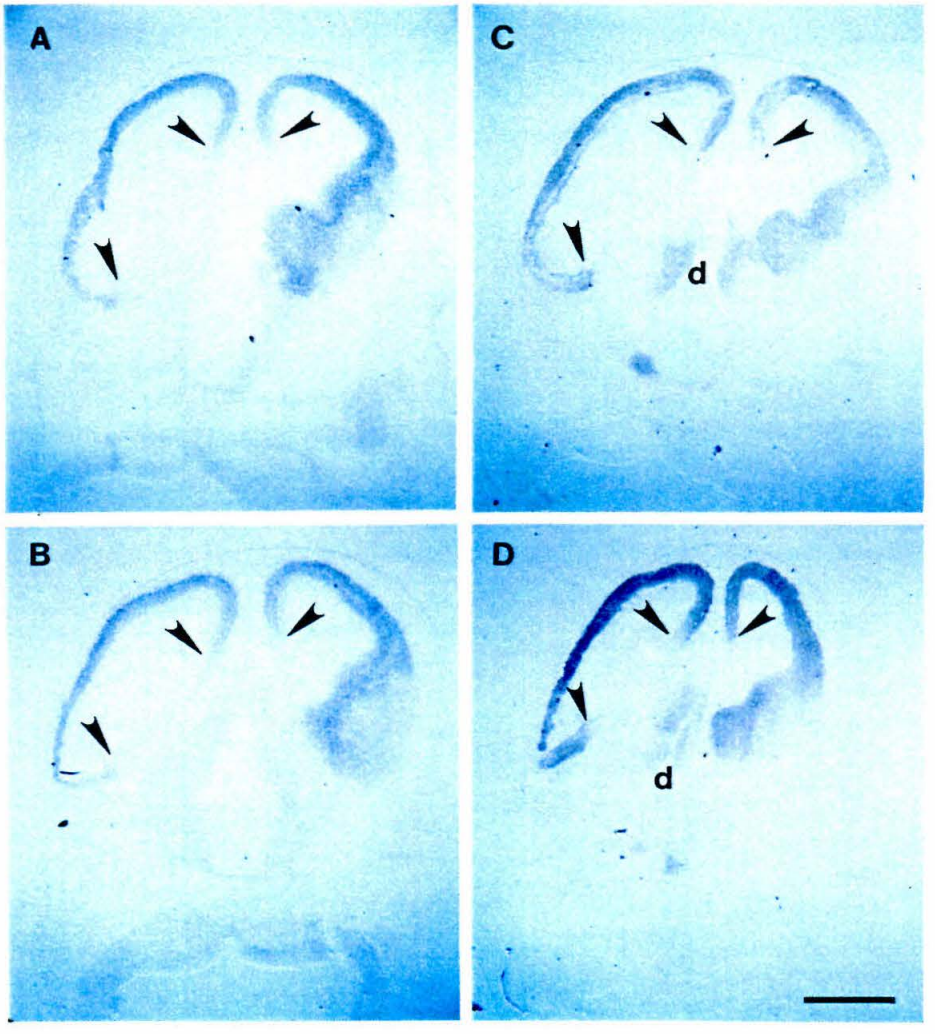
L). DT, dorsal thalamus; EMT, eminentia thalami; Hb, hindbrain; MA, mammillary area; Mb, midbrain; MGE, medial ganglionic eminence; np, nasal pits; op, optic placode; p1-5,prosomeres of Bulfone et al. (1993); r, retina; SM, stria medullaris; SPV, supraoptic paraventricular area; VT, ventral thalamus; zli, zona limitans intrathalamica. Scale bar is 2mm.

Figure 4: High magnifications of sections from figure 3. (A-H), serial pairs of sagittal sections of an E13.5 embryo, hybridized with the antisense probe to Dlx-2 (A-D), or labeled with FORSE-1 (E-H). (A, E) are high magnification pictures of a portion of Fig. 3 (C, I). (A, E), (B, F) and (D, H) all show Dlx-2 and FORSE-1 labeling in the ventral thalamus, while only FORSE-1 is seen in the dorsal thalamus. The zli in between is indicated by a double headed arrow. In addition, (D, H), high magnification pictures of a portion of Fig. 3 (E, K), show the FORSE-1-negative stria medullaris (arrow) near the junction of the telencephalon and diencephalon. (C, G) show the Dlx-2-positive medial ganglionic eminence (MGE). FORSE-1 is also present in the MGE, except for a sub region, indicated by the asterix. DT, dorsal thalamus; dv, diencephalic ventricle; MGE, medial ganglionic eminence; tv, telencephalic ventricle; VT, ventral thalamus; zli, zona limitans intrathalamica. Scale bar is 100μm.

Figure 5: FORSE-1 and Dlx-2 expression in the E13.5 CNS. (A, C) and (B,D), serial pairs of sections of an E13.5 embryo, cut as shown in the schematic, hybridized with the antisense probe to Dlx-2 (A,B), or labeled with FORSE-1 (C, D). FORSE-1 is present in the dorsal and ventral thalamus, but not the zli in between, while Dlx-2 is detected in the ventral thalamus (zli, arrows, A and C). The ventral limit of the ventral thalamus (dotted line, A) marks the boundary between the alar and basal plate of the diencephalon, and FORSE-1 labels a dorsoventrally-restricted region in this basal plate. The ventral most region of the basal plate is FORSE-1-negative (arrowheads, C). Both Dlx-2 and FORSE-1 are present in the LGE and MGE, though FORSE-1 is restricted within the MGE, and the sections in B and D pass through the FORSE-1-positive and FORSE-1-negative areas of the MGE (see schematic). Further ventrally, FORSE-1 labels both the POA and the POP, while Dlx-2 is not present in the POP (POP, region between dotted lines in B and D). FORSE-1 and Dlx-2 both respect the boundary between the POP and SCH (lower dotted line in B and D), FORSE-1 being present in the POP but not ventral to it, and Dlx-2 being detected in the SCH, but not immediately dorsal to it. FORSE-1 labeling of the nasal pits is also

seen in D. DT, dorsal thalamus; ET, epithalamus; Hb, hindbrain; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; np, nasal pits; POA, anterior preoptic area; POP, posterior preoptic area; SCH, suprachiasmatic area; VT, ventral thalamus; zli, zona limitans intrathalamica. Scale bar is 1mm.

Figure 6: A schematic representation of FORSE-1, Dlx-2, and BF-1 labeling in the E13.5 rat CNS. The FORSE-1 pattern is represented in blue, with the lighter shade representing a decreased amount of FORSE-1. The boundaries in the schematic are as proposed by Bulfone et al. (1993). 5A illustrates the FORSE-1 pattern overlaid with that of Dlx-2, in red, showing that FORSE-1 and Dlx-2 share several distinct boundaries and that FORSE-1 further subdivides some areas of the forebrain, like the MGE, and the basal plate of the diencephalon. 5B shows the FORSE-1 pattern overlaid with that of BF-1, in green, demonstrating the close correspondence between FORSE-1 and BF-1 in the telencephalon. AH, anterior hypothalamus; DT, dorsal thalamus; ET, epithalamus; EMT, eminentia thalami; Hb, hindbrain; HCC, hypothalamic cell cord; LGE, lateral ganglionic eminence; MA, mammillary area; Mb, midbrain; MGE, medial ganglionic eminence; p1-6, prosomeres of Bulfone et al. (1993); PEP, posterior entopeduncular area; POA, anterior preoptic area; POP, posterior preoptic area; RCH, retrochiasmatic area; RM, retromamillary area; SM, stria medullaris; SPV, suprapotic paraventricular area; SCH, suprachiasmatic area; TU, tuberal hypothalamus; VT, ventral thalamus; zli, zona limitans intrathalamica.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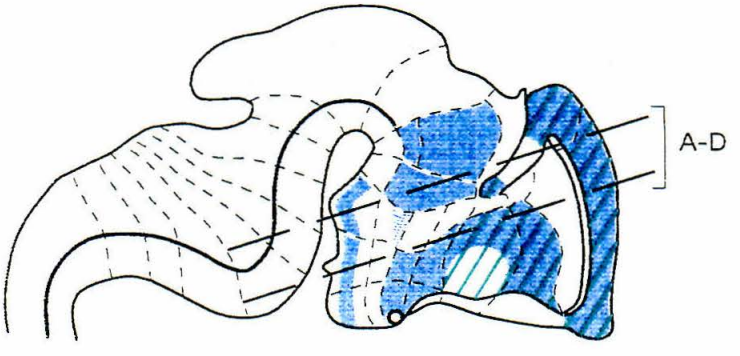
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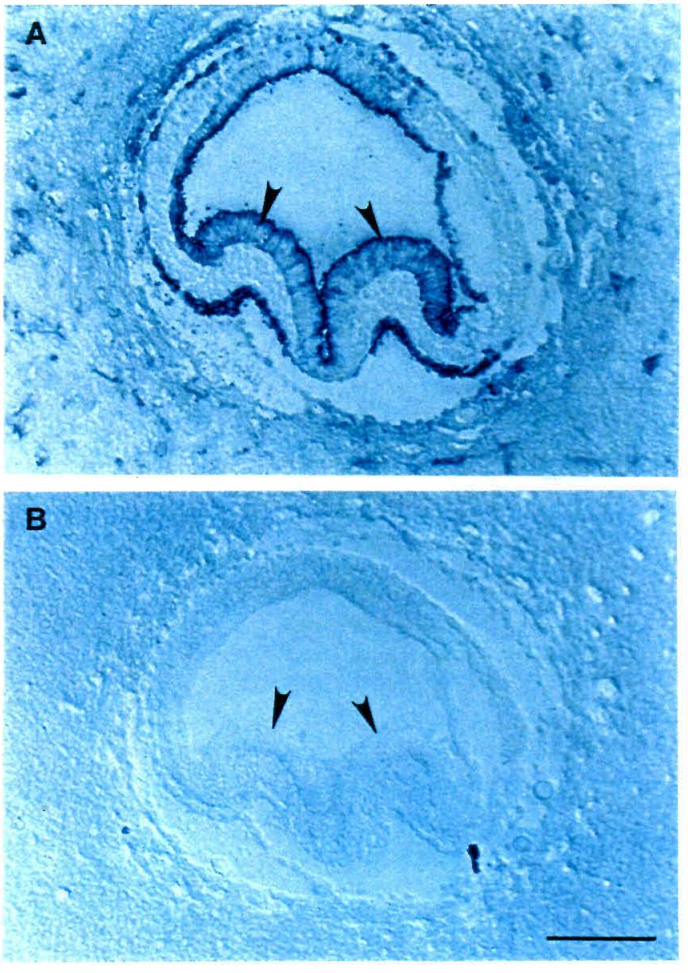
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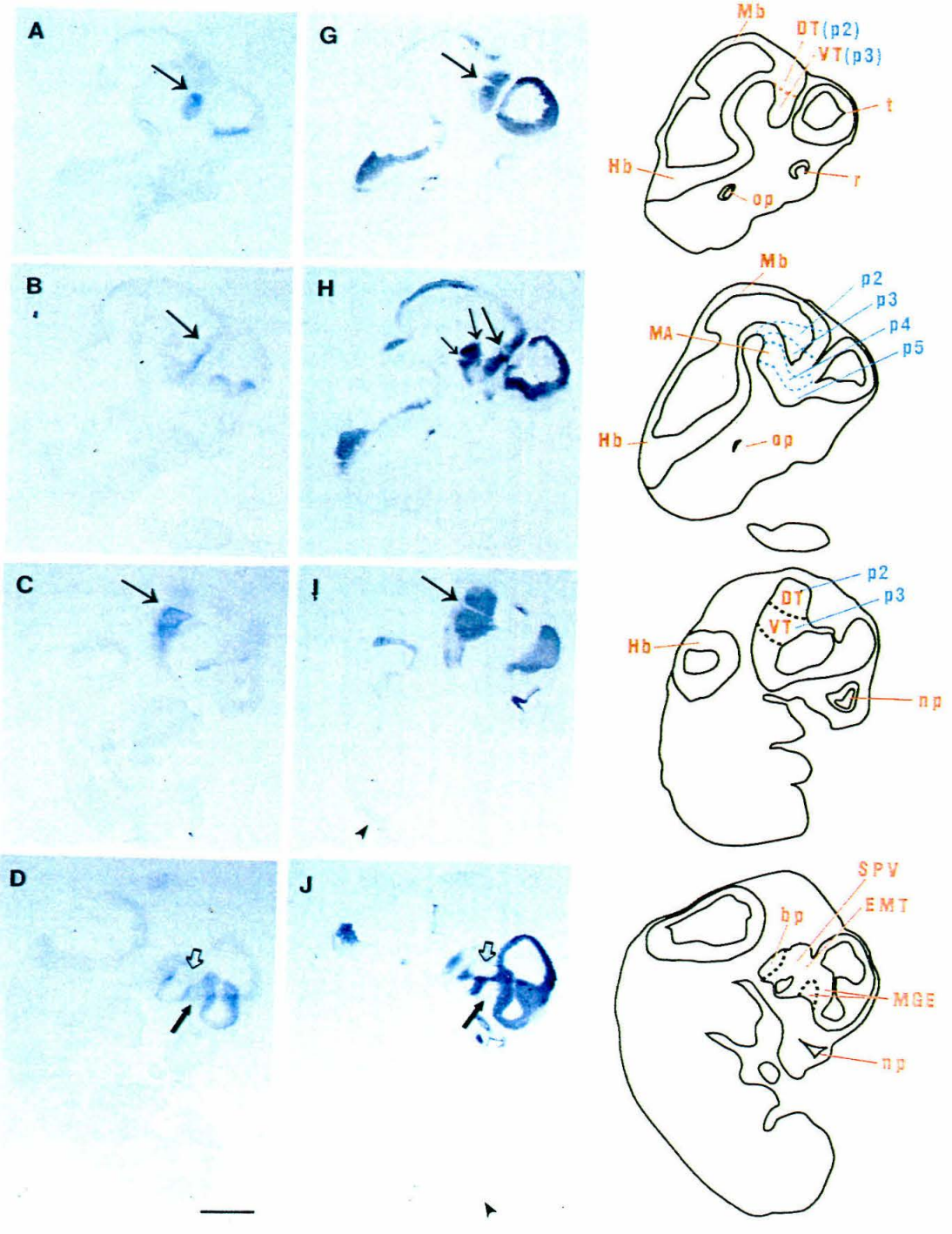
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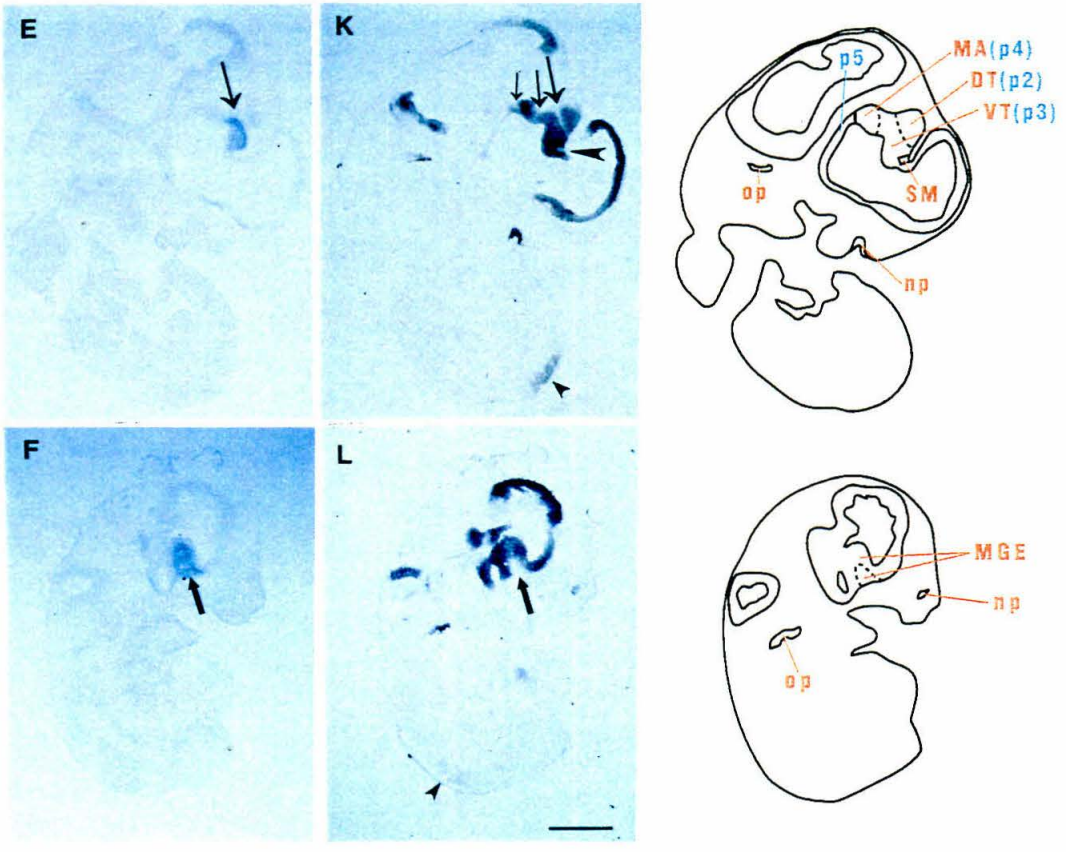
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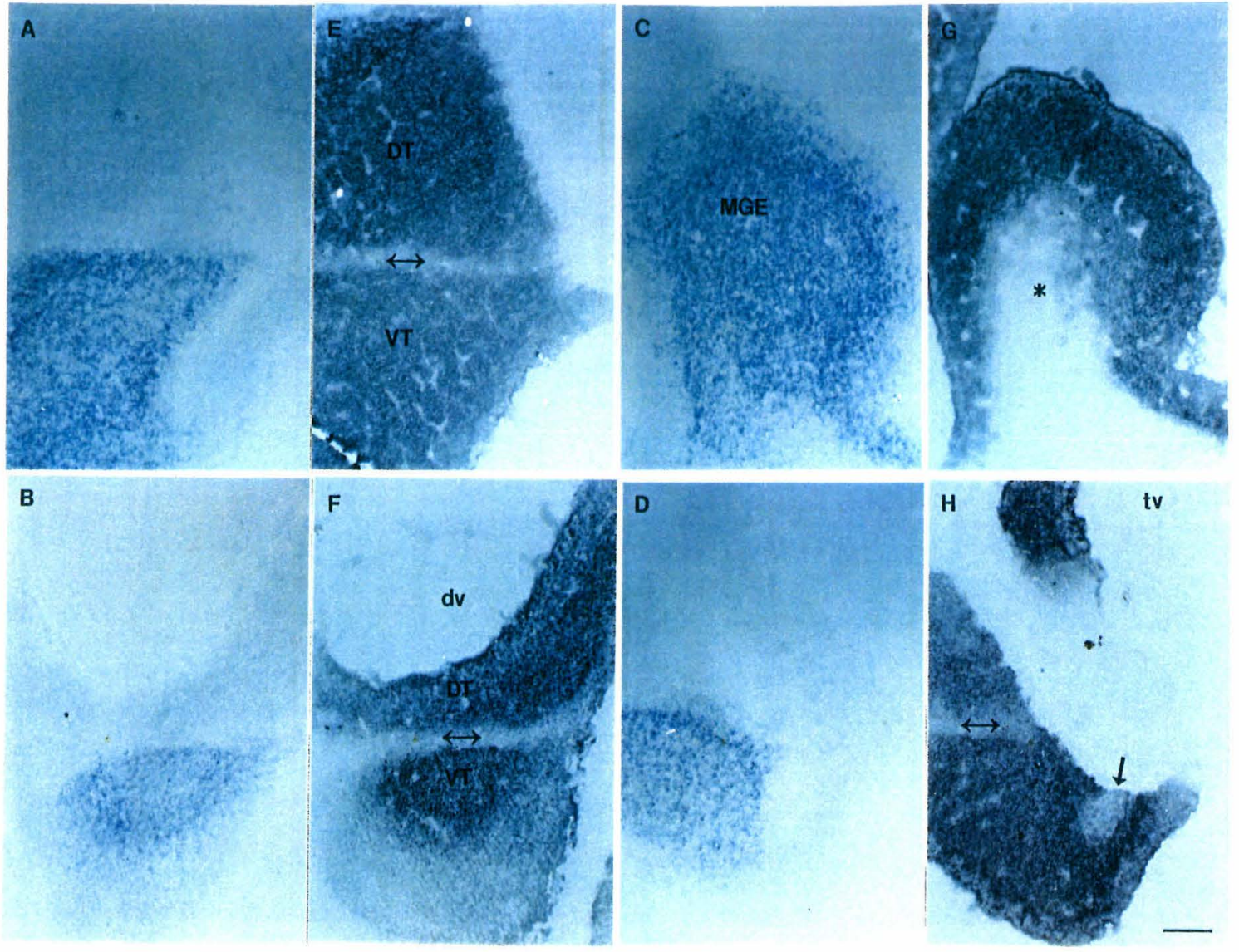
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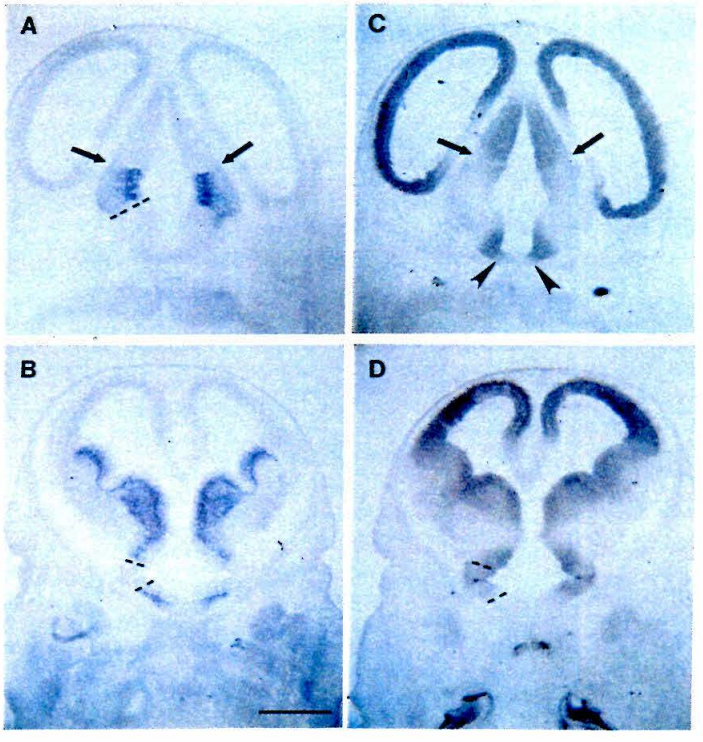
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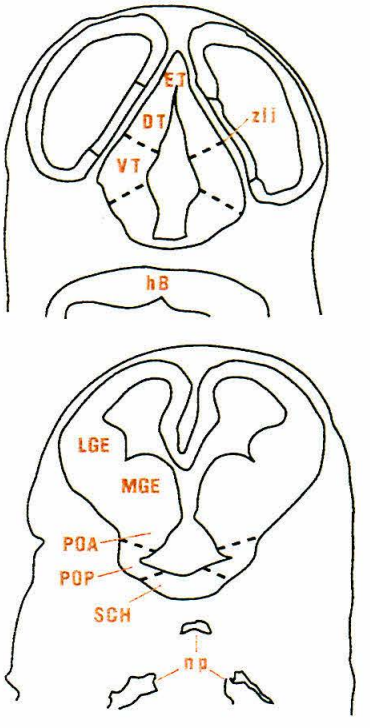
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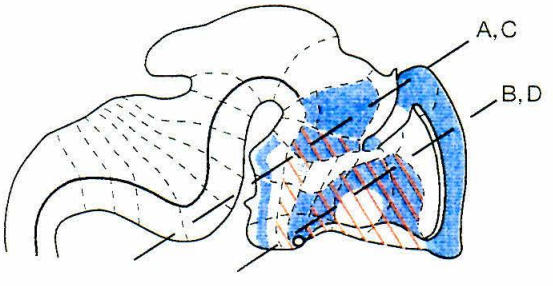
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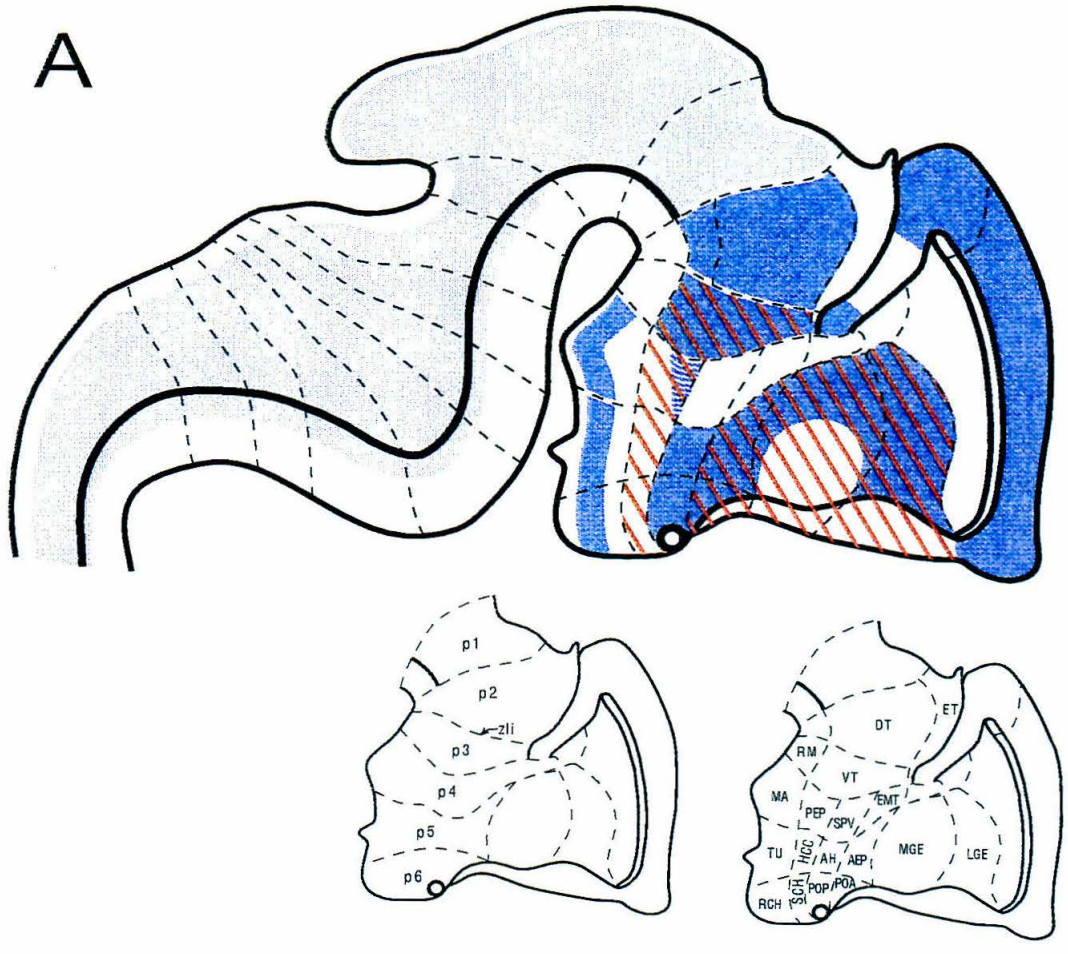
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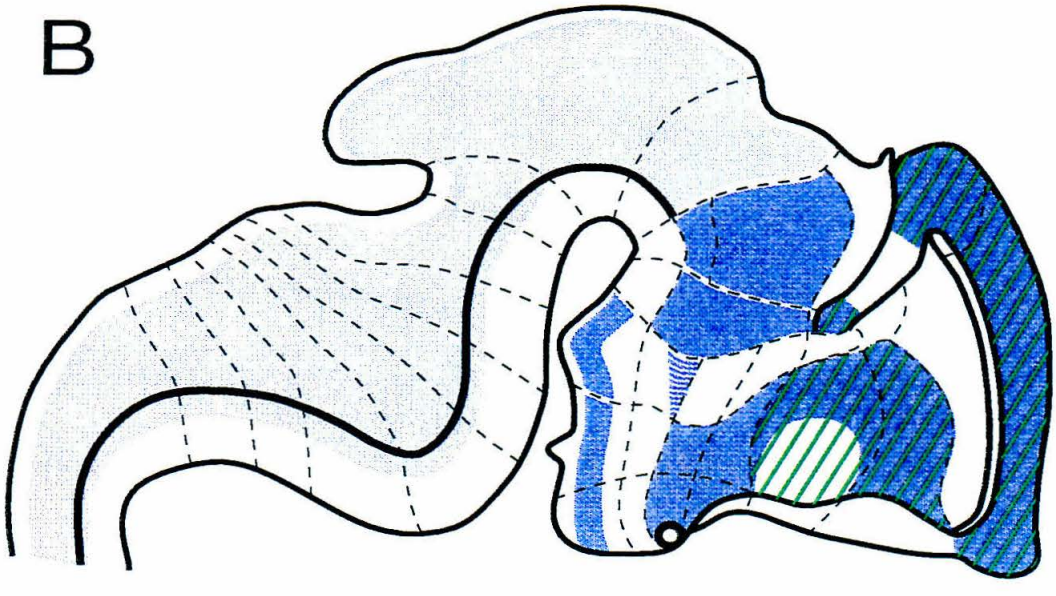
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Chapter 3

The FORSE-1 labeling pattern in other vertebrates

Introduction

All vertebrates share certain basic structural and functional features of the CNS in common, and the structural similarity is most pronounced in development. Moreover, the strategies for generating these features, e.g., mesodermal induction of neurectoderm, neural crest migration and placode induction, are conserved in all vertebrates. Comparative studying can yield insights into the hierarchy of developmental mechanisms. For example, notochord induction of dorsoventral patterning in the neural tube is found to be invariant across all vertebrates, and is therefore likely to be a fundamental part of the developmental plan. A feature found exclusively in one class of vertebrates, in contrast, is likely to serve a more specialized purpose. Furthermore, the study of different species may yield an opportunity for analyzing systems that are better developed or more accessible in a given species. Finally, studies of embryology and evolution can be mutually reinforcing, providing critical insights to the function and plasticity of the system.

In order to ask if the regionally restricted features of FORSE-1 labeling are conserved in vertebrates, we carried out a developmental study of FORSE-1 expression in the chick embryo, comparing, in particular, FORSE-1 labeling in the avian versus the mammalian telencephalon. Neocortex, a specialized mammalian dorsal telencephalic derivative, is absent in birds. Instead, a dorsal pallial structure, the dorsal ventricular ridge (DVR) is thought to perform some of the corresponding functions, along with the rest of the dorsal pallium. The DVR does not contain layers of neurons, like neocortex, but rather, has nuclei of cells thought to process thalamic input with a pattern of connectivity analogous to that between various cortical layers (Karten 1991). Thus the dorsal pallium of the telencephalon gives rise to a very different, though functionally analogous structure in birds. The rest of the CNS is structurally very similar in birds and mammals: basal telencephalon gives rise to basal ganglia; diencephalon forms various thalamic and hypothalamic structures; hindbrain and spinal cord generate neural crest and process sensory and motor activity.

We also examined the regulation of FORSE-1 staining in the spinal cord, by notochord, a powerful inducer of dorsoventral patterning.

This study forms the basis for future experimental manipulation of FORSE-1 expression in the chick.

Results and Discussion

FORSE-1 labeling in the developing chick CNS

There are many similarities in the FORSE-1 labeling pattern between the developing chick and rat. There is a rostrocaudal gradient of FORSE-1 labeling in which the chick forebrain is very intensely labeled, whereas hindbrain and spinal cord display weaker label (Fig. 1). Within the forebrain, there is regional restriction in the FORSE-1 pattern similar to that seen in rat, with some interesting differences. The chick telencephalon does not label until St. 21, while the diencephalon shows intense label in specific areas at St. 17 (Fig. 2). At St. 28 (Fig. 3), the telencephalon is strongly labeled in the basal region, with weak or undetectable label in the medial walls, a pattern very similar to the rat (Tole et al., 1994). Different from the rat, however, is the relative weakness of labeling of the dorsolateral walls of the telencephalic vesicles in the chick. There is a sharp transition between strong basal and weak dorsal label in the chick telencephalon, which correlates with the boundary between the dorsal and ventral pallium. Regions that give rise to the basal ganglia are located in the basal pallium, and regions that give rise to the DVR and other dorsal structures are found in the dorsal pallium. There is preliminary evidence that the dorsal-ventral pallial boundary may be a barrier to cell migration (Montgomery et al., 1993). Later in development, FORSE-1 reveals a difference in the ventricular zones (vz) of the dorsal and ventral pallium, illustrated in sections at St. 37 (Fig. 5), where the dorsal pallial vz shows intense, compact staining that appears to line the cavity of the ventricle as well as penetrate into the vz; the ventral vz, in contrast, displays more diffuse label that does not appear to line the cavity of the ventricle. In addition, postmitotic neurons in the rest of the telencephalon label weakly for FORSE-1, similar to the pattern seen in the rat CNS after E18 (Tole et al., 1994).

The chick diencephalon displays FORSE-1-positive and -negative regions from St. 17 (Fig. 1). This pattern persists through St. 29 (Fig. 4). As in the rat, the FORSE-1-negative areas are often narrow gaps between two well-labeled areas (arrows, Fig.1-4), and may correspond to the cell migration boundaries observed in chick by Figdor and Stern (1993). One difference between the chick and rat diencephalon is that FORSE-1 labeling is continuous along the vz of the chick diencephalon, and is often seen even in

the vz of the otherwise FORSE-1-negative gap. In rat, FORSE-1 labeling contributes to the intricate pattern of subdivisions in which forebrain regions display unique patterns of gene expression (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Tole and Patterson, 1994). The lack of detailed localization of markers identifying these areas in chick prevents us from unambiguously identifying the FORSE-1-positive and -negative parts of the forebrain. In addition, morphologic landmarks are somewhat obscured in our sections because of the use of fresh-frozen, unfixed embryos; the FORSE-1 epitope is sensitive to fixatives that are used for whole embryos.

The chick midbrain labels well with FORSE-1. Label is seen in the entire neuroepithelium at St. 17, and in varying intensities in specific layers of the St. 29 and St. 37 tectum (Fig. 6). The hindbrain and spinal cord show significantly less staining than the forebrain. The intensity of labeling diminishes along the rostrocaudal axis. As in rat, staining in both hindbrain and spinal cord is dorsoventrally restricted (Fig. 1-4). In order to test if the dorsoventral domain of expression is regulated by notochord, we collaborated with Dr. Andrew Groves to remove a caudal section of the notochord in the region of the caudal neural plate, prior to closure at St. 10. The embryos were allowed to develop an additional 5 days to reach approximately St. 26, and were examined for FORSE-1 expression in the spinal cord in the region of the operated site. Successful excision of the notochord was confirmed by labeling adjacent sections with FP1, a floorplate marker, which is not induced in the absence of notochord (Yamada et al., 1991). Sections rostral to the operated site appear normal, displaying ventral motor columns and the FORSE-1-positive centrum around the notochord. FP1 labeling confirms the presence of the floorplate in adjacent sections. In the operated, notochordless region, the neural tube appears rounded, without differentiated motor columns. FP1 labeling is undetectable, confirming that the floorplate was not induced. Since the corresponding mesoderm was excised along with the notochord, the FORSE-1-positive centrum is missing. Despite these changes, we observe no difference in the extent or intensity of the dorsoventral restriction of FORSE-1 label in the spinal cord. Thus, unlike *dorsalin-1, Pax-3 and Pax-6* (Basler et al., 1993; Goulding et al., 1993), FORSE-1 appears to be regulated by a notochord-independent mechanism. Such a mechanism would mask any possible regulation of FORSE-1 by the notochord itself. These results suggest that

dorsoventral patterning in the spinal cord is the result of a complex regulatory mechanism.

Conclusions

FORSE-1 labeling in the developing chick shares many key features with that in the developing rat, including rostrocaudal and dorsoventral restriction, as well as regional specificity in the forebrain. There are, however, a few interesting differences in telencephalic labeling between the two species. An additional novel finding is that FORSE-1 may be dorsoventrally restricted in the spinal cord by a notochord-independent mechanism.

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Figure Legends

Figure 1: FORSE-1 labeling in the St. 19 chick. A-E are fresh frozen sections of a St. 19 chick embryo stained with FORSE-1 (A, C-E) showing label in parts of the forebrain (A), hindbrain (C) and spinal cord (D, E). B is a phase contrast view of the section in A. The intensity of staining diminishes along the rostrocaudal axis. The eye cups (e), seen in A, do not label with FORSE-1.

Figure 2: FORSE-1 labeling in the St. 17 chick. A-F are acetone-fixed sections of a St. 17 chick embryo stained with FORSE-1. In the forebrain, the telencephalon (t) does not label with FORSE-1 while parts of the diencephalon (d) show strong label (A-C). The hindbrain (hb) and spinal cord (sc) label in a dorsoventrally restricted manner (A-F). The eye cups (e), seen in A, do not label with FORSE-1.

Figure 3: FORSE-1 labeling in the St. 28 chick. Fresh frozen sections of a St. 28 chick labeled with FORSE-1 reveal staining in the basal region of the telencephalon (t) and parts of the diencephalon (d). In the telencephalon, FORSE-1 label is almost undetectable in the medial and dorsolateral walls. The staining of the pigment layer of the retina is non specific. Neg. indicates negative control sections labeled with an irrelevant primary antibody.

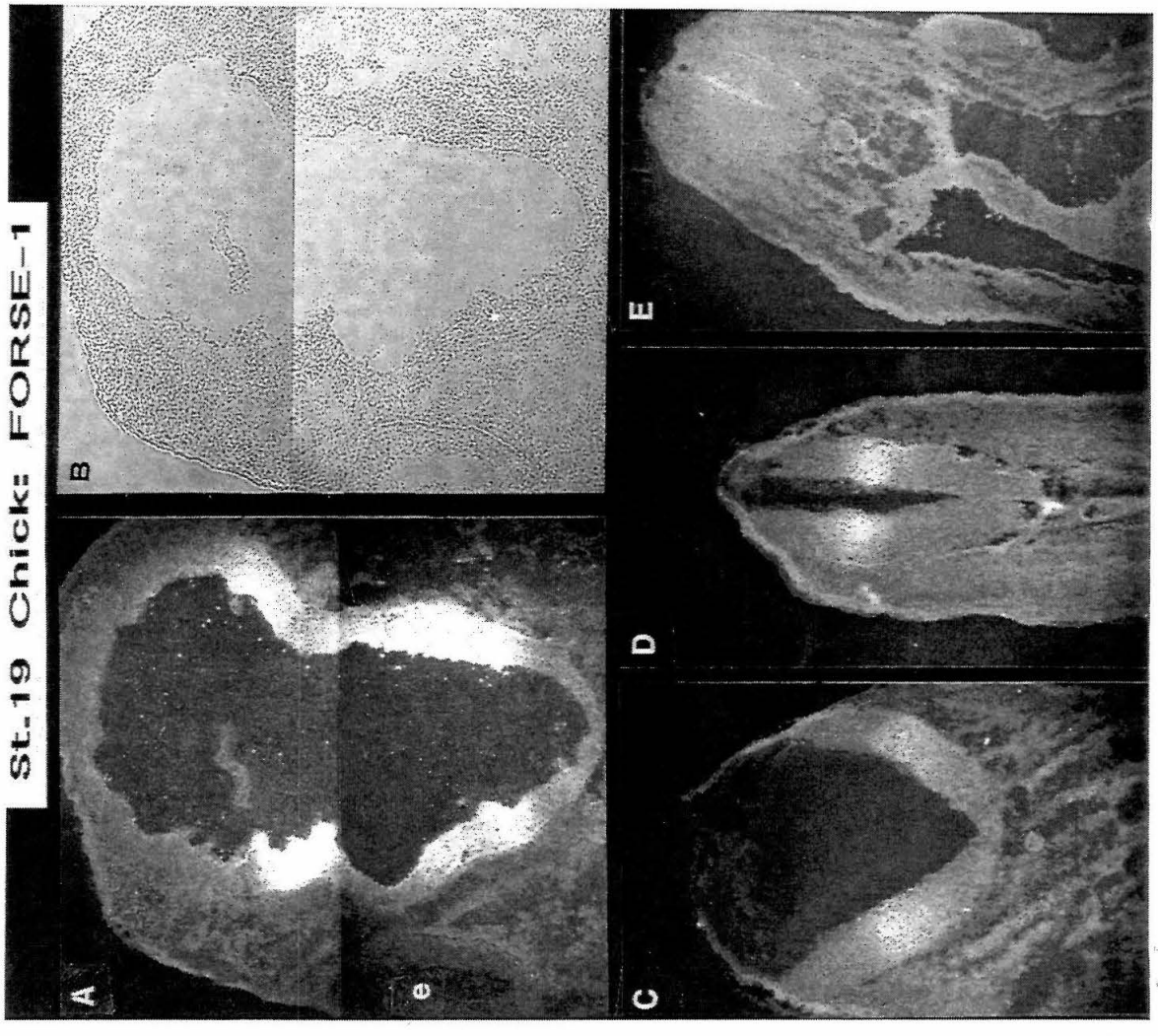
Figure 4: FORSE-1 labeling in the St. 29 chick. A-F, acetone-fixed sections of a St. 29 chick embryo reveal labeling in parts of the telencephalon and diencephalon, but the hindbrain (hb), also present in these sections, is labeled only in the region lining the ventricle. Forebrain labeling is punctuated by gaps of reduced or undetectable staining, indicated by thick arrows. The dorsal part of the telencephalon labels less intensely than the basal region, while the medial walls (thin arrows) do not label at all at specific levels of sectioning (D-F).

Figure 5: FORSE-1 labeling in the St. 37 chick. A-C, acetone-fixed sections of a St. 37 chick brain reveal weak label throughout the tissue and strong label in the ventricular zone (vz). The vz of the DVR labels intensely while that of the basal telencephalon (bt) labels more diffusely.

Figure 6: FORSE-1 labeling in the midbrain. A-C, acetone-fixed sections of midbrain (mb) of different stages. At St. 17, the entire neuroepithelium labels intensely. At St. 29 and 37, different layers of the tectum label with varying intensities.

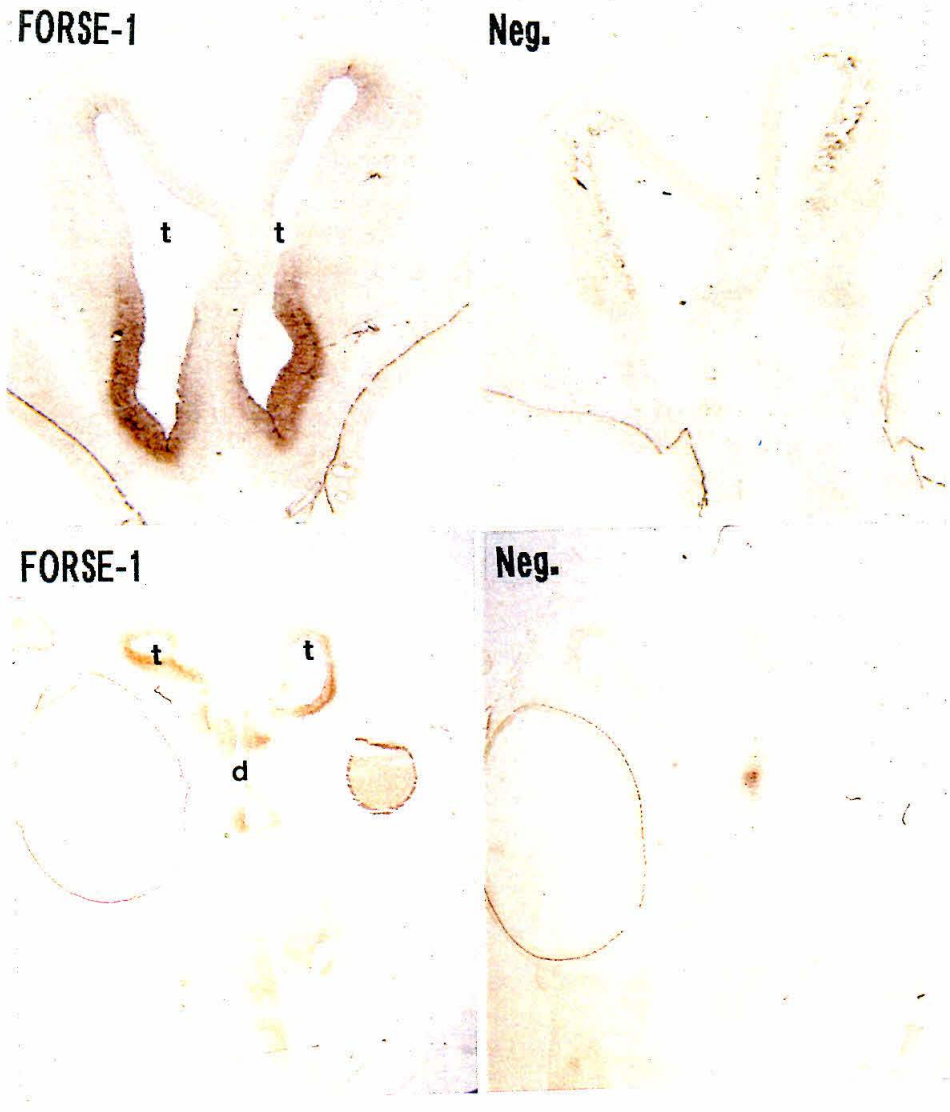
Figure 7: FORSE-1 labeling in the St. 26 spinal cord. A (brightfield) and B (DIC) are acetone fixed sections of St. 26 chick spinal cord, showing a dorsoventrally restricted region of staining, while the rest of the spinal cord is unlabeled. FORSE-1 also labels the cartilage of the centrum around the notochord (n).

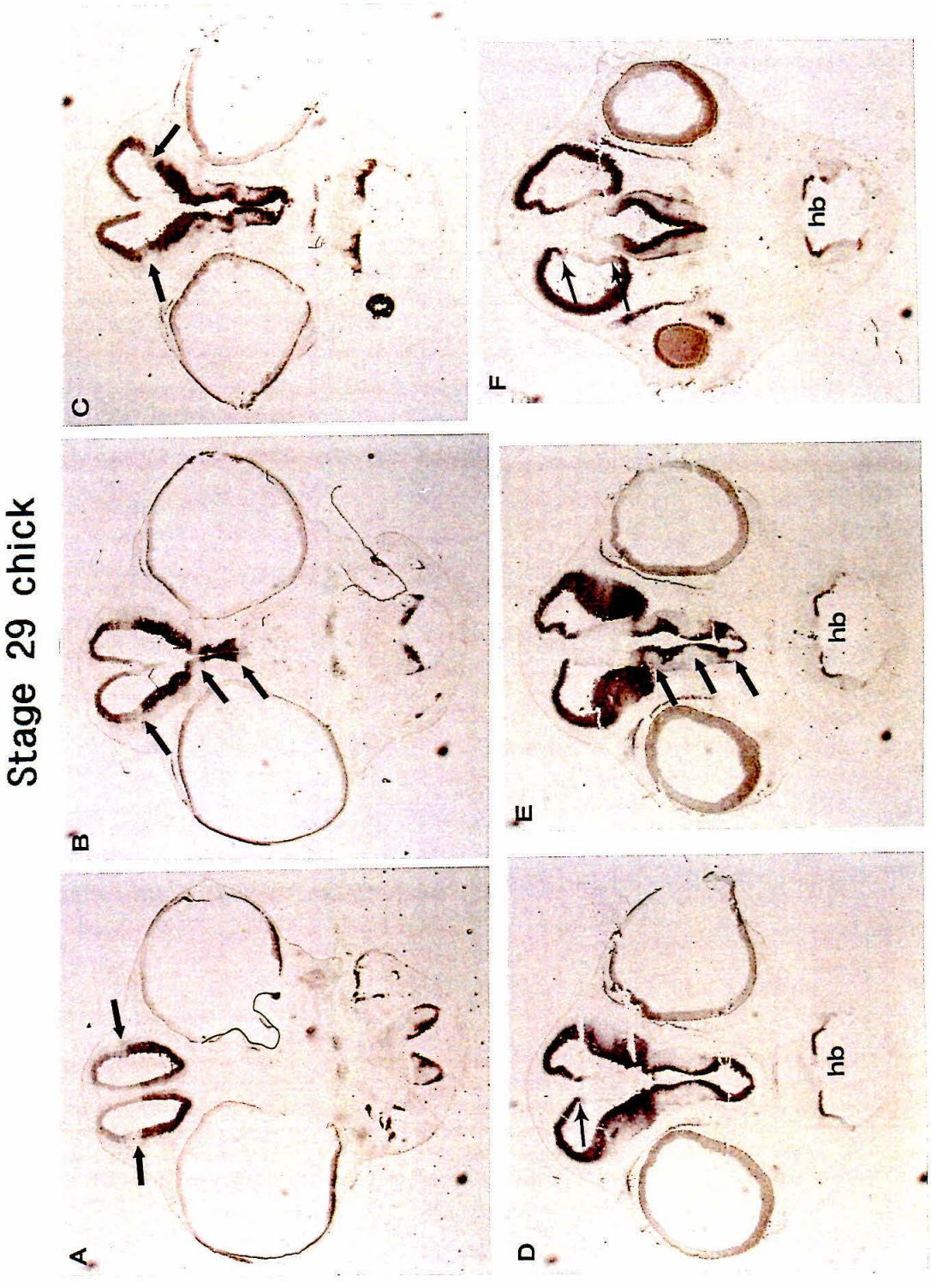
Figure 8: FORSE-1 labeling in the operated chick spinal cord. A-D, acetone-fixed sections of St. 26 chick embryos through a normal region (A, C) and through the operated, notochordless region (B, D). A and B are labeled with FORSE-1, and show similar dorsoventrally restricted patterns of staining. The FORSE-1-positive centrum as well as the notochord (n) are absent in B. C and D are serial sections to A and B respectively, labeled with a combination of FORSE-1 and FP1. The sections have high background in the spinal cord, but the FP1-positive floorplate, intensely labeled in C, is absent in D.


St.19 Chick: FORSE-1
A
B
C
D
E

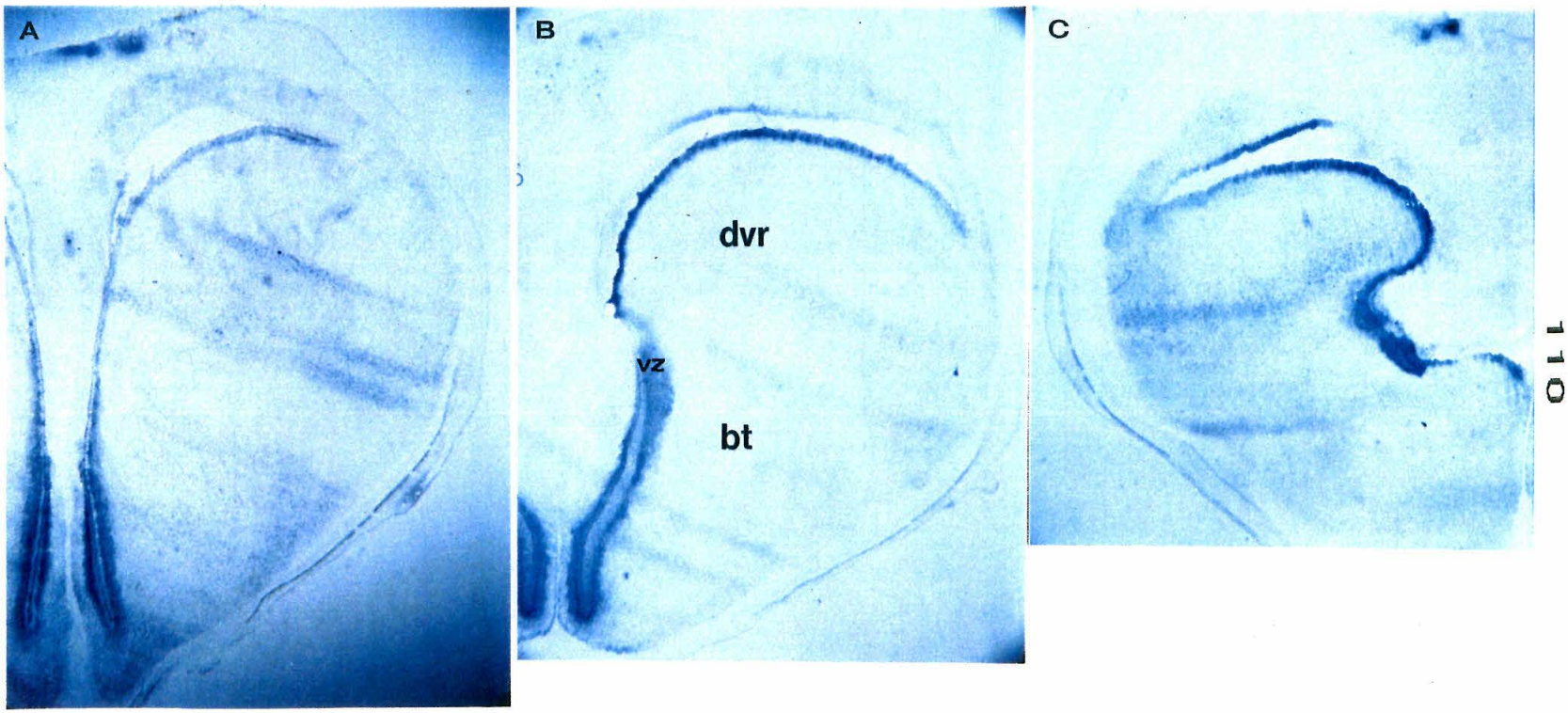
Stage 17 Chick: FORSE-1
A
t
B
t
d
C
e
hb
D
hb
E
F
sc

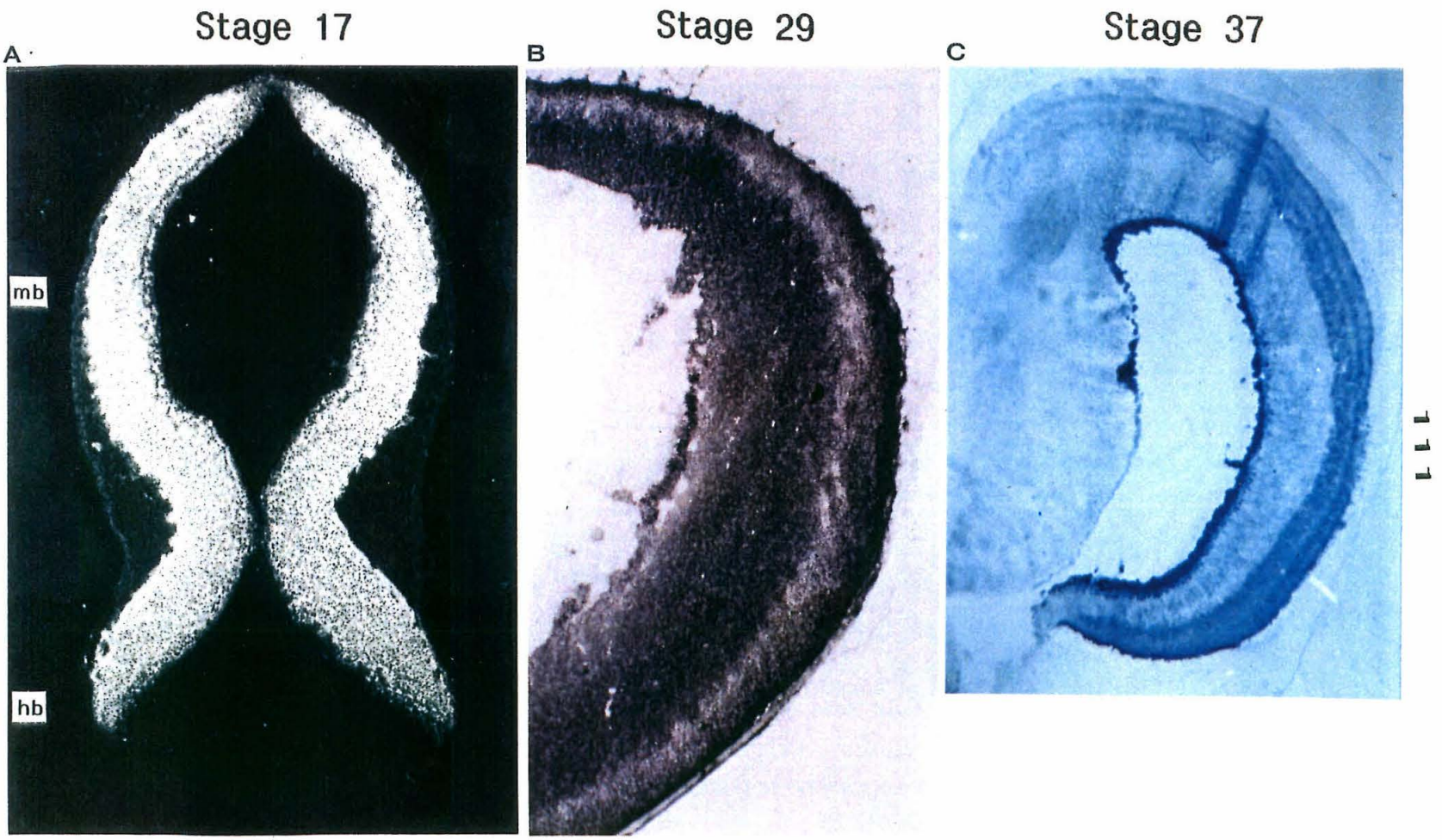
Stage 28 chick



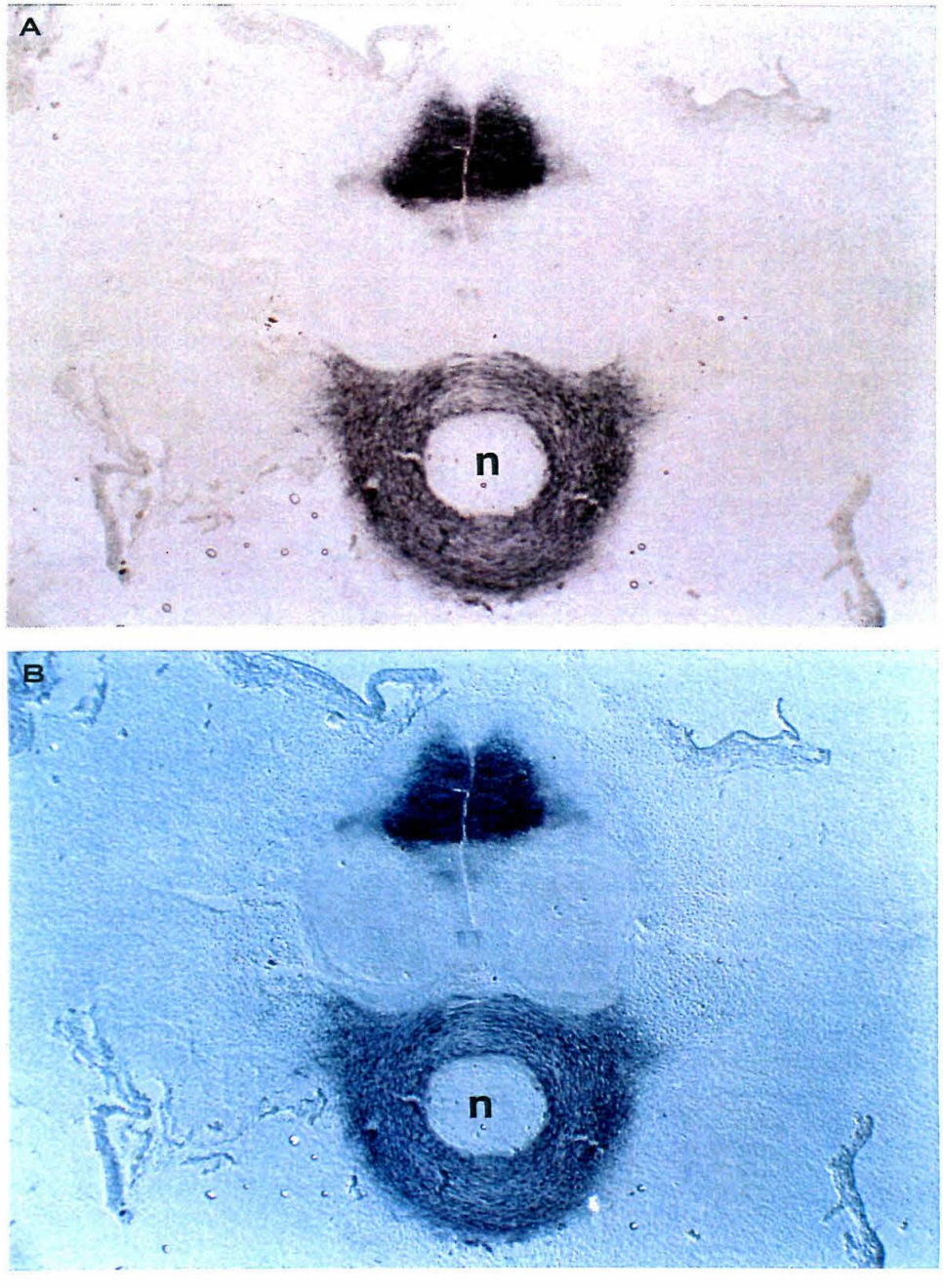

Stage 29 chick
A
B
C
D
E
F
hb
hb
hb

Stage 37 chick



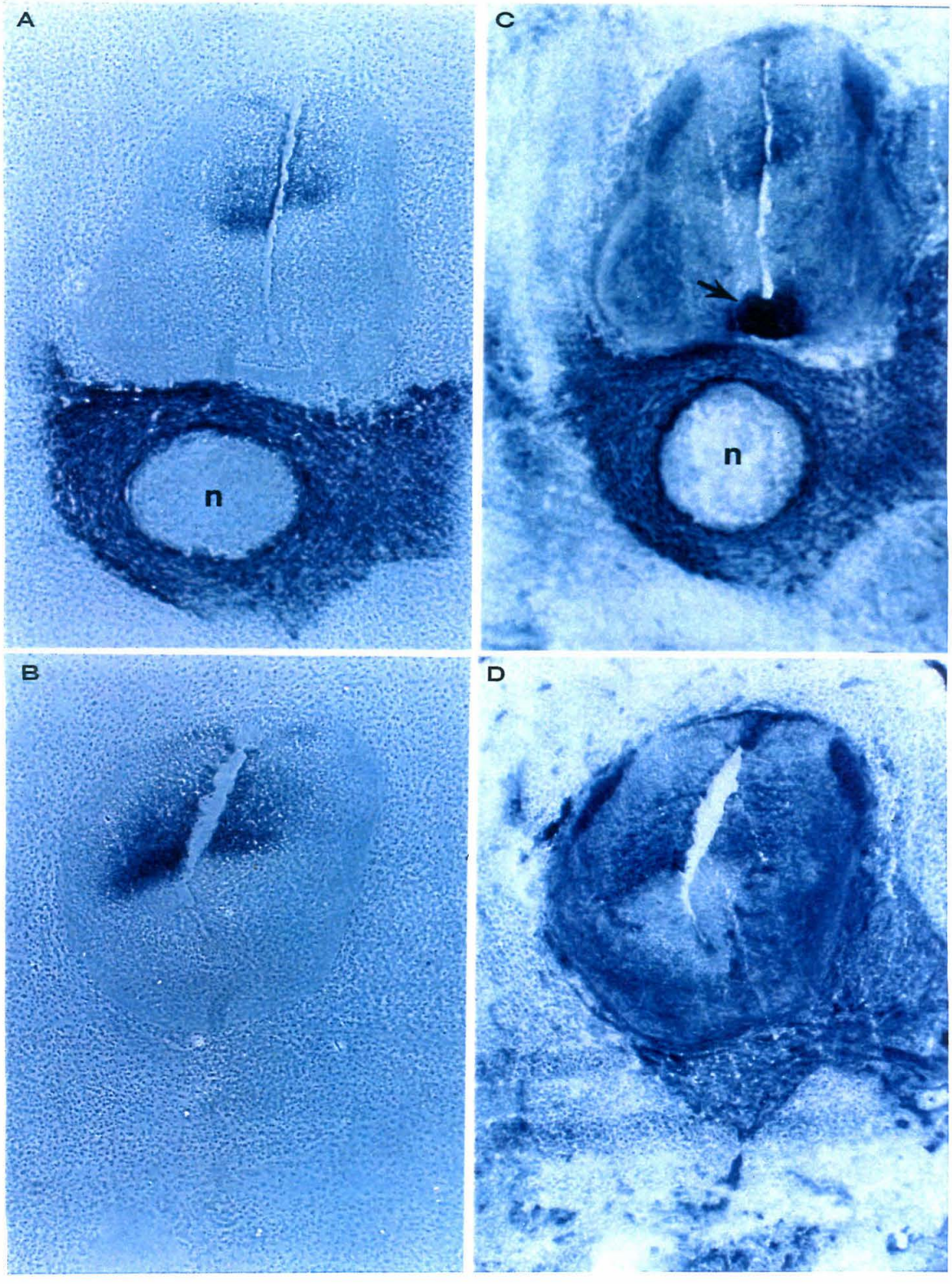

Stage 17
Stage 29
Stage 37
A
B
C
mb
hb

FORSE-1



FORSE-1

FORSE-1 & FP-1



Chapter 4

Forward

In this project, we focused on the adult sympathetic system to identify molecules that could be involved in specifying rostro-caudal position.

The sympathetic division of the autonomic nervous system displays a well characterized specificity in the innervation of sympathetic neurons in the ganglia by preganglionic axons from the spinal cord (Langley, 1892; Purves and Lichman, 1978). Rostral preganglionic axons tend to innervate rostral ganglia while caudal preganglionic axons tend to innervate caudal ganglia, even after transection in the adult (Nja and Purves, 1977). This specificity is not merely a matter of closer access of the preganglionic axons to ganglia at the corresponding rostrocaudal level. Purves et al. (1981), demonstrated that caudal sympathetic ganglia transplanted to rostral levels are reinnervated by a more caudal population of preganglionic axons than the original rostral ganglion that has been removed.

To identify molecules that may play a role in this specificity, mAbs were generated using the cyclophosphamide immunosuppression protocol. Membrane preparations from caudal ganglia were injected into mice followed by the immunosuppressive agent, cyclophosphamide; subsequently, the mouse was immunized with membrane preparations from rostral ganglia. One mAb from this fusion, ROCA1 (ROstroCAudal-1), labels sections of adult rostral sympathetic ganglia intensely, and the labeling becomes progressively weaker in caudal ganglia (Suzue et al, 1990). The antigen responsible for the immunohistochemical gradient has been purified, and the cDNA cloned and sequenced. It is the rat homolog of CD9 (Kaprielian and Patterson, 1993), a 4-transmembrane domain protein previously identified in the hematopoietic system. Kaprielian and Patterson (1993) showed that the protein is not differentially distributed in sympathetic ganglia from different axial levels. The ROCA1 epitope is graded, however, possibly because of differential masking at various rostrocaudal levels. Other mAbs against rat CD9, ROCA2 and B2C11 recognize epitopes that are equally well labeled in sections of rostral and caudal sympathetic ganglia. The ROCA2 and B2C11 findings support the uniform distribution of CD9 in all axial levels, and highlight the rostrocaudal gradient of the ROCA1 epitope.

In the hematopoietic system, CD9 has been implicated in intercellular signaling. Anti-CD9 mAbs cause activation and aggregation in platelets, a phenomenon involving an association between CD9 and the GPIIb-IIIa

integrin. (Slupsky et al., 1989; Jennings et al., 1990; Masellis-Smith et al., 1990; Forsyth et al., 1991; Griffith et al., 1991). In the nervous system, immobilized anti-CD9 mAb B2C11 specifically causes adhesion of Schwann cell lines, primary Schwann cells and PC12 cells. In addition, the same mAb promotes robust proliferation of S16 Schwann cells (Hadjiargyrou et al., 1994).

Based on these *in vitro* studies, CD9 is likely to be a surface molecule that can be involved in a variety of functions depending on where and when it is expressed. In this chapter, I describe which cell types express the protein in the embryo, and determine if CD9 is present early enough to participate in intercellular communication in development.

An important question that was part of the original motivation for this study is whether the ROCA1 epitope is rostrocaudally graded in embryonic sympathetic ganglia. This issue cannot be resolved due to additional complexity involving the ROCA1 epitope. This epitope is also present on peripherin (Kaprielian and Patterson, 1993), an intermediate filament protein present in the cytoplasm of peripheral neurons (Parysek and Goldman, 1987). In embryonic as well as early postnatal ganglia, ROCA1 binds well to cytoplasmic peripherin (Tole, unpublished observations; Kaprielian and Patterson, 1993), obscuring any possible gradient in the labeling of cell surface CD9. In immunohistochemical labeling of adult sympathetic sections, however, ROCA1 does not bind to cytoplasmic peripherin, but only binds to cell-surface CD9. That these neurons do contain peripherin can be shown by labeling with anti-peripherin antiserum (Tole, unpublished observations; Kaprielian and Patterson, 1993).

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Distribution of CD9 in the Developing and Mature Rat Nervous System

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ABSTRACT **CD9 is a cell surface protein implicated in intercellular signaling that has been identified in selected cell types of the hematopoietic system. To begin a study of the role of CD9 in the developing and adult nervous system, we used the anti-rat CD9 monoclonal antibody ROCA2 to determine the distribution of this protein. The identity of the antigen in these tissues was confirmed by immunoblotting and peptide sequencing. Early embryonic sympathetic and dorsal root ganglion sensory neurons and adrenal chromaffin cells all express CD9. ROCA2 also labels the somas, axons, and growth cones of cultured sympathetic and sensory neurons. In the central nervous system (CNS), CD9 is transiently and specifically expressed in embryonic spinal motorneurons. In the adult, central and peripheral glia intensely express CD9. Thus, CD9 is developmentally regulated in a variety of peripheral and central neurons and glia, including proliferating progenitors as well as mature cells. These findings suggest that CD9 may have diverse roles in the nervous system.**

Key words: Sympathoadrenal, Sensory neuron, Motorneuron, Schwann cell

INTRODUCTION

ROCA2 is a monoclonal antibody (mAb) derived from a fusion used to identify positional markers in the peripheral nervous system (Suzue et al., 1990). Recent work has shown that ROCA2 binds to a 26 kD protein identified as CD9 (Kaprielian and Patterson, 1993). The identification of the ROCA2 antigen as CD9 indicates that this integral membrane protein is expressed by peripheral glia and adrenal chromaffin cells of the adult rat (Suzue et al., 1990; Kaprielian and Patterson, 1993). Prior studies had shown that CD9 is also present on the surfaces of human platelets, pre-B cells, and monocytes (Kersey et al., 1981; von dem Borne et al., 1989), as well as in several non-hematopoietic tissues (Jones et al., 1982; Boucheix and Benoit, 1988). Functional experiments indicate that CD9 is involved in the activation and aggregation of platelets and pre-B cells (Jennings et al., 1990; Masellis-Smith et al., 1990; Griffith et al., 1991), and there is evidence that this occurs through an interaction of CD9 with the GPIIb-IIIA integrin, within the plane of the platelet membrane (Boucheix et al., 1983; Higashihara et al., 1985; Miller et al., 1986; Slupsky et al., 1989).

Since CD9 could have a related signaling role in the nervous system, it is important to determine more precisely where, and over what developmental time course, it is expressed. In addition to our work on the peripheral nervous system, antibodies to CD9 have been shown to bind to bovine retina (Martin-Alonso et al., 1992), to certain retinoblastoma and neuroblastoma cell lines (Kemshead et al., 1982; Komada et al., 1983), and to white matter in the brain (von dem Borne et al., 1989). Using immunohistochemistry and immunoblotting, we here show that CD9 is present in very early embryonic rat sympathetic neuroblasts and adrenal chromaffin cells; later in development it is expressed at high levels in dorsal root ganglia and peripheral glia. Cultured sympathetic neurons display CD9 on the surfaces of their somas, as well as on axonal and growth cone membranes. There is also an interesting transient expression of CD9 in the embryonic spinal cord. The heterogeneity in cell types that express CD9, as well as its developmental regulation, suggest that CD9 may have more than one function in the nervous system.

RESULTS AND DISCUSSION

Since the peripheral nervous system largely arises from the neural crest, our observations are initiated at an age prior to neural crest migration (E10.5), and several neural crest derivatives are followed in development. The central nervous system (spinal cord) is also studied at each age. Migrating neural crest cells are identified by mAb 192 IgG, which binds to the low affinity nerve growth factor receptor (NGFR; Bernd, 1986). Cells of the sympathoadrenal lineage are identified by mAb SA2 (Carnahan and Patterson, 1991). Neurons of the peripheral nervous system are identified by anti-peripherin (Parysek and Goldman, 1987), as are motorneurons in the spinal cord. Neurons of both the central and peripheral nervous system are identified by a mAb against the 68 kD subunit of neurofilament (NF68). Myelinating Schwann cells are identified by a mAb against myelin basic protein (MBP; Martini and Schachner, 1986).

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E11.5 Embryo

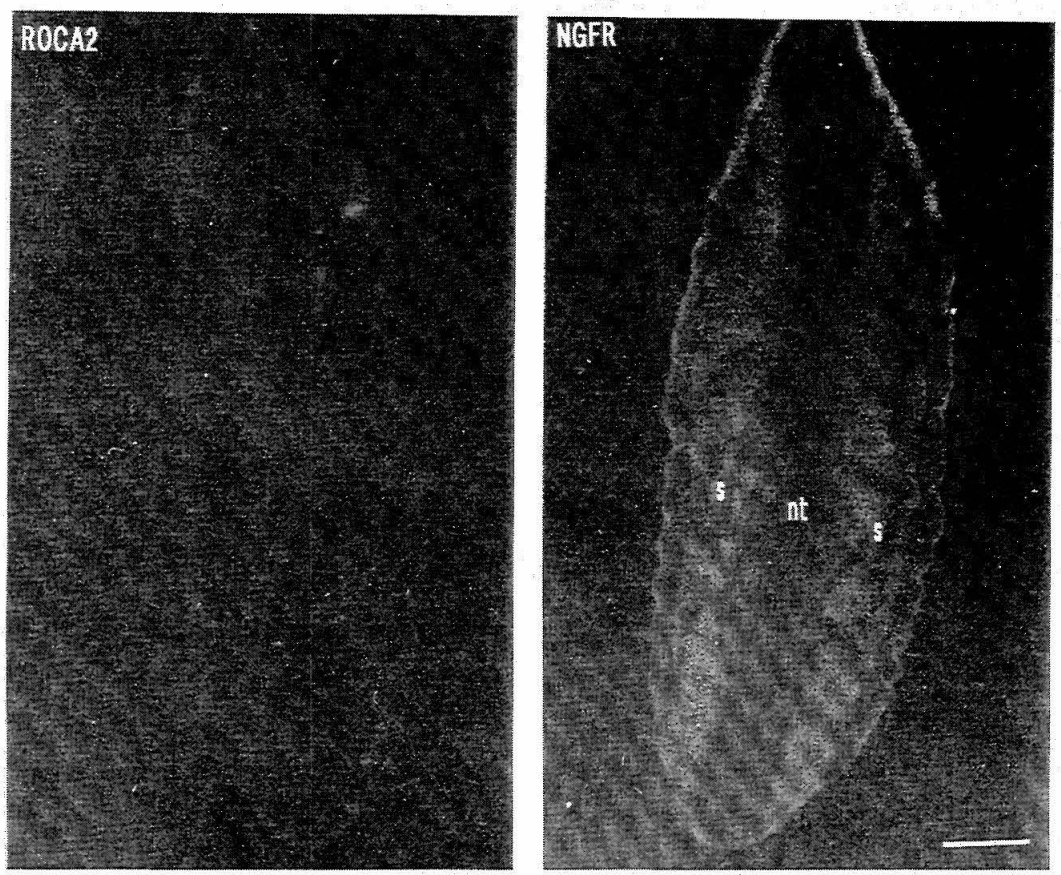


Fig. 1. ROCA2 does not appear to label migrating neural crest cells. Serial sections of an E11.5 embryo, cut in the horizontal plane, and labeled with ROCA2 and 192 IgG (NGFR) show NGFR positive neural crest cells in the rostral half of the somites, on either side of the neural tube. ROCA2 does not detectably label these cells. Scale bar is 200 μm. nt, neural tube; s, somite. Rostral is toward the top of the picture.

Neural Crest

Since ROCA2 binds adult sympathetic ganglia intensely (Suzue et al., 1990), we determined when this labeling begins in embryonic development. Premigratory (E10.5) and migrating (E11.5) neural crest cells, identified by the binding of mAb 192 IgG against the low affinity NGFR, do not detectably bind ROCA2 (Fig. 1).

Sympathetic and Sensory Ganglia

The earliest appearance of ROCA2 staining in the embryonic peripheral nervous system is in the forming sympathetic ganglia (E12; Fig. 2). This parallels the labeling of mAb SA2, which is also undetectable on migrating crest cells and labels forming sympathetic ganglia brightly (Carnahan and Patterson, 1991). Three lines of evidence indicate that the cells labeled in these ganglia are neuroblasts. First, $CD9^+$ cells are also labeled by an antiserum against the peripheral neuron-specific intermediate filament protein, peripherin (Parysek and Goldman, 1987) (Fig. 2). Second, $CD9^+$ cells are also stained by mAb SA2, a marker specific for sympathoadrenal progenitors in sympathetic ganglia and the adrenal medulla (not shown; Carnahan and Patterson, 1991). Third, lineage studies suggest that glial progenitors do not proliferate in these ganglia until embryonic day 16 (E16; Hall and Landis, 1992), and therefore are not a major proportion of the cells in the ganglion at E12. At E12, ROCA2

appears to label the entire SA2$^+$/peripherin$^+$ sympathetic population (not shown; Fig. 2). Since CD9 is an integral membrane protein and peripherin is a cytoplasmic intermediate filament, the labeling of the sympathetic ganglion in Figure 2 is not identical for ROCA2 and peripherin, but it is clear that the majority of the staining is on sympathetic neuronal progenitors. ROCA2 continues to label sympathetic ganglia throughout development, and into adulthood. Staining of live, cultured neonatal sympathetic neurons reveals cell surface labeling, with specific ROCA2 binding on cell bodies, axons, and growth cones (Fig. 3). Staining is maintained for at least 3 weeks in culture. Neonatal sympathetic neurons also bind ROCA2 when stained live, and freshly dissociated (not shown), as do chromaffin cells (Fig. 3; see below). Therefore, embryonic and neonatal sympathetic neurons express cell surface CD9. Non-neuronal cells (satellite cells and Schwann cells) also label with ROCA2 and the intensity of labeling increases with myelination (see below) so that in the adult, the sympathetic ganglion labels extremely intensely with ROCA2 (Suzue et al., 1990). Due to this strong non-neuronal cell surface labeling, it is not possible to determine, using immunohistochemistry, if adult sympathetic neurons also label with ROCA2.

Dorsal root ganglia are not labeled by ROCA2 until E15 (Fig. 2), even though neurons are present and label with anti-peripherin antiserum at E12 (Fig. 2; Escurat et al., 1990). As in sympathetic ganglia, most of the ROCA2 staining at E15 appears to be on the surfaces of neurons, since live, freshly dissociated as well as cultured sensory neurons bind ROCA2 on their surfaces (not shown). It is curious that ROCA2 labels sympathetic but not dorsal root ganglia at E12, even though the latter ganglia are well formed at that stage. Both sympathetic and dorsal root ganglion neurons are neural crest derivatives, and at least some neurons in each type of ganglion come from a common progenitor in the crest (Bronner-Faser and Fraser, 1988). It has been demonstrated, however, that sympathetic neurons differentiate and acquire neuronal markers while they are still dividing, while dorsal root ganglion neurons differentiate only after they become postmitotic (Rohrer and Thoenen, 1987; DiCicco-Bloom et al., 1990). This interpretation portrays CD9 as a differentiation marker in these cells, sympathetic neuroblasts staining very early with ROCA2, while dorsal root ganglion neurons are not stained until E15, when most of the neurons are postmitotic. A marker with a complementary expression would be the helix-loop-helix protein Id, whose expression declines in cultured neurons during differentiation, and is present in dorsal root but not sympathetic ganglia in the E15 mouse (Benezra et al., 1990).

Adrenal Chromaffin Cells

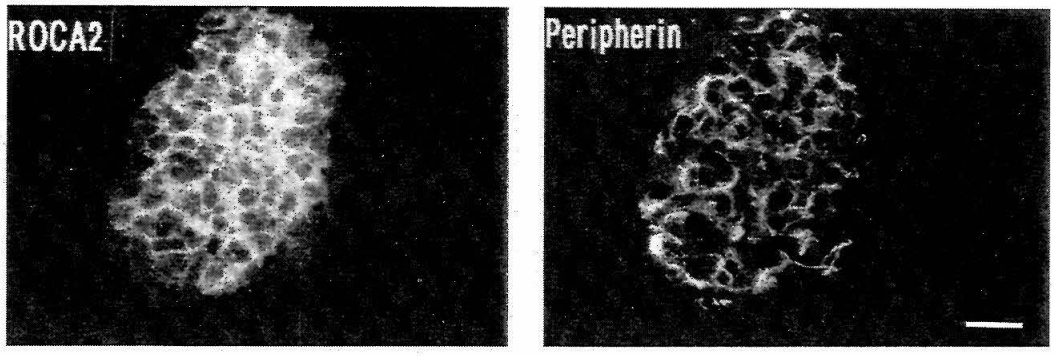
Like sympathetic and dorsal root ganglion neurons, adrenal chromaffin cells are derived from the neural crest. Moreover, chromaffin cells share the SA2$^+$ sympathoadrenal progenitor with sympathetic neurons (Carnahan and Patterson, 1991; Anderson et al., 1991). Therefore, it is not surprising that chromaffin cells label strongly with ROCA2 from E14.5 through adulthood. In serial sections of E18.5 adrenal medullae, we find that ROCA2 appears to label the entire SA2$^+$ and TH$^+$ population in the adrenal medulla (Fig. 4; not shown). This staining is on the cell surface, because freshly dissociated, living chromaffin cells from the adult rat also bind ROCA2 very well (Fig. 3) In separate experiments using freshly dissociated chromaffin cells, we have confirmed that the ROCA2$^+$ cells are also SA2$^+$ and TH$^+$ (not shown). The chromaffin cell staining does indicate, however, that CD9 expression is not restricted to neurons in the neural crest lineage.

Peripheral Glia

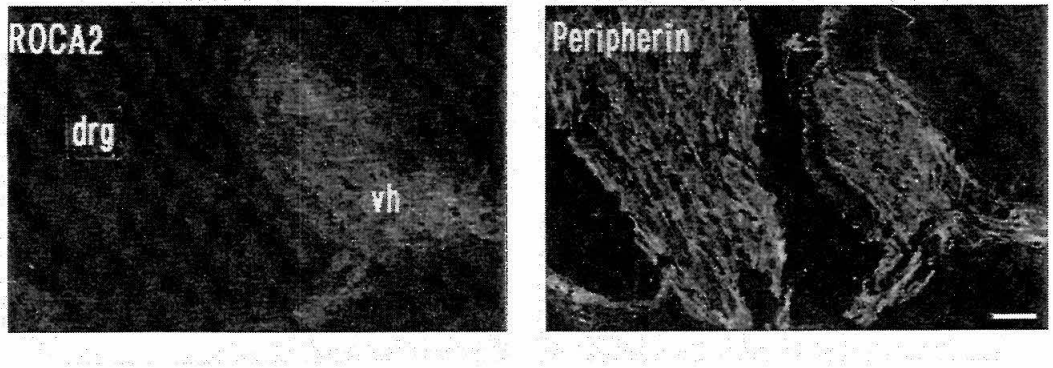
Immature glia in the peripheral nervous system (satellite cells in ganglia and Schwann cells in nerves) label weakly with ROCA2 during late embryonic development (E17, data not shown; P0, Fig. 5). Staining increases in intensity postnatally, and is very strong in the adult (Fig. 5). In mature nerves, ROCA2 binds the abaxonal (between Schwann cell and basal lamina) and the adaxonal surfaces (between Schwann cell and axon) of Schwann cells, and is not detectable in the compacted myelin lasers (Fig. 5; bottom panel). In these micrographs, it is not possible to determine if axonal surface membranes are also staining with ROCA2. These observations are consistent with a role for CD9 in the initial association of Schwann cells with axons and with basal lamina. A 1:1 association between Schwann cells and axons is required for myelination, and the interaction with basal lamina is thought to enable Schwann cells to express polarized, epithelial features (Bunge and Bunge, 1983), and to provide an anchor for the Schwann cell as it wraps around the axon (Bunge et al., 1989). Its continued presence may also imply a role for CD9 in Schwann cell-axon interaction in maturity. CD9 expression is also very high in satellite cells of adult peripheral ganglia (surrounding neuronal cell bodies; Fig.6), implying

Fig. 2. ROCA2 labeling in the early embryonic CNS and PNS. A transverse section of an E12 embryo through a sympathetic ganglion, double labeled with ROCA2 and anti-peripherin, shows cells throughout the ganglion labeling strongly for both antigens, while surrounding tissue is negative for both antigens. A transverse section of an E12 embryo in the trunk region, double labeled with ROCA2 and anti-peripherin, shows the ventral horn of the spinal cord labeled with both ROCA2 and anti-peripherin, while the adjacent DRG is negative for ROCA2 and positive for anti-peripherin. The rest of the spinal cord is negative for both antigens. At E12, sympathetic ganglia label much more intensely with ROCA2 than the ventral horn. Serial, transverse sections of an E15 embryo, labeled with ROCA2 and monoclonal anti-neurofilament-68, show the DRG is positive for ROCA2 at this age, while the ventral horn of the spinal cord is negative. Scale bars are 50 μm for all DRG and ventral horn pictures; 25 μm for sympathetic ganglion. drg, dorsal root ganglion; vh, ventral horn.

E12 Sympathetic Ganglion



E12 DRG and Ventral Horn



E15 DRG and Ventral Horn

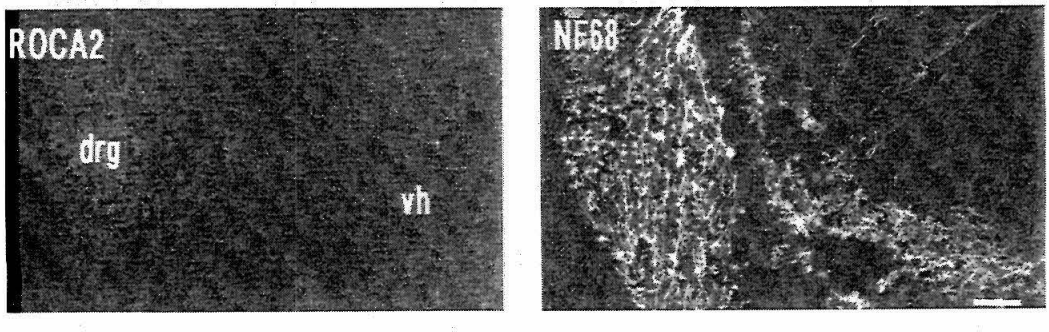


Fig. 2.

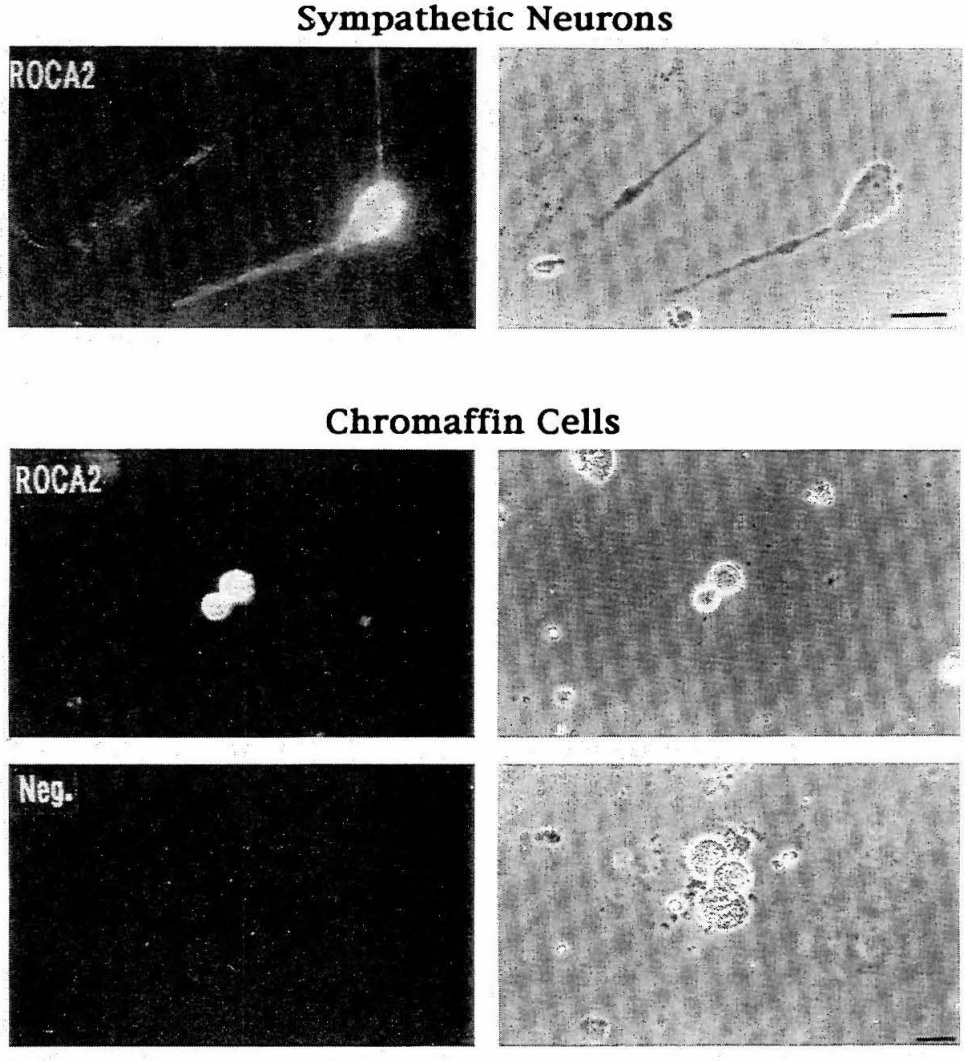


Fig. 3. Surface labeling of sympathetic neurons and chromaffin cells by ROCA2. Sympathetic neurons stained live with ROCA2 after 3 weeks in culture display surface label on the cell body, axon, and growth cone. A phase contrast image of the same fields is on the right. Adult chromaffin cells, freshly dissociated and stained live with ROCA2, show surface staining. Neg. is a negative control, with no primary antibody added. Phase contrast pictures of the same fields are on the right. Scale bar is 25 μm for sympathetic neurons, and 20 μm for chromaffin cells.

a function in maturity unrelated to the saltatory conduction in axons.

Central Nervous System

There are parallels between some of the ROCA2 staining patterns in the PNS and the in central nervous system (CNS). For example, there is widespread labeling of glia in the adult CNS, and there is labeling of a selected neuronal population during the period of initial axon outgrowth. The earliest detectable ROCA2 labeling in the embryonic CNS is in the ventral horn of the E11 spinal cord (staining at E12 is illustrated in Fig. 2). This staining appears to be on the motorneuron cell bodies and on their axons exiting through the ventral roots. ROCA2 labeling is transient, however, becoming undetectable by E15 (Fig. 2). CD9 expression

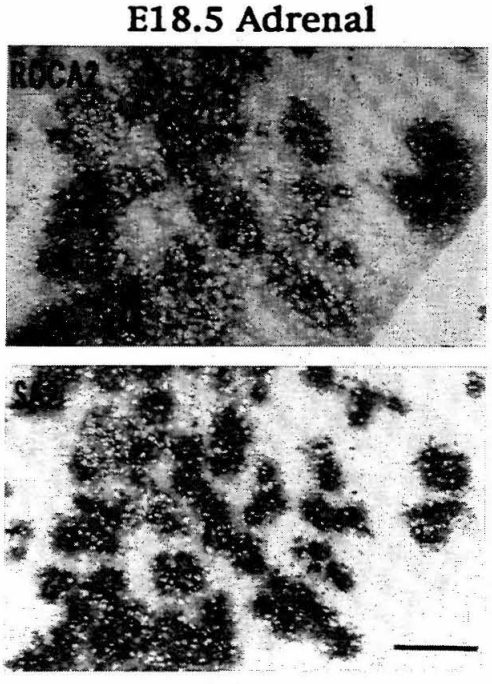


Fig. 4. ROCA2 labels embryonic adrenal chromaffin cells. Serial sections of an E18.5 adrenal gland labeled with ROCA2 and SA2 using peroxidase labeling show that SA2-positive chromaffin cells also label with ROCA2. Scale bar is 100 μm.

appears to be correlated, therefore, with the period of early axonal outgrowth in motorneurons, corresponding to a similar developmental stage in sympathetic (E12) and sensory (E15) neurons. CD9 expression also parallels another surface protein transiently expressed by motorneurons from E11 to E14, TAG-1 (Dodd et al., 1988).

The rest of the embryonic spinal cord labels poorly or not at all with ROCA2, at all embryonic ages, except in a ventral part of the ventricular zone where glia are generated (Altman and Bayer, 1984; Warf et al., 1991). The correlation between CD9 expression and gliogenesis is not precise, however. ROCA2 labeling first appears at E17.5, and persists into postnatal life, when glia throughout the spinal cord begin to label (Fig. 7). In contrast, Warf et al. (1991) demonstrated the ability of ventral but not dorsal spinal cord to give rise to oligodendrocytes in culture at E14.5. At later ages,both ventral and dorsal cord contain oligodendrocyte precursors, possibly due to migration of these from their ventral origin. Altman and Bayer (1984) find ^{3}H-thymidine incorporating cells along the ventral but not dorsal ventricular zone of the spinal cord at E17–E19. They also find scattered incorporation in all areas of

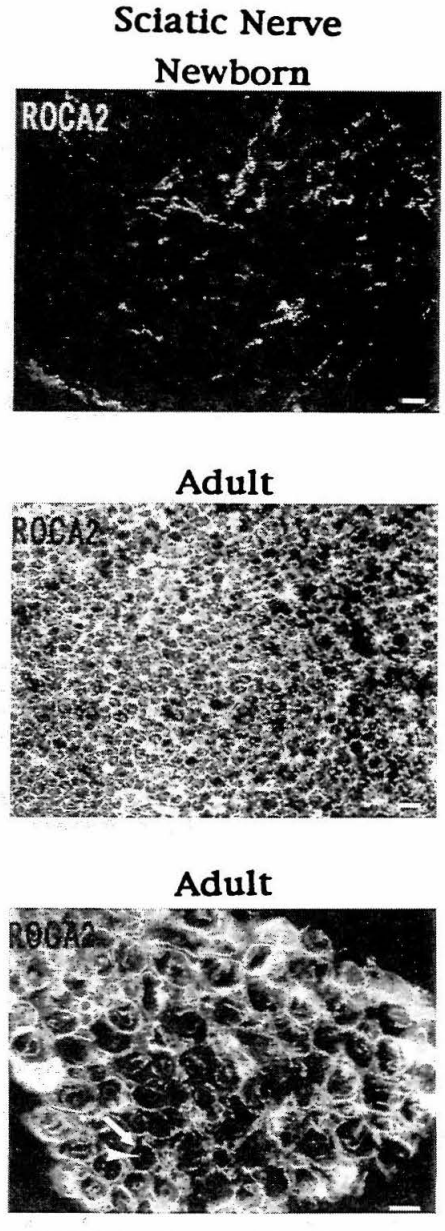


Fig. 5. ROCA2 labels newborn and adult sciatic nerve. A section of newborn rat sciatic nerve labeled with ROCA2 shows weak label throughout the section, with a few isolated areas of stronger labeling. Sections of adult sciatic nerve, in contrast, label very strongly with ROCA2 (**middle** panel). The **bottom** panel is a higher magnification of an adult sciatic nerve labeled with ROCA2, showing label in the abaxonal (arrow) and adaxonal (arrowhead) surfaces. Scale bar is 20 μm for **upper** and middle panels, and 10 μm for bottom panel.

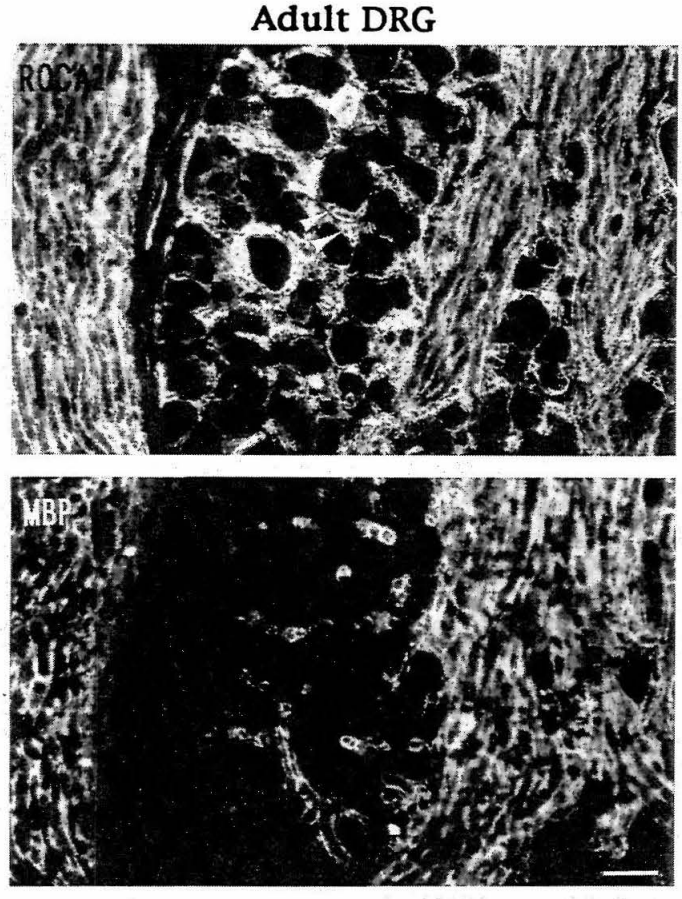


Fig. 6. ROCA2 labels satellite cells in peripheral ganglia. Serial sections of adult dorsal root ganglia labeled with ROCA2, and anti-MBP show ROCA2 labeling small cells (arrowheads) surrounding large neuronal cell bodies. These are satellite cells that do not form myelin. A few of these cells, labeled by anti-MBP, are likely to be myelinating Schwann cells. Nerve fibers that pass through the ganglion and are present in these sections (on the right and left of each picture) have myelinating Schwann cells, and label with both ROCA2 and anti-MBP. Scale bar is 50 μm.

the spinal cord, suggesting migration and local proliferation of oligodendrocyte precursors. In contrast, we find ROCA2 label only in the ventral ventricular zone at these ages, while the rest of the spinal cord is negative (Fig. 7). Therefore, while presumptive oligodendrocyte precursors from the ventral ventricular zone appear to label with ROCA2 late in embryonic life, these cells do not label once they have migrated to dorsal and non-ventricular areas of the spinal cord, until postnatal life, when the spinal cord shows scattered label throughout the grey matter (Fig. 7). In the adult, the spinal cord is brightly labeled throughout with ROCA2 (Fig. 8), probably because glia constitute a considerable cell population in the adult. We cannot, however, rule out ROCA2 staining of neurons in the adult spinal cord.

Excluding the transient labeling of the ventral horn motor neurons, the time course of ROCA2 labeling in the brain is similar to that of the spinal cord in that there is poor labeling throughout embryonic life, and bright labeling of the white matter in the adult. After the 1st week of postnatal life, there is an upregulation of CD9 expression in the CNS and PNS by non-neuronal cells, as detected by in situ hybridization (Z. Kaprielian, in preparation). Thus CD9 is upregulated as glia mature.

That glia in the adult CNS label is indicated by strong staining of the adult cerebellum, where the

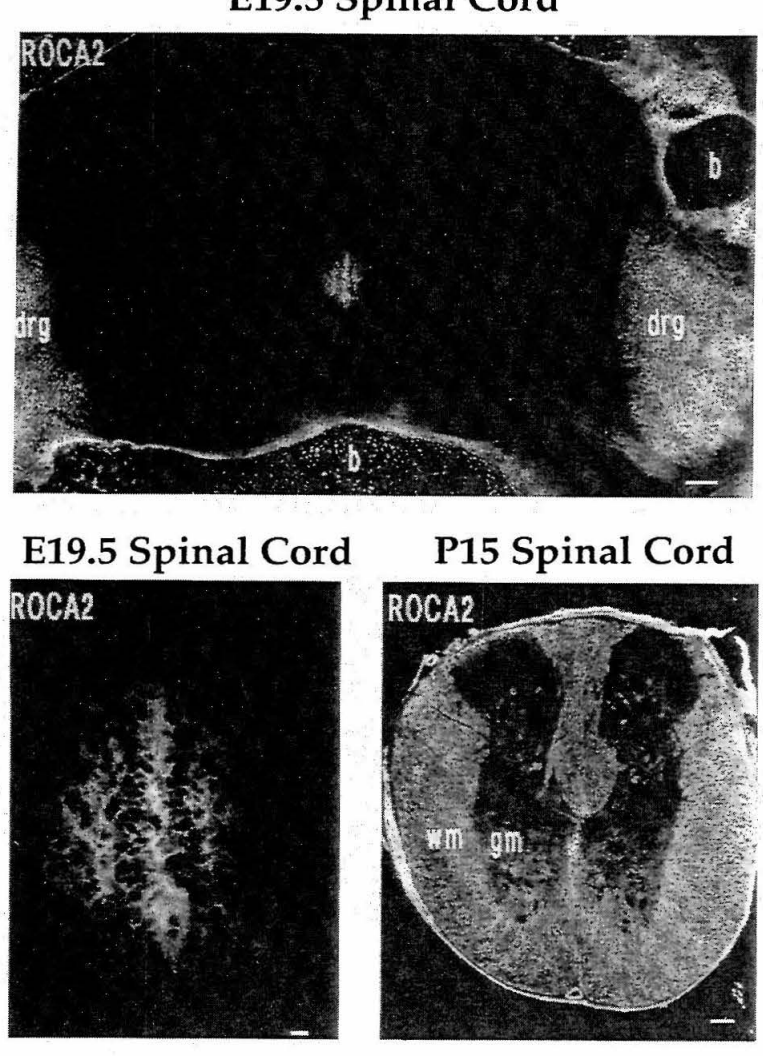


Fig. 7. ROCA2 labeling in the developing spinal cord. A transverse section of an E19.5 embryo **(top)** stained with ROCA2 shows label in the spinal cord, in a restricted ventral part of the ventricular zone. A high magnification of this area is shown in the lower **left** panel. The rest of the spinal cord does not label with ROCA2, while the dorsal root ganglia (drg) on either side of the spinal cord, as well as bone (b), do. A transverse section of P15 spinal cord shows extensive label in the white matter (wm) and disperse label in the grey matter (gm). Scale bars are 100 μm for the top and lower right panels, and 10 μm for lower left panel.

white matter and the granule cell layer, but not the molecular layer, are positive for ROCA2 (Fig. 8). This white matter staining indicates that oligodendrocytes label, and express CD9 (see below). The label in the granule cell layer is probably on oligodendrocytes and velate protoplasmic astrocytes. The latter are found predominantly in the granule cell layer, in contrast with the smooth protoplasmic astrocytes, which are found in both the granule cell and molecular layers. The third type of protoplasmic astrocyte, the Golgi epithelial cell, is found in the Purkinje and molecular layers (Palay and Chan-Palay, 1974), and probably

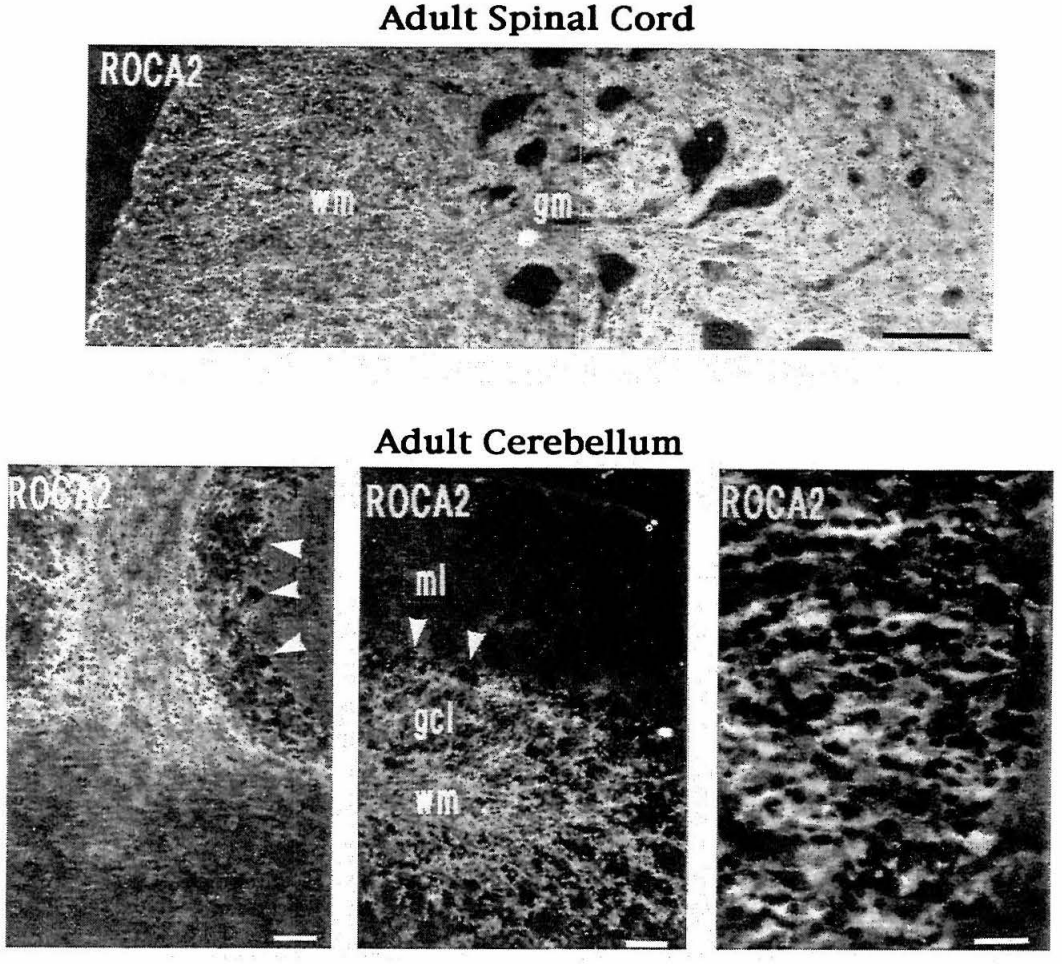


Fig. 8. ROCA2 labels adult CNS tissues. Transverse sections of adult spinal cord stained with ROCA2 show label in the white matter and around neurons in the ventral horn. Sections of adult cerebellum stained with ROCA2 show label in the white matter (wm) and granule cell layer (gcl). The **lower left** and **center** panels contain white matter bordered by granule cell layer, with the Purkinje cell layer between the granule cell layer and the molecular layer; **lower right** is a higher magnification of the granule cell layer. Scale bars are 100 μm for spinal cord; 50 μm for low mag. cerebellum (lower left and center); 25 μm for high mag cerebellum (lower right). wm, white matter; gm, gray matter; ml, molecular layer; gcl, granule cell layer; arrowheads point to Purkinje cells.

does not label. The granule cell layer staining is probably too sparse to represent CD9 expression by the granule cells themselves, which are present in large numbers in this layer. The lack of staining in the molecular layer also indicates that other cerebellar neurons are similarly negative in the adult. Neonatal cerebellum does not label with ROCA2 (data not shown).

Non-Neural Tissues

As previously reported for human CD9 (Jones et al., 1982; Bouchiex and Benoit, 1988), ROCA2 labels epithelia in the adult rat, e.g., esophagus (Kaprielian and Patterson, 1993) and urinary bladder. These tissues are stained in the embryo as well (data not shown), as is the embryonic lung and skin (Fig. 9). In the intestine, the muscularis externa is stained by ROCA2 (data not shown). The myenteric plexus of Auerbach, which consists of vagal, sympathetic, and enteric fibres, is present in this layer and may label as well, but the bright staining of the muscularis externa makes this difficult to determine. Also consistent with results using human material, ROCA2 intensely stains iso-

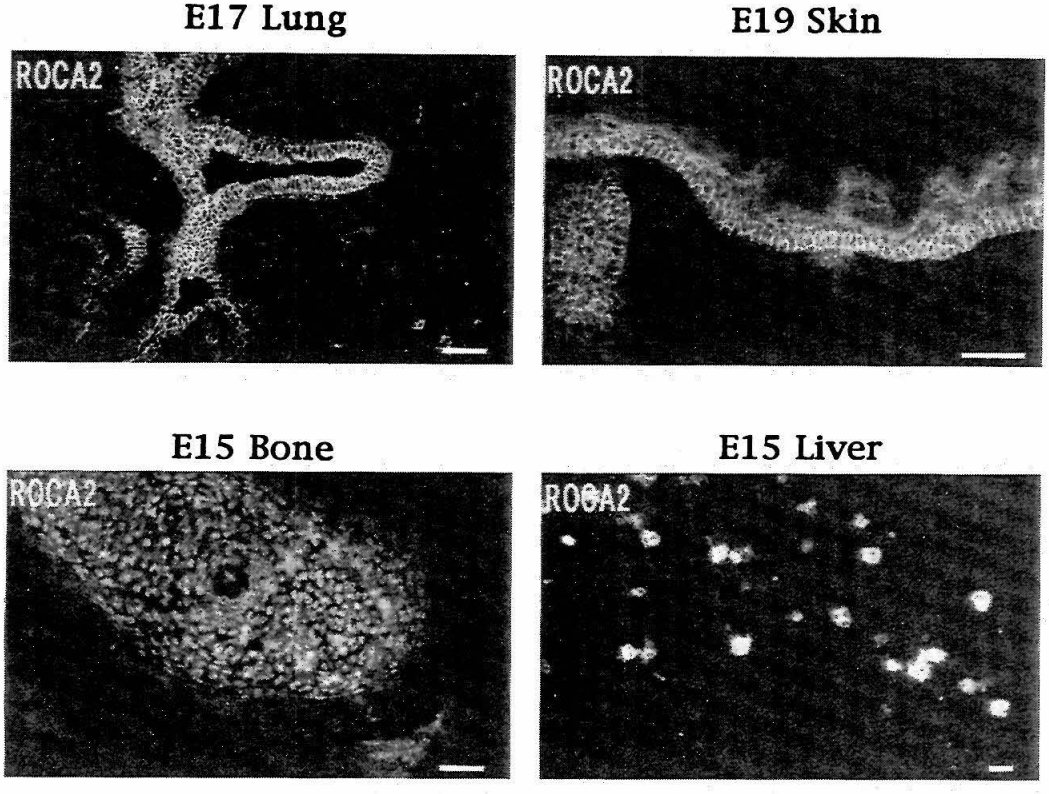


Fig. 9. ROCA2 labels embryonic non-neural tissues. E17 lung and E19 skin show staining in the columnar and squamous epithelium, respectively, with ROCA2. E15 centrum, labeled with ROCA2 shows bright staining. A section of E15 liver labeled with ROCA2, shows isolated clusters of a few cells each labeling intensely. Scale bars are 50 μm for skin, lung, bone; 25 μm for liver.

lated small clusters of a few cells each in the E12–16 rat liver (Fig. 9). These are likely to be hematopoietic cells (Jones et al., 1982). The adult rat liver is entirely negative (data not shown). Although not yet reported in humans, we find that CD9 is expressed by embryonic rat bone as well (Fig. 9).

Biochemical Characterization of the ROCA2 Antigens

To determine if the ROCA2 staining in the developing nervous system indeed reflects the expression of CD9, immunoblots were performed on membrane extracts from embryonic (E16) rat sympathetic and dorsal root ganglia, as well as from adrenal glands (Fig. 10). Each lane was loaded with a 1% Triton-X extract of a membrane fraction prepared as described in Experimental Procedures. Equal protein was loaded in the sympathetic ganglion and skeletal muscle lanes (120 μg), and in the dorsal root ganglion, adrenal, and brain lanes (40 μg). The sympathetic ganglion and skeletal muscle lanes contain more protein because the sympathetic ganglion tissue preparation includes some surrounding, ROCA2-negative tissue that would decrease the concentration of sympathetic ganglion-specific proteins. Sympathetic ganglia, dorsal root ganglia, and adrenal glands contain a 26 kD protein that is specifically labeled by ROCA2, while brain and skeletal muscle show no detectable levels of this band. In other work, we have shown by peptide sequencing that a protein of the corresponding size purified from adult peripheral nerves is, in fact, CD9 (Kaprielian and Patterson, 1993). Adult CNS tissues also show the presence of a 26 kD band that binds ROCA2 (Z. Kaprielian, unpublished).

CONCLUSIONS

We have shown, using immunohistochemistry and biochemical methods, that CD9 is present very early in specific cell types in both the central and peripheral

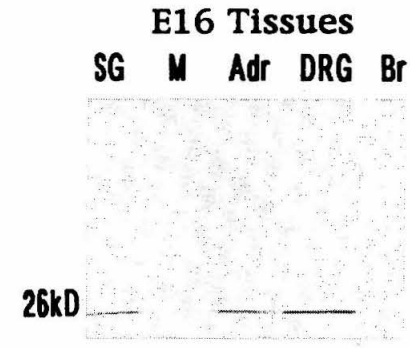


Fig. 10. ROCA2 binds a 26 kD band from embryonic sympathetic ganglia (SG), dorsal root ganglia (DRG), and adrenal (Adr) membranes. An immunoblot of E16 tissues probed with ROCA2 shows the presence of a ROCA2-positive, 26 kD band in sympathetic ganglion, dorsal root ganglion, and adrenal lanes, but not in skeletal muscle or brain. Each lane was loaded with a 1% Triton-X extract of a membrane fraction prepared as described in Experimental Procedures. Equal protein was loaded in the sympathetic ganglion and skeletal muscle lanes (120 μg), and in the dorsal root ganglion, adrenal, and brain lanes (40 μg). The sympathetic ganglion and skeletal muscle lanes had 3 times as much protein as the others because the sympathetic ganglion tissue preparation included some surrounding, ROCA2-negative, tissue that would decrease the concentration of sympathetic ganglion-specific proteins. SG, sympathetic ganglion; M, skeletal muscle; Adr, adrenal; DRG, dorsal root ganglion; Br, brain.

nervous systems, i.e., neural crest derivatives like sympathetic neurons and chromaffin cells, and in motorneurons in the CNS. CD9 is detectable later in embryonic sensory neurons, and is very highly expressed in adult central and peripheral glia. Table 1 summarizes our findings. Our results suggest that CD9 is subject to regulation at different times in all these different cell types in the rat nervous system, which may be taken to signify varied roles for CD9 in development and maturity.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

Pregnant female Sprague-Dawley rats and Long Evans hooded rats (the latter are partially pigmented, and were used to enable identification of melanocytes only) were obtained from Simosen Laboratories (Gilroy, CA). The morning after the day of mating is designated as day 0.5. Whole embryos and embryonic and adult tissues were directly frozen without fixation in Tissue-tek O.C.T., on dry ice. Sections (10 μm) were cut using a Bright cryostat (Hacker Instruments, Inc.), and placed on gelatin-coated slides. After air drying, the slides were either stained immediately or stored at −80°C. For staining with anti-peripherin, the slides were fixed in ice-cold acetone for 10 min prior to the staining procedure given below.

The sections were preadsorbed with 2% goat serum in PBS, and then incubated in the primary antibody in the form of hybridoma supernatant (for ROCA-2 peripherin double labeling, anti-peripherin was diluted in ROCA2 supernatant at 1:250, and acetone-fixed sections were used; similar results were obtained if ROCA2 labeling was performed first on unfixed sections, and then followed by acetone fixation and anti-peripherin labeling) for 1 hr at room temperature (RT), washed twice with PBS, and then incubated in the secondary antibody that was either FITC-conjugated (Hi-F goat anti-mouse IgG, Antibodies Inc., used at 1:100 in PBS) or biotinylated (Vector Mouse IgG ABC kit). In the latter case, the Vector ABC procedure was followed without blocking endogenous peroxidase, using a negative control (no primary antibody) to assess background staining. For double labeling, goat anti-mouse IgG-Texas red (Southern Biotech.) and goat anti-rabbit IgG-FITC (Sigma Immunochemicals) were used as secondary antibodies. FITC and Texas-red-labeled slides were mounted in glycerol containing n-propyl gallate (8 mg/ml final concentration, dissolved in 0.1 M Tris-HCl pH9). Peroxidase-labeled slides were mounted in Aquamount mountant (BDH limited, Poole, England). Slides were observed and photographed using a Zeiss ICM 405 microscope, Kodak Tri-X Pan 400 film for fluorescence pictures, and Kodak Plus-X Pan 125 film for brightfield pictures.

Anti-peripherin antiserum was obtained from Dr. L. Parysek, 192 IgG from Dr. D. J. Anderson, monoclonal anti-MBP from Boehringer-Mannheim, and monoclonal anti-neurofilament-68 from Sigma Immunochemicals.

Staining of Living, Freshly Dissociated, and Cultured Cells

Adult adrenal medullae were dissected in Ca^{++}/Mg^{++} free Hanks'-A solution, and incubated in 1 mg/ml collagenase (Worthington Biochemical Corp.) in Hanks'-A for 1 hr at 37°C. The enzyme was then blocked by the addition of serum. The cells were washed free of collagenase by pelleting at 1,000 rpm in a table top centrifuge and resuspending in culture medium (repeated twice). Undissociated clumps were broken up by gentle trituration using a flame-polished Pasteur pipette that had been preadsorbed with serum. The cells were centrifuged again, resuspended in primary antibody hybridoma supernatant, and incubated for 1 hr at RT. Then, cells were washed and resuspended in FITC-conjugated secondary antibody for 30 min at RT. After a final wash, cells were resuspended in a small volume of PBS and allowed to settle on a polylysine-coated glass slide for 30 min. Cells were then fixed in 3.7% formaldehyde for 10 min, washed, and mounted in glycerol containing n-propyl gallate.

Cultured cells were stained in a similar manner, except that they remained in the tissue culture dish. The incubations, washes, and fixation were performed in the dish itself. Finally, 1 ml of glycerol containing n-propyl gallate was added to the dish, and cells were

TABLE 1. CD9 Expression in the Rat[a]

Tissue	E11.5	E12	E14.5	E16	E19	Neonatal	Adult
Crest derivatives							
Sympathetic neurons	+	+	+	+	+	+	+?
DRG neurons		−	−	+	+	+	+?
Chromaffin cells			+	+	+	+	+
Melanocytes					−		
Glia				±	+	+	+

Tissue	Embryonic	Adult
CNS:brain		
Neurons	−	+?
Glia	±	+
CNS:spinal cord		
Neurons	+[†]	+?
Glia	+	+
Epithelia		
Esophagus	+	+
Skin	+	
Lung	+	
Bladder	+	
Hematopoietic[b]		
Liver	+	−
Bone/ cartilage	+	

[a]+ = ROCA2 staining; ± = weak, almost undetectable staining; − = undetectable staining; +? = uncertain; cannot distinguish neuronal from glial staining; +[†] = ventral horn of the spinal cord.
[b]Presumed hematopoietic cells in liver.

observed using an inverted fluorescence microscope (Zeiss ICM 405).

Tissue Culture

Twenty adrenal glands from 4 day old rat pups were dissected under sterile conditions, and placed in Ca^{++}/Mg^{++} free Hanks'-A solution. The medullae were dissected out, and dissociated in 1 mg/ml collagenase in Hanks'-A for 1 hr at 37°C. The enzyme was then blocked by the addition of serum-containing culture medium. Cells were washed free of collagenase by centrifuging at 1,000 rpm and resuspending in culture medium (repeated twice). Undissociated clumps were broken up by gentle trituration using a flame-polished Pasteur pipette that had been preadsorbed with serum. Cells were plated on polylysine-laminin coated tissue culture dishes (Corning, 35 mm) at a density of 10^5 cells per dish.

L15 CO2 medium (Hawrot and Patterson, 1979) supplemented with 10% FBS was used as the tissue culture medium. For chromaffin cells, dexamethasone (1 mM, Sigma) was added from a 1,000× stock in 95% ethanol. Ara-C (10 mM) was added for 5 days to eliminate cortical cells. Chromaffin cells grown in this medium for 10 days were converted into sympathetic neurons by replacing dexamethasone with nerve growth factor (0.1 mg/ml, Boehringer-Mannheim), and culturing for 10 days.

Immunoblotting

Embryonic and adult tissues were dissected and frozen directly on dry ice. After homogenization in TBS (50 mM Tris HCl, 50 mM NaCl, pH 7.5) containing protease inhibitors, the nuclei were removed by centrifugation at 1,000 rpm. The supernatant was centrifuged at 100,000*g* in a Beckman ultracentrifuge. The membrane fraction obtained as the pellet was resuspended in homogenization buffer containing 1% Triton X-100 and centrifuged at 100,000*g*, yielding a Triton-extractable component as the supernatant. The protein content was determined using BCA reagent (PIERCE). SDS-PAGE was performed as described by Laemmli (1970), using a 1.5 mm thick, 10% separating gel. Following electrophoresis, the gel was equilibrated for 15 min in transfer buffer (12.5 mM Tris, 96 mM glycine, pH 8.5, 20% v/v methanol), and the proteins within the gel were transferred to a nitrocellulose membrane (0.45 μm, Schleicher and Schuell) overnight at 50 V. The nitrocellulose strips were then preadsorbed with 5% nonfat dry milk in TBS (50 mM Tris-HCl, 0.9% NaCl, pH 7.5) containing 0.1% Tween-20 (TBST) for 30 min. Incubation with purified ROCA2 (50 μg/ml in 5% nonfat dry milk in TBST) for 5 hr at RT was followed by 3 washes in TBST, and then incubation with a peroxidase-conjugated goat-anti-mouse IgG (Chemicon, 1:500) for 1 hr at RT. After 3 washes (the last wash in TBS without Tween-20), immunoreactivity was developed using 0.5 mg/ml 4-chloro-1-naphthol in 0.02% H_2O_2 for 20 min at RT. The nitrocellulose was then air dried and photographed.

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