A Fibronectin-like Molecule Is Present in the Developing Cat Cerebral Cortex and Is Correlated with Subplate Neurons

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Abstract. The subplate is a transient zone of the developing cerebral cortex through which postmitotic neurons migrate and growing axons elongate en route to their adult positions within the cortical plate. To learn more about the cellular interactions that occur in this zone, we have examined whether fibronectins (FNs), a family of molecules known to promote migration and elongation in other systems, are present during the fetal and postnatal development of the cat's cerebral cortex. Three different anti-FN antisera recognized a single broad band with an apparent molecular mass of 200–250 kD in antigen-transfer analyses (reducing conditions) of plasma-depleted (perfused) whole fetal brain or synaptosome preparations, indicating that FNs are present at these ages. This band can be detected as early as 1 mo before birth at embryonic day 39. Immunohistochemical examination of the developing cerebral cortex from animals between embryonic day 46 and postnatal day 7 using any of the three antisera revealed that FN-like immunoreactivity is restricted to the subplate and the marginal zones, and is not found in the cortical plate. As these zones mature into their adult counterparts (the white matter and layer 1 of the cerebral cortex), immunostaining gradually disappears and is not detectable by postnatal day 70.

Previous studies have shown that the subplate and marginal zones contain a special, transient population of neurons (Chun, J. J. M., M. J. Nakamura, and C. J. Shatz. 1987. *Nature (Lond.).* 325:617–620). The FN-like immunostaining in the subplate and marginal zone is closely associated with these neurons, and some of the immunostaining delineates them. Moreover, the postnatal disappearance of FN-like immunostaining from the subplate is correlated spatially and temporally with the disappearance of the subplate neurons. When subplate neurons are killed by neurotoxins, FN-like immunostaining is depleted in the lesioned area. These observations show that an FN-like molecule is present transiently in the subplate of the developing cerebral cortex and, further, is spatially and temporally correlated with the transient subplate neurons. The presence of FNs within this zone, but not in the cortical plate, suggests that the extracellular milieu of the subplate mediates a unique set of interactions required for the development of the cerebral cortex.

THE mammalian cerebral cortex, the multilayered structure of the paired cerebral hemispheres, is derived from a simple neural tube. During fetal development, the proximal portion of the tube (the telencephalon) expands through successive cell division and migration, giving rise to the cerebral hemispheres. The location of the telencephalon is shown schematically in Fig. 1 *A* (*TEL*), a horizontal section through the embryonic central nervous system. The cellular organization of the developing telencephalon is very different from that found in the adult, and several distinct embryonic zones have been identified (see Fig. 1 and Boulder Committee, 1970).

Two of these embryonic zones, the subplate and marginal zones, are remarkable for several cellular elements and interactions that occur there. Positioned in the subplate and marginal zones is a special population of cells, the subplate and marginal zone cells. They are the earliest generated cells of the telencephalon, having undergone their last round of DNA synthesis and cell division between embryonic days (E)[1] 24 and 30 in the cat (gestation is 65 d in the cat) based on [^{3}H]thymidine administration (Luskin and Shatz, 1985*a*; see Materials and Methods). Initially forming a single band within the developing cerebral cortex, the population is subsequently split apart by intervening cells destined for the various layers of the adult cortex. Thus, cells from the same early generated population take residence in the subplate and marginal zones. During fetal life, these cells are present in large numbers in the subplate (and to a lesser extent in the marginal zone) but disappear postnatally as the subplate matures into the white matter (and the marginal zone matures into cortical layer 1) of the adult (Luskin and Shatz, 1985*a*). The subplate and marginal zone cells are neurons based on ultrastructural, neuroanatomical, and immunohistochemical

1. *Abbreviations used in this paper*: E, embryonic day; FN, fibronectin; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; P, postnatal day.

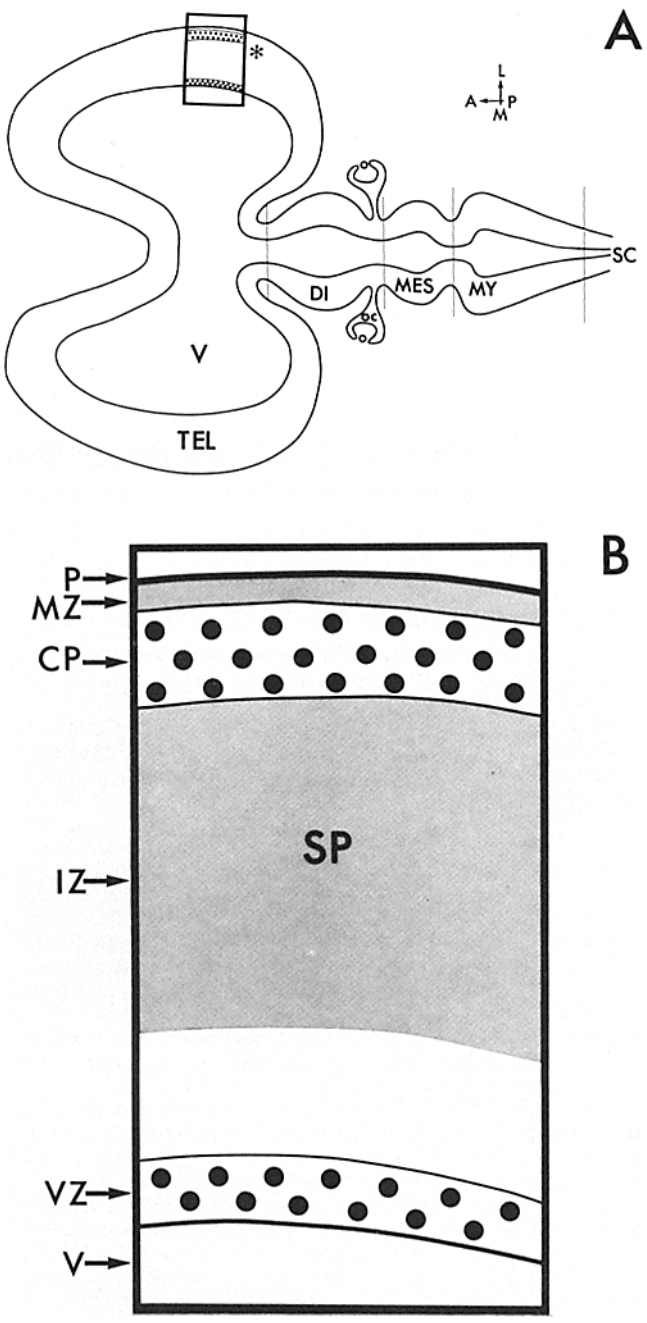

Figure 1. The location of the telencephalon in the fetal brain is shown in a schematic drawing (horizontal plane of section) in *A*. The telencephalon (*TEL*) includes two symmetric lobes (the cerebral hemispheres), which enclose the ventricles (*V*) containing cerebral spinal fluid and the choroid plexus. Further posterior are the diencephalon (*DI*), mesencephalon (*MES*), myelencephalon (*MY*), and spinal cord (*SC*). (*OC*, optic cup). (*B*) An enlargement of the region enclosed in the box of *A** shows that during development the telencephalic wall can be subdivided into several embryonic zones. Closest to the ventricle (*V*) is the ventricular zone (*VZ*) or future ependyma. Next is the intermediate zone (*IZ*) the future white matter, that contains as a subdivision the subplate zone (*SP*) indicated by shading. The subplate is subjacent to the cortical plate (*CP*) or future cortex. Immediately above the cortical plate is the marginal zone (*MZ*) or future layer 1. The pial (*P*) surface of the brain is outermost. *A*, anterior; *P*, posterior; *M*, medial; *L*, lateral.

data (Chun et al., 1987); many of them are immunoreactive for neuropeptides, and all are immunoreactive for the neuron-specific protein microtubule-associated protein 2 (MAP2; Chun et al., 1987; see Figs. 8 and 9).

The subplate also contains other postmitotic neurons that, having undergone their final round of DNA synthesis at the ventricular zone, are migrating en route to the cortical plate. These cells must pass through the subplate, apparently moving along radial glia positioned there (Rakic, 1972). Many growing axonal systems also pass through the subplate to ultimately innervate their cortical targets. In so doing, these developing axonal systems do not directly innervate the cortical plate, but rather wait for several weeks within the subplate before finally growing into the cortical plate itself (Rakic, 1977; Lund and Mustari, 1977; Wise and Jones, 1978; Crandall and Caviness, 1984; Shatz and Luskin, 1986). Amongst these waiting afferents are found transient synapses that also disappear from the subplate when afferent systems grow into the cortical plate (Molliver et al., 1973; Kostovic and Molliver, 1974; Cragg, 1975). The presence of the subplate (and marginal zone) neuron is therefore correlated with the presence of migrating neurons, waiting axons, and transient synapses.

Since the subplate is a region in which extensive cell migration and axon elongation occurs, it may contain extracellular matrix molecules essential for these functions. One such family of molecules are the adhesive glycoproteins known as fibronectins (FNs; Hynes and Yamada, 1982; Hynes, 1985). In other systems, FNs can promote a variety of cellular events such as cell migration (Hynes and Yamada, 1982; Hynes 1985; Thiery and Duband, 1986) and neurite extension (Akers, 1981; Rogers et al., 1983, 1985). However, it is uncertain whether FNs are present within the developing brain, and if so, whether they are found in the correct neuroanatomical locations at the appropriate times to mediate functions such as cell migration or axon elongation. Several previous immunohistochemical studies addressed the presence of FNs by examining the developing rodent brain, but the results have been contradictory (FN was not detected by Schachner et al., 1978; Minier et al., 1981; Hynes et al., 1986; but see Hatten et al., 1982; Stewart and Pearlman, 1987). Here we have investigated whether FN-like molecules are present during the development of the cat's brain by means of antigen-transfer analysis and immunohistochemistry. We were especially interested in examining the subplate and marginal zones in view of the wealth of available information mentioned above indicating the presence of developmental events that could be mediated by extracellular matrix molecules. The prolonged time course of development in the cat as compared with rodents increased the likelihood that such molecules could be detected. In particular, we wished to examine whether the subplate and marginal zone neurons might be correlated with the regulation and/or expression of this extracellular matrix molecule during development.

Materials and Methods

41 cat brains between the ages of E39 (gestation is 65 d) and adulthood were examined using immunohistochemical techniques combined with [^{3}H]thymidine autoradiography. These two approaches were also combined with neurotoxin injections. Table I summarizes the experimental permutations used. Fetal ages were determined by timed breedings as described previously (Luskin and Shatz, 1985*a*).

Table I. Experimental Age and Analysis Performed

Age* at examination	No. of animals	Antigen-transfer analysis	Immunohisto-chemistry
E39	1	+	
E46‡	1		+
E48	1	+	
E50	2		+
E50‡	2		+
E52	2	+	
E54	1		+
E56	1	+	
E56‡	2		+
P1	1		+
P7	1	+	
P7‡	1		+
P7	1	+	
P21	1		+
P28‡	1		+
P49‡	1		+
P70	1		+
P178‡	1		+
P401(ADULT)‡	1		+
ADULT	1	+	
ADULT	2		+

* E, Embryonic day (65-d gestation); P, postnatal day.
‡ [^{3}H]thymidine was administered at E27 and tissue autoradiographed at the examination age.

Surgical Procedure

All procedures included sterile surgical technique to externalize fetuses by cesarian section for study. Pregnant cats in which fetuses were of known gestational age were initially anesthetized by inhalation of halothane (1–2% in O_2) or by intramuscular injection of ketamine hydrochloride (20 mg/kg) and acepromazine (0.2 mg/kg). Anesthesia was maintained by halothane (0.5–2% in O_2) administered through an endotrachial tube, and the appropriate level was monitored by ventilation and heart rate. A 5% dextrose/saline i.v. drip was maintained throughout the surgery. Terbutaline sulfate was administered (0.03 mg/kg i.v.) before each fetal manipulation. After surgery, the animals were given analgesics and monitored in the 24-h/d intensive care unit for at least 36 h. Further details of the surgical procedure can be found in Luskin and Shatz, 1985*a*.

Injections

The subplate and marginal zone neurons were identified by labeling them on their birthdates with an intrauterine injection of [^{3}H]thymidine (NET O27Z, 76.1 Ci/mmol; DuPont Co., Wilmington, DE) delivered on E27 or E28, a time well before neurons of the adult cortical layers are generated (see Luskin and Shatz, 1985*a*, *b*). All neurons undergoing DNA synthesis at the time of the injection will incorporate [^{3}H]thymidine, but only those that undergo their final round of cell division to become forever postmitotic (the cell's birthdate) will be heavily labeled at all subsequent times. Therefore, if [^{3}H]thymidine is given at E27 or E28, and brains are subsequently analyzed with autoradiography at later ages, the only heavily labeled neurons are those of the subplate and marginal zones.

Subplate neurons were eliminated by exposure to the neurotoxins kainic acid (No. K0250; Sigma Chemical Co., St. Louis, MO; Coyle et al., 1978) or ibotenic acid (No. I0382; Sigma Chemical Co.; Schwarcz et al., 1979; see also Jonsson, 1980). Each toxin was diluted (10 mg/ml) in 0.9% sterile saline and used within 2 h. For animals at E44 and older, a sterile dental drill was used to remove a portion of the cranium overlying the targeted areas in order to insert the injection needle. With the brain surface exposed, an array of two to six injections of 0.1 μl were made with a 1-μl syringe (Unimetrics, Shorewood, IL) at a depth of 1–2 mm in a parasagittal array across the cerebral cortex of the right hemisphere. The left hemisphere was treated identically except that vehicle solution (0.9% sterile saline) was injected. Animals were then returned to the uterus, developing an additional 6–16 d before cesarian section delivery and tissue preparation. Table II shows the ages at which fetuses were injected and subsequently examined.

Table II. Experimental Age of Neurotoxin Exposure and Examination

Age at injection	No. of animals	Neurotoxin* injected	Age at examination
E40	1	K	E50
E40‡	1	I	E54
E40§	2	I	E56
E43	1	I	E51
E44	1	I	E50
E45	2	K	E52
E50§	2	K	E58
E50	2	K	E58
E50	2	K	E60
P4	1	K	P6

* K, Kainic acid; I, ibotenic acid.
‡ [^{3}H]thymidine was administered at E27 and tissue autoradiographed at examination age.
§ [^{3}H]thymidine was administered at E40 and tissue autoradiographed at examined age.

Tissue Preparation for Histology

Fetuses were anesthetized transplacentally through maternal anesthesia with halothane, and postnatal animals were deeply anesthetized with ketamine hydrochloride (20 mg/kg) plus acepromazine (0.2 mg/kg) delivered intramuscularly or subcutaneously, followed by Nembutal (50 mg/kg) injected intraperitoneally. Animals were perfused transcardially using a peristaltic pump with flow rates varying from 3.5 ml/min for fetuses to 30 ml/min for adult animals. The descending aorta was ligated in animals of length greater than 12 cm. Perfusion commenced with Ringer's solution (5 min, 25°C; 124 mM NaCl, 5 mM KCl, 1.15 mM KH_2PO_4, 1.15 mM $MgSO_4$ ($7H_2O$), 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, pH 7.4; Shatz and Kirkwood, 1984) followed immediately by fresh 4% paraformaldehyde in 0.1 M monobasic sodium phosphate buffer, pH 7.4 (30 min, 25°C). The crania were then removed and brains were placed into fresh, circulating fixative at 4°C for 12–18 h. Brains were prepared for either cryostat or vibratome sectioning. Material to be sectioned by cryostat was removed from fixative and placed into a circulating solution of PBS (20 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4) plus 6% sucrose and 0.025% sodium azide at 4°C for 18–24 h. Material to be sectioned on the vibratome was processed identically except that sucrose was omitted from the buffer. For cryostat sectioning, the brain was then frozen by immersion into liquid freon (cooled with dry ice), and either sectioned immediately or stored at −60°C and sectioned within 48 h. An average of 80 8–10-μm thick sections were cut through the telencephalon in the horizontal (for fetuses) or coronal (some fetuses and all postnatal animals) plane, with a particular emphasis directed at future visual (or adult visual) cortical areas (lateral and suprasylvian gyri; see Tusa et al., 1978). Sections were mounted on subbed slides, stored at −20°C, and processed within 4 h for immunohistochemistry and/or autoradiography. Material to be vibratome sectioned was embedded in 30% albumin plus 0.5% gelatin cross-linked with 5% vol/vol of 50% glutaraldehyde and cut at 80 μm (fetal material) or 50 μm (postnatal day [P] 7 and older) in the horizontal or coronal plane. Sections were stored in PBS plus 0.025% sodium azide at 4°C.

Immunohistochemistry

Antibodies against the following antigens were used. MAP2 (rabbit anti-bovine polyclonal diluted 1:2,500 and mouse monoclonal anti-bovine MAP2-3 diluted 1:30) was a gift from Drs. W. Theurkauf, F. Luca, and R. Vallee (Worcester Foundation for Experimental Biology, Shrewsbury, MA); glial fibrillary acidic protein (GFAP, rabbit anti-human polyclonal diluted 1:500) was a gift from Dr. L. Eng (Stanford University, Stanford, CA). Antibodies against fibronectins were commercially obtained as follows. Antiserum a: rabbit anti-human fibronectin, No. 341643, diluted 1:500; Calbiochem-Behring Corp., La Jolla, CA. Antiserum b: rabbit anti-human fibronectin, No. A245, diluted 1:2000; Dakopatts, Copenhagen, Denmark. Antiserum c: rabbit anti-bovine fibronectin, No. 341632, diluted 1:1,000; Calbiochem-Behring Corp. In addition, two monoclonal antibodies (showing a qualitatively similar but less intense pattern of staining and not characterized using antigen transfer analysis) were used: mouse

monoclonal anti-human fibronectin, ascites fluid, No. 9523SA, 1:500; and mouse monoclonal anti-chick cellular fibronectin, No. 9425SA, 1:500; both from Bethesda Research Laboratories, Gaithersburg, MD). Human and bovine plasma fibronectin were purchased from Sigma Chemical Co. Bound antibody was visualized using Vectastain ABC kits (Vector Laboratories, Burlingame, CA) and the chromagen 3,3'diaminobenzidine (DAB; grade II, No. D-5637; Sigma Chemical Co.).

Sections cut by cryostat or vibratome were processed similarly. Sections were first preincubated at 25°C for 2 h in incubation buffer containing PBS plus 2.5% BSA (No. A-4503, fraction V; Sigma Chemical Co.) and 0.3% Triton X-100 (No. T-6876; Sigma Chemical Co.). Triton X-100 was not necessary to observe staining but increased staining intensity. This solution was replaced by fresh incubation buffer containing diluted antisera/antibody and incubated for 15–24 h at 25°C (free-floating sections were gently agitated during incubation). After incubation, sections were washed in PBS without sodium azide (four changes over 30 min) followed by 60 min in secondary antibody (1:200), then washed again (four changes over 30 min), and finally incubated for 40 min in Advidin Biotin Complex solution (HRP; 1:100). The sections were then washed (four changes over 30 min) with 50 mM Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and reacted with the addition of 0.5 mg/ml (3,3' Diaminobenzidine) with 0.0075% H_2O_2. The sections were then washed again (20 min with two changes of TBS). Vibratome sections were mounted on subbed slides, air dried, and processed along with cryostat sections through three washes of distilled water followed by dehydration in graded ethanols. All sections were then immersed in 100% xylene for 8 h, rehydrated, and dipped in 0.4% osmium tetroxide in distilled water for 30 s to intensify the reaction product and prevent fading. The sections were then either again dehydrated and cover slipped with Permount (Fisher Scientific Co., Fairlawn, NJ) or washed in three changes of distilled water and air dried. They were then dipped, exposed, and developed for autoradiography as previously described (Luskin and Shatz, 1985*a*).

Antigen Transfer Analyses

Fetal (E52) and postnatal (P7, adult) brains prepared for antigen transfer analysis (Western blots) were first perfused with Ringer's solution as described above. The entire brain was then rapidly removed from the cranium, immediately immersed in boiling sample buffer (30 mM dithiothreitol [DTT], 10% wt/vol SDS, 0.1 M Tris-HCl, pH 7.5, 50% vol/vol glycerol), and homogenized using a motor-driven, teflon–glass homogenizer at 400 rpm. Additional animals (E39, E48, P7) were processed through the Ringer's perfusion step, then further homogenized in a Hepes buffer (4 mM Hepes, 2 mM EGTA, 2 mM EDTA, 0.32 M sucrose, 1.0 mM phenylmethyl sulfonyl fluoride), and differentially centrifuged to yield a crude synaptosome preparation (Huttner et al., 1983). The crude synaptosome pellet was solubilized in boiling sample buffer. Once solubilized, both preparations were stored at −20°C before electrophoresis. Another preparation was made in which, after perfusion with Ringer's solution, the brain was removed and immediately frozen in liquid nitrogen. The frozen brain was split in the saggital plane and the entire surface was abraded with a file to a depth of approximately 2 mm in an effort to remove the pia. While still frozen, the brain was broken to reveal parts of the choroid plexus, which were removed. This pia- and choroid-depleted brain was then processed as above.

Samples containing adult cat plasma fibronectin were obtained by collecting cat plasma and diluting it 4:5 with boiling sample buffer. Adult human and bovine plasma FN were obtained from Sigma Chemical Co. and solubilized in sample buffer. Discontinuous SDS-PAGE (Laemmli, 1970) of the desired material was carried out in 1.5-mm thick, 9.5-cm long, 4–15% gradient slab gels with a 5-cm, 2.75% stack. After electrophoresis, the material in the gels was either cut out and stained with Coomassie Blue or processed for antigen transfer analysis (Towbin et al., 1979; Bittner et al., 1980) on 0.45-μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After transfer, the nitrocellulose was incubated for 8 h at 25°C in TBS plus 2.5% BSA with 0.025% sodium azide, then incubated in the primary antibody at concentrations one-tenth of those used for immunohistochemistry (1:10,000–1:20,000). Blocking controls were conducted by simultaneous incubation with native bovine plasma FN (No. F-4759; Sigma Chemical Co.) or human plasma FN (No. F-2006; Sigma Chemical Co.) at 23 nM or 10 μg/ml. Normal rabbit serum controls were conducted at twice the concentration of that used for FN antisera. After a 12–15-h incubation at 25°C, the bound antibody was visualized in identical fashion to immunohistochemically visualized material, except that all washes made use of TBS, wash times were doubled, and the chromogen 4-chloro-1-napthol (No. C-8890; Sigma Chemical Co.) was used in a 0.5% solution in 17% methanol, 0.15% H_2O_2 in TBS (modified from Vector Laboratories, Burlingame, CA).

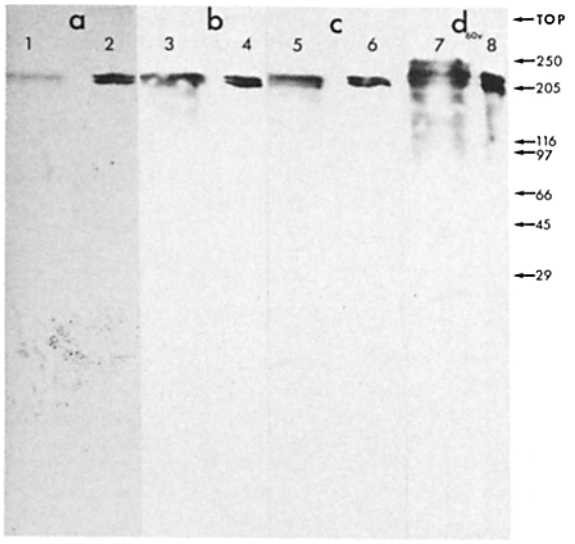

Figure 2. Three different antisera against plasma fibronectins (*FNs*) recognize both adult cat plasma FN (200–220-kD doublet) and a molecule from plasma-depleted embryonic brain (200–250-kD broad band) on antigen transfer analyses (reducing conditions). Even-numbered lanes contain cat plasma FN, while odd-numbered lanes contain preparations of plasma-depleted (buffer perfused) E52 whole brain. *a*, *b*, and *c* correspond to FN antisera a, b, and c (see Materials and Methods for details). All lanes were analyzed using 4–15% SDS-PAGE; lanes *1–6* were subjected to electrophoresis at 150 V; lanes *7* and *8* were subjected to electrophoresis at 60 V. At 60 V (*d*, using antiserum b), brain preparations contained an FN-immunoreactive broad band ranging between 200 and 250 kD (lane *7*), a molecular mass higher than plasma FN (lane *8*). Lower molecular mass products of less staining intensity that appear to be proteolytic fragments are also observed (lane *7*). No bands were found in controls in which 23 nM FN was added to antiserum, or in which antiserum was replaced by normal rabbit serum. No immunoreactivity was observed either at the dye front or in the stacking gel. The positions of molecular mass standards are indicated (250 kD is calculated).

Results

The results are presented in three parts. First, the specificity of the anti-FN antisera is evaluated with antigen transfer analyses of plasma and brain preparations. Next, the histological localization of FN-like immunoreactivity in fetal and postnatal brain is determined using the same antisera. Finally, the relationship between FN-like immunostaining and the subplate neurons is examined.

Antigen Transfer Analyses

To examine the specificity of the anti-FN antisera used in this study, we first characterized antisera a, b, and c (see Fig. 2, *a*, *b*, and *c*; see Materials and Methods for identifications) using antigen transfer analyses of adult cat plasma FN. As shown in Fig. 2 (lanes *2*, *4*, and *6*) all three antisera recognize two protein bands (a doublet) with an apparent molecular mass around 200–220 kD in a preparation of adult cat plasma analyzed by 4–15% gradient SDS-PAGE at 150 V. No other major bands were observed. The doublet comigrates with both human and bovine plasma FN, is not present on transfers exposed to FN antisera with 23 nM FN incubated simultaneously, and cannot be reproduced by normal rabbit

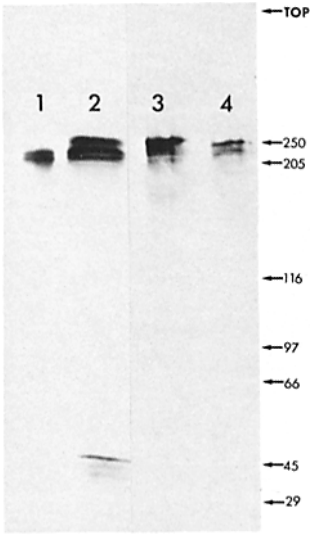

Figure 3. Antigen transfer analysis reveals that brains at several different ages contain similar FN-like molecules. Adult plasma FN (lane *1*), plasma-depleted whole E52 brain (lane *2*), and plasma-depleted crude synaptosome preparations (E48 in lane *3*, E39 in lane *4*) were analyzed using 4–15% SDS-PAGE at 60 V (see Materials and Methods for further details). Preparations at all three ages contain immunoreactive bands of higher molecular mass than in plasma (*top arrow* 250 kD; *bottom arrow* 220 kD). The positions of molecular mass markers are indicated (250 kD is calculated).

serum controls at any dilution (not shown). The apparent molecular mass is identical to that reported in previous studies of plasma FN (reviewed by Hynes and Yamada, 1982; Hynes 1985).

These same three antisera were used to examine whether FN or a molecule with similar immunologic and electrophoretic properties (i.e., an FN-like molecule) is present in fetal brain at E52, a time when extensive neuronal migration and axonal elongation is occurring in the cat telencephalon (Luskin and Shatz, 1985*b*; Shatz and Luskin, 1986). Whole brain homogenates were prepared first in an effort to include all the possible molecules present within brain that show immunologic similarity to FN. The brains were perfused with Ringer's solution before homogenization in order to minimize contamination of the preparation by FNs in fetal plasma. Lanes *1*, *3*, and *5* of Fig. 2 show that E52 whole fetal brain contains a protein, recognized by all three antisera, with a molecular mass nearly identical to that of adult plasma FN when subjected to electrophoresis at 150 V. When the electrophoretic voltage is decreased (Fig. 2 *d*; 60 V), the immunoreactive material in the E52 brain sample migrates as a broad band with an upper limit molecular mass of ~250 kD (lane *7*), always higher than plasma FN under identical conditions (lane *8*). This higher molecular mass material was not eliminated in pia- and choroid plexus–depleted brain (not shown; see Materials and Methods). We did not observe any other bands of similar intensity in the gel or in the stack. The immunoreactive broad band was not present when 23 nM FN was added in blocking controls, or in normal rabbit serum controls (not shown). A consistent finding was the presence of FN-immunoreactive products of molecular mass <200 kD (see lane *7*), although these products were always of lesser intensity compared with the broad band. While all three FN antisera behaved in similar fashion, the rabbit anti–human FN antiserum b appeared most sensitive and it was therefore used more extensively for subsequent antigen transfer analyses.

A slightly modified approach was to examine plasma-depleted (perfused) crude synaptosome preparations for FN-like molecules as compared with plasma and whole brain homogenate preparations in order to gain further insight into the subcellular distribution of FN-like immunostaining. In Fig. 3, lane *1* contains a preparation from adult plasma and lane *2* from E52 whole brain, while lanes *3* and *4* are from synaptosome preparations at ages E48 and E39, respectively (subjected to electrophoresis at 60 V). The 200–220-kD apparent molecular mass of adult plasma FN in lane *1* contrasts with the 200–250 kD broad bands of lanes *2–4*. These antigen transfer results combined with those in Fig. 2 show that each of three anti-FN antisera recognizes not only an adult plasma molecule with the same 200–220-kD molecular mass doublet reported for plasma FNs, but also a 200–250-kD broad band from plasma-depleted whole fetal brain (both forms of FN are reviewed in Hynes and Yamada, 1982; Hynes, 1985). Similar forms are also present in E48 and E39 plasma-depleted synaptosome preparations.

Distribution of FN-like Immunoreactivity

The same three antisera used in the antigen transfer analyses (antisera a, b, and c in Fig. 2) were next applied to cryostat sections of brain to examine the distribution of FN-like immunostaining within E50 brain. At this age, as shown in the cresyl violet–stained section of Fig. 4 *d*, the telencephalon can be subdivided histologically into the several embryonic layers identified in Fig. 1. When adjacent sections were stained with antisera a, b, or c, the same general pattern of staining was observed in each case (Fig. 4, *a–c*; panel letters correspond to antisera). The FN-like immunostaining is present in the marginal zone and the subplate region of the intermediate zone (cf. Fig. 4, *a–c* and *d*), but is absent from the cortical plate and ventricular zone. It is also present, although with less intensity, in the lower part of the intermediate zone. The pia mater (Fig. 4 *a*, *Pia*) as well as the choroid plexus (Fig. 4 *a*, *Chr*) are also immunostained. Normal rabbit serum controls did not reproduce this staining pattern (Fig. 4 *e*) and staining also could be blocked completely for all three antisera by the addition of 23 nM FN incubated simultaneously with antisera (shown in Fig. 4 *f* for antiserum b). Since all three antisera always showed the same staining patterns with respect to each other at every age studied, results obtained using antiserum b will be presented in the subsequent figures.

While FN-like immunostaining in the fetal brain is concentrated in the subplate, the immunohistochemical pattern in the adult is completely different, with no staining in the white matter. Fig. 5 *a* shows the adult pattern of anti-FN immunostaining observed by P70 and complemented by two older ages, P178 (Fig. 5 *b*) and >1-y-old (Fig. 5 *c*). Fig. 5, *a–c* shows that FN-like immunostaining is absent from the white matter. The controls shown in Fig. 5, *d–f* indicate that the staining of the cortex and other areas of adult neuropil in Fig. 5, *a–c* is nonspecific (Fig. 5, *d* and *e* are FN-blocking controls; Fig. 5 *f* is a normal rabbit serum control). We have previously observed nonspecific staining in areas of high cell density using other non-FN antisera on vibratome cut sections and this phenomenon has been previously reported for similarly prepared adult brain (Hendry et al., 1984). The nonspecific staining does serve to clearly demarcate the cortical layers from the unstained white matter. Since, in our experience, vibratome sections provided greater sensitivity at fetal ages than cryostat sections for detecting FN-like immunoreactivity, vibratome sections were preferred over

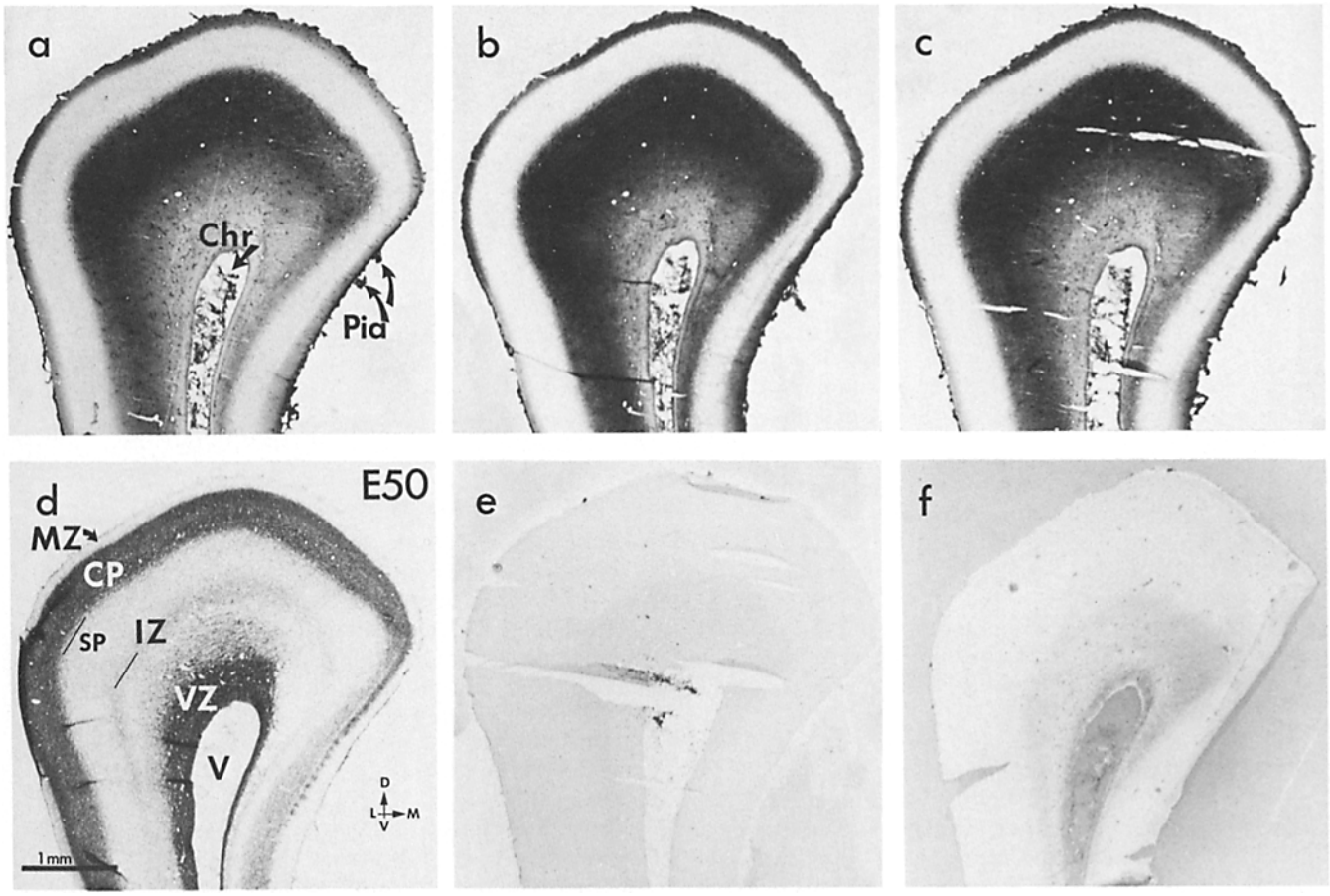

Figure 4. The three anti-FN antisera (*a–c*) show identical patterns of tissue immunostaining at E50. Adjacent 8-μm thick coronal sections through the telencephalon in the region of future visual cortex at E50 were stained with the same three antisera analyzed in Fig. 2. Fig. 4 *d* is an adjacent cresyl violet–stained section to illustrate the histological organization of the telencephalon at this age (see also Fig. 1). (*e*) A control using normal rabbit serum; (*f*) a blocking control in which 23 nM FN was added. The pia (*Pia* or leptomeninges) and choroid plexus (*Chr*) are immunostained. Immunostaining is also present in the marginal zone (*MZ*) (cf. *d*) and the subplate (*SP*), but not in the lower part of the intermediate zone (*IZ*) or in the ventricular zone (*VZ*). Note the clear absence of immunostaining from the cortical plate (*CP*). Orientation of sections is given in *d*: *D*, dorsal; *L*, lateral; *M*, medial; *V*, ventral.

those cut by cryostat for postnatal and adult ages. (Cryostat sections at all ages showed qualitatively identical results). In addition to the absence of white matter staining, note that blood vessels are also unstained for FN in the cerebral cortex of adult animals (Fig. 5, *a–c*). We have never observed any adult cerebral cortical blood vessel FN-like immunostaining by the three antisera used in this study in either vibratome- or cryostat-sectioned material.

The presence of FN-like immunostaining at E50 and its absence in the adult suggest that this immunostaining is transient. To examine the time course of disappearance of FN-like immunostaining, a developmental series was prepared. Fig. 6 *a* is a cresyl violet–stained section and can be compared with Fig. 6 *b*, which shows that by E46, the earliest age examined histologically, the subplate and marginal zones are already immunostained. FN-like immunostaining of the subplate is maintained through E52 (Fig. 6 *c*) and E60 (Fig. 6 *d*), although marginal zone immunostaining is not detected after late E50 to early E60 (only the pia mater shows superficial staining at these later ages). As the subplate gradually matures to become the white matter, immunostaining in this zone declines, as can be seen by P7 (Fig. 6 *f*). The disappearance of subplate/white matter staining is uneven, resulting in the persistence of islands of FN immunostaining at the bases of the cortical gyri (cf. Fig. 6 *f* with Fig. 6 *g*; in *g*, the most dorsal island is enclosed in a box). These islands of FN-like immunostaining have vanished by P49 (Fig. 6 *h*), when only a faint rim of staining separates layer 6 from underlying white matter (Fig. 6 *h*, *arrows*). By P28, the previously noted nonspecific background staining increases, thereby making it difficult to identify unequivocally specific immunostaining in some brain regions such as the thalamus (asterisks in Fig. 6, *g* and *h*). However, control experiments at P28 and P49 indicate that the FN-like immunostaining within the white matter is specific.

In addition to the cerebral cortex, FN-like immunostaining is observed developmentally within the cell-poor regions of the hippocampus (*H* in Fig. 6, *a*, *d*, and *e*, compared with *b* and *c*), the thalamus (*L* indicates the lateral geniculate nucleus of the thalamus in Fig. 6, *a* and *e*, compared with *b*, *c*, and *f*), and the midbrain (*MB* in Fig. 6 *d*). Like the neocortex, regions of high cell density are devoid of FN-like immunostaining. Thus, Fig. 6 shows that by E46, FN-like immunostaining is present within cell-poor regions of the

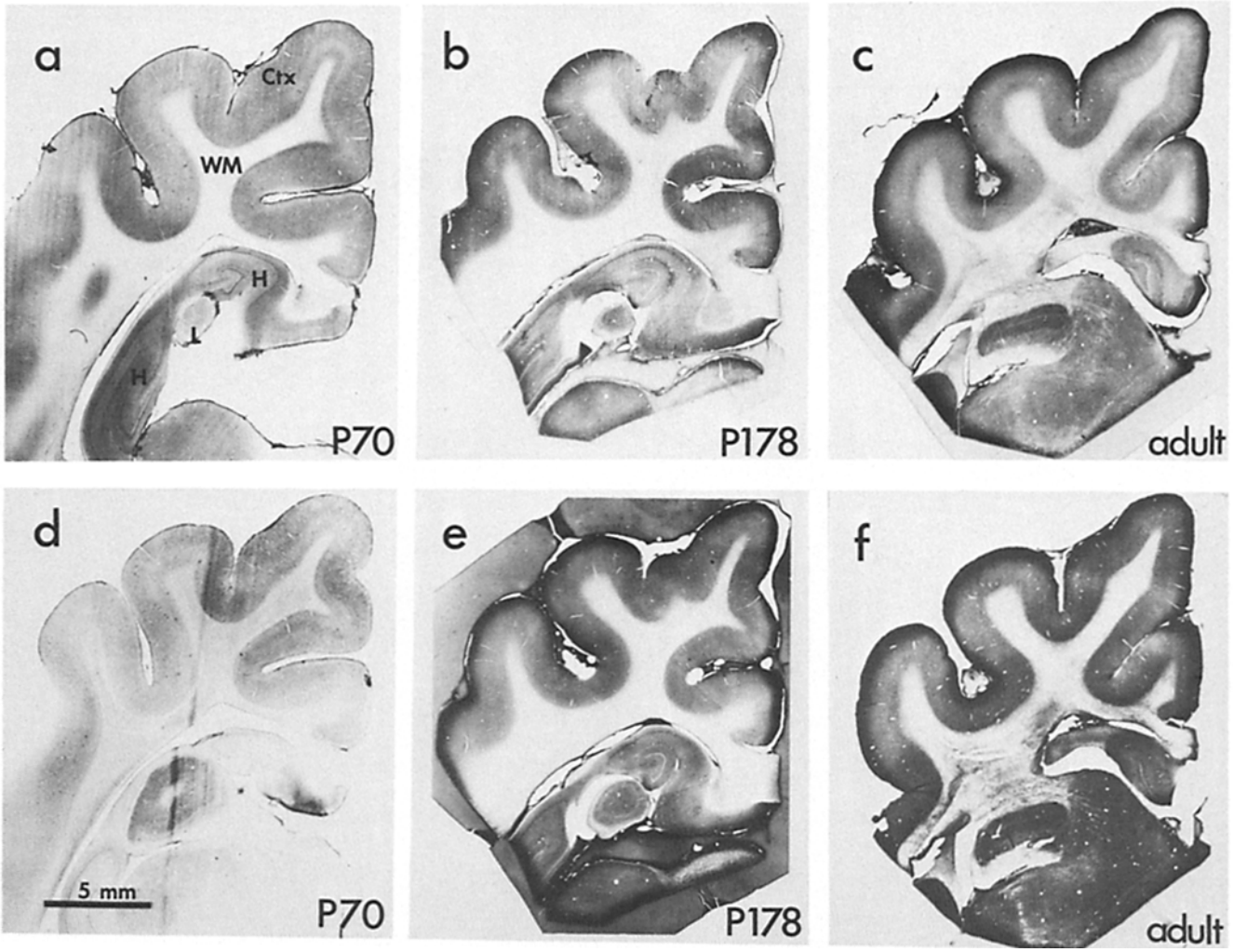

Figure 5. FN immunostaining is not present in the white matter (*WM*) of adult animals nor by P70. In these 50-μm thick vibratome sections cut in the coronal plane through visual cortex, immunostaining (*a–c*) of cortex (*Ctx*), hippocampus (*H*), and thalamus (not labeled) is not specific since it is also present in FN blocking controls of matched sections (cf. *d* and *a*; *e* and *b*), and in normal rabbit serum controls (cf. *f* and *c*; see text for further details). Note the absence of blood vessel staining in these sections, which are all prepared from buffer-perfused brains. Orientation is identical to that shown in Fig. 4 *d*.

brain, particularly in the subplate and marginal zones and is absent from cell-dense regions such as the cortical plate. This pattern persists into the early postnatal period, after which immunostaining first clears from the white matter near sulci, leaving islands at the bases of the gyri. By P49 most of the islands have also disappeared so that by P70 (Fig. 5) no FN-like immunostaining can be detected in the white matter.

Correlation between FN-like Immunostaining and Subplate Neurons

These observations raise the issue of what cellular element or elements might be responsible for producing the FN-like immunostaining seen in the subplate and marginal zones of the developing cerebral cortex. In analyzing the distribution of FN-like immunoreactivity, we were struck by the similarity in location and time course of disappearance between the FN-like immunostaining in the subplate and marginal zones, and the subplate and marginal zone neurons. For example, Fig. 7 *a* is a cresyl violet–stained cryostat section showing selected embryonic zones. Fig. 7, *b* and *c* are micrographs of the same field from a section adjacent to 7 *a*, immunostained for FN; Fig. 7 *b* shows the section observed with darkfield optics (immunostaining appears white) and Fig. 7 *c* is the same section in brightfield optics (immunostaining appears black). As indicated in Fig. 7, *a* and *b*, the subplate can be divided into an upper (SP_U) and lower (SP_L) part, both of which contain the early generated subplate neurons as identified by [^{3}H]thymidine autoradiography. The higher packing density of cell somata, however, distinguishes the more compact upper subplate from the less dense lower subplate. In Fig. 7 *b*, the position of the upper subplate can be accurately determined by noting the clusters of silver grains over the nuclei of the subplate neurons (note arrows) that were [^{3}H]thymidine labeled at E27 (Luskin and Shatz, 1985) and subsequently examined here at E56 with a combination of FN immunohistochemistry followed by autoradiography. Comparison with Fig. 7 *c* illustrates that the relatively cell-poor lower subplate contains extensive FN-like immunostaining. Conversely, FN-like immunostaining does not

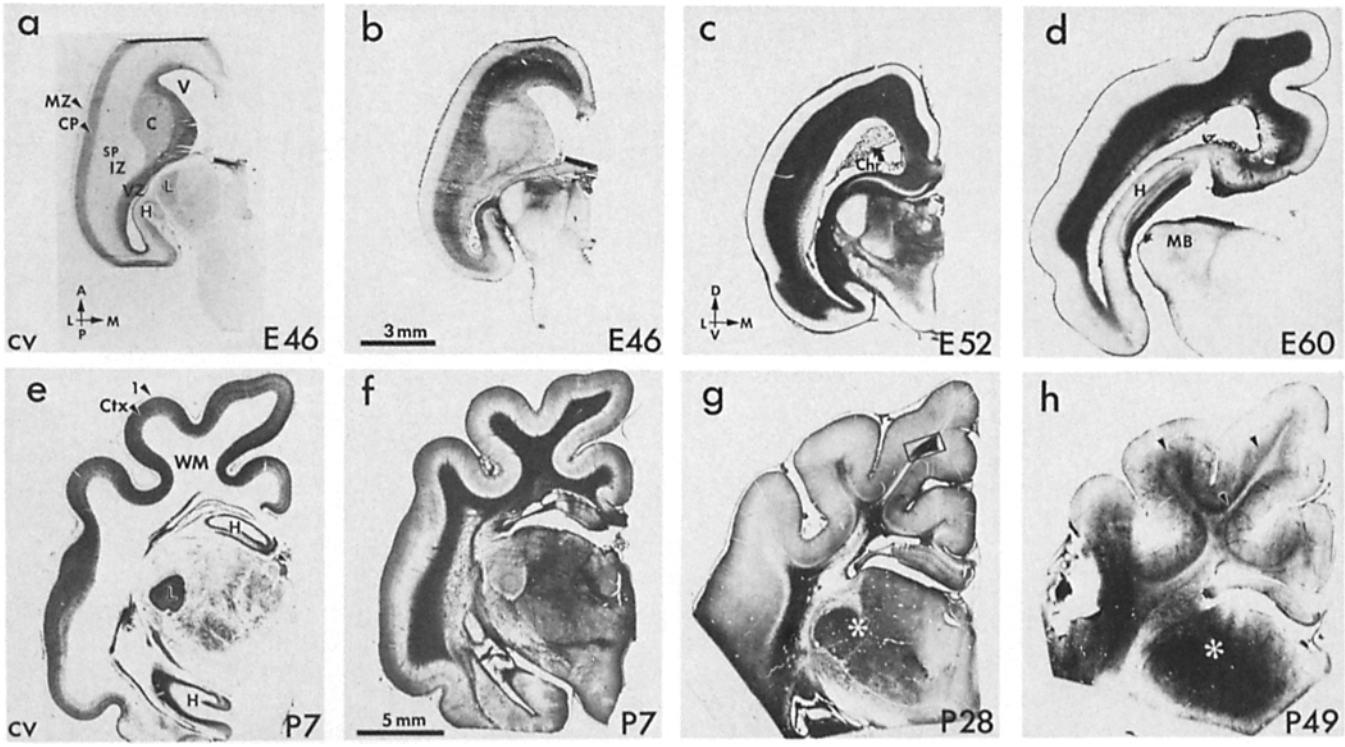

Figure 6. FN immunostaining is present prenatally in the subplate (*SP*) and diminishes postnatally in the white matter (*WM*). Specific immunostaining fills the subplate at E46, E52, and E60 (*b–d*), and white matter at P7 (*f*). At P28 (*g*) and P49 (*h*), immunostaining begins to decrease in the white matter. *a* and *e* are cresyl violet–stained sections adjacent to those of *b* and *d*, to show the histological organization of the brain at E46 (*a* and *b*) and P7 (*e* and *f*). Immunostaining is also present in the marginal zone at E46 and E52, but cannot be detected there by E60 (*d*). Cell poor regions of the thalamus such as those surrounding the lateral geniculate nucleus (*L* in *a* and *e*) and of the hippocampus (*H*) are also immunostained between E46 and P7. However, by P28 nonspecific immunostaining (asterisks in *g* and *h*) increases, making specific immunostaining difficult to detect. All sections are cut coronally except those in *a* and *b*, which are horizontal sections. Orientation of *a* and *b* is given in *a*: *L*, lateral; *M*, medial; *A*, anterior; *P*, posterior. All other sections are oriented as shown in *c*: *D*, dorsal; *V*, ventral. Identified neuroanatomic structures: marginal zone (*MZ*), layer 1 (*1*), cortical plate (*CP*), cortex (*Ctx*), subplate (*SP*), ventricular zone (*VZ*), caudate (*C*), hippocampus (*H*), lateral geniculate nucleus (*L*), midbrain (*MB*), and choroid plexus (*Chr*). Rectangle in *g* encloses an island of FN-like immunostaining shown at higher magnification in *a*. Arrows in *h* indicate residual FN-like immunostaining at the cortex–white matter border. Bars: (*a–d*) 3 mm; (*e–h*) 5 mm.

appear to invade substantially the cell-rich upper subplate. Thus, as shown in Fig. 7, *b* and *c*, the heaviest FN-like immunostaining is present in the lower subplate, and this staining declines near the base of the upper subplate.

To examine further the correlation between FN-like immunostaining and the position of subplate neurons in the lower subplate, thicker (80-μm vibratome) sections were stained with FN antisera to obtain more complete information on subplate neuron morphology. As shown in Fig. 8 *a*, the typical appearance of subplate neurons at E50 can be revealed clearly by immunostaining them with an antibody against MAP2. Many have pyramidal shaped somata (large arrows) with one or more dendrites directed into the lower subplate and towards the ventricular zone (curved arrows). Cells with remarkably similar morphologies are also FN-immunostained, as shown in Fig. 8 *b* (large arrows). In addition, a blood vessel is immunostained (asterisks in Fig. 8, *b*, *c'*, and *c''*) along with more diffuse subplate labeling. At least some of the FN-like immunostained somata such as those shown in Fig. 8 *b* are subplate neurons as confirmed by [^{3}H]thymidine birthdating of subplate neurons at E27, followed by immunostaining at E50. The result of this experiment, shown in Fig. 8, *c* and *c''* demonstrates that some FN-immunoreactive cells can also be labeled with [^{3}H]thymidine, thus identifying them as subplate neurons.

The time course and pattern of disappearance of FN-like immunostaining is also correlated with that of the subplate neurons. As the subplate neurons gradually disappear from the white matter during early postnatal life, so too does FN-like immunostaining, leaving islands of stain such as those observed at P28 (Fig. 6 *g*, boxed area). Fig. 9 *a* shows an enlarged photo of the island of FN-like immunostaining enclosed by the box in Fig. 6 *g*. The dark, diffuse, FN-like immunostaining within the white matter of Fig. 9 *a* contrasts with the white, unstained region of neocortex. When an adjacent section (Fig. 9 *b*) was immunostained with MAP2 to reveal the location of the subplate neurons, MAP2-immunoreactive cells were also found in similar locations within the islands. (It should be noted that at this postnatal age, MAP2 also stains the neurons of the cerebral cortex). Proof that the MAP2-immunoreactive cells are indeed subplate neurons is provided in Fig. 9 *c*, a higher magnification of the boxed area in Fig. 9 *b*. In this experiment, a [^{3}H]thymidine injection was made at E27 to label some of the subplate neurons,

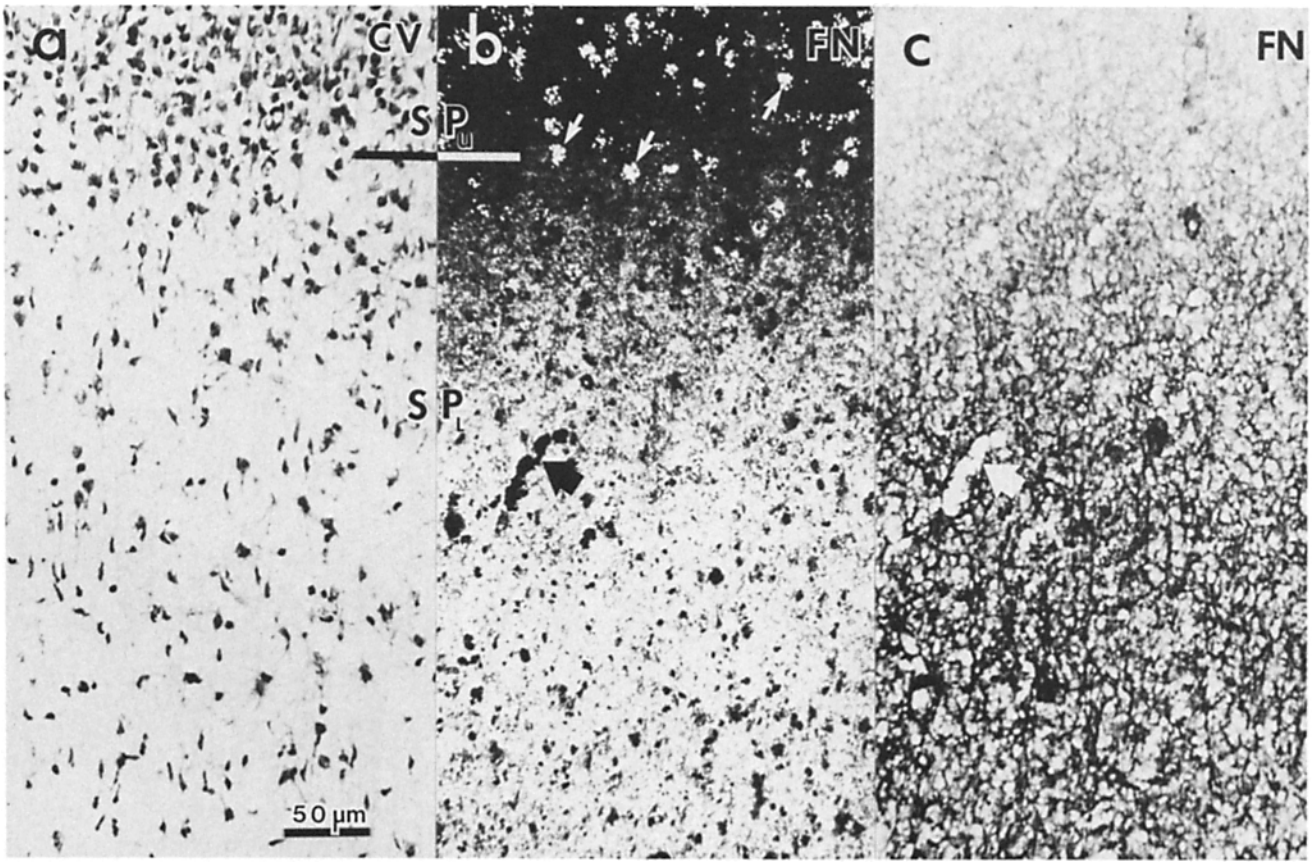

Figure 7. FN-like immunostaining at E56 is located primarily within the lower subplate. (*a*) A cresyl violet (*CV*)–stained cryostat section showing the histological subdivision between the lower (SP_L) and upper (SP_U) parts of the subplate. (*b*) A darkfield photomicrograph of an adjacent cryostat section immunostained for FN and then prepared for autoradiography to reveal [^{3}H]thymidine-labeled neurons of the SP_U that had been birthdated at E27. Note that the neurons of the upper subplate (white arrows indicate silver grains over nuclei) sit just above the region of the most dense FN-like immunostaining. (*c*) A brightfield photomicrograph of the same section as in *b*. Note that FN-like immunoreactivity also labels a blood vessel (*large arrow* in *b* and *c*). All sections are oriented with pial surface to the top of the page.

which were subsequently examined at P28 by both MAP2 immunostaining and autoradiography. The curved arrows in Fig. 9 *c* mark MAP2-immunostained cells with silver grains over their nuclei, thereby identifying them as subplate neurons. Thus, FN-like immunostaining not only correlates with the presence of subplate neurons during fetal life, (the lower subplate neurons in particular) but also with the time course and pattern of their disappearance during early postnatal life.

To learn more about the correlation between FN-like immunoreactivity and subplate neurons, subplate neurons were partially eliminated to examine the effect of this perturbation on the distribution of FN-like immunoreactivity. Subplate neurons were killed during fetal life by making injections of the excitatory neurotoxins kainic acid (Coyle et al., 1978) or ibotenic acid (Schwarcz et al., 1979) at E40, E43, or E45 and then examining the consequences 1–3 wk later (see Table II for exact ages). The effectiveness of neurotoxin treatment was evaluated by using anti-MAP2 to detect remaining subplate neurons. Both neurotoxins destroyed subplate neurons, although kainic acid injection produced a larger, less localized lesion than that from ibotenic acid. Fig. 10 shows the results of an experiment in which kainic acid was injected at E45 and the brains were examined at E52. A cresyl violet–stained section (in Fig. 10 *a*) reveals that the subplate is markedly hypercellular (Fig. 10 *a*; subplate borders are indicated by arrows) as compared with the control hemisphere (Fig. 10 *e*). An adjacent section from the lesioned hemisphere stained with anti-MAP2 (Fig. 10 *b*) shows markedly decreased staining compared with the control hemisphere (Fig. 10 *f*), indicating that, except for a rim of cells at the cortical plate-subplate border, the majority of subplate neurons were eliminated. Confirmation that the loss of MAP2 immunostaining reflected subplate neuron deletion was established by another experiment (not shown) in which [^{3}H]thymidine-labeled subplate neurons were also eliminated from neurotoxin lesioned, MAP2-depleted areas.

Adjacent sections stained with anti-FN revealed that FN-like immunostaining in the lesioned region is also markedly decreased (Fig. 10 *c*) as compared with the control hemisphere (Fig. 10 *g*). Paralleling the rim of surviving subplate neurons labeled by MAP2 (Fig. 10 *b*), a rim of FN-like immunostaining remained at the cortical plate–subplate border (Fig. 10 *c*). Note that despite the elimination of FN-like im-

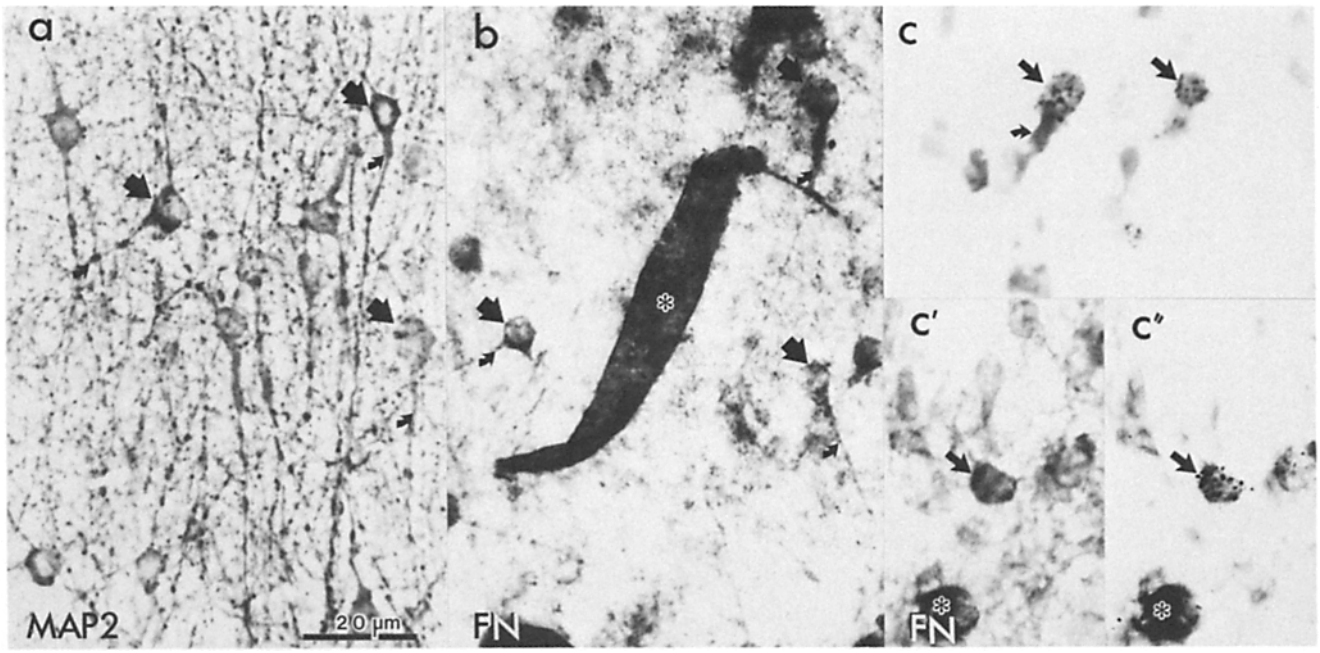

Figure 8. Subplate neurons are delineated by FN-like immunostaining. The subplate neurons have characteristic morphologies, as revealed with MAP2 immunohistochemistry. (*a*) A photomicrograph of the lower subplate from an E50 brain immunostained for MAP2. Large arrows indicate several somata; curved arrows indicate the often beaded dendritic processes. When similar tissue is immunostained for FN, morphologically similar cells can be observed (*large* and *curved arrows* as in *a*); a large blood vessel (*asterisk*) is also FN immunoreactive. (*c*–*c''*) Some of the FN immunostained cells are subplate neurons, since they can also be labeled by [^{3}H]thymidine administered on their birthdate at E27. In *c* and *c''*, the arrow points to silver grains over subplate neuron nuclei; *c'* is at a different focal plane from the same cell shown in *c''* (asterisks indicate a labeled blood vessel). Orientation of all photos is with pial surface towards the top of the page.

munostaining from the subplate, blood vessel immunostaining is still present within the lesioned region (Fig. 10 *c*). To assess whether the treatment was selective for SP neurons or whether glia were also eliminated, an antiserum against GFAP (found in radial glia and astrocytes; Eng et al., 1971; Bignami and Dahl, 1974) was also applied to adjacent sections. In the lesioned hemisphere (Fig. 10 *d*), GFAP immunostaining appeared increased compared with its low levels in the control hemisphere, indicating that GFAP-positive glia are still present. Thus, after neurotoxin elimination of subplate neurons, FN-like immunostaining decreases within the subplate in a similar regional pattern. This result is consistent with the temporal and spatial correlation between FN-like immunostaining and the subplate neurons drawn earlier (see above).

Discussion

In this study, antigen transfer analyses using antisera raised against FNs show that a molecule (or complex of molecules) with an apparent molecular mass of 200–250 kD is present within fetal cat brain. The molecule has immunologic and electrophoretic similarities to FNs, cellular FNs in particular. These same antisera used immunohistochemically recognize not only fetal blood vessels, pia mater (leptomeninges), and choroid plexus, but also stain the neuropil of the subplate and marginal zones within the developing cerebral cortex. As the cortex matures, FN-like immunoreactivity gradually disappears from the marginal zone (by late prenatal life) and subplate (postnatally) so that by P70 no specific immunostaining remains in the white matter (the adult pattern). Moreover, the presence and subsequent disappearance of FN-like immunoreactivity is spatially and temporally correlated with the presence and disappearance of the subplate neurons, both in normal development and when the subplate neurons are artificially removed by neurotoxin injections, suggesting that the subplate neurons play an important role in the regulation and expression of FNs.

Identity of the In Situ Molecule(s)

The interpretation of the results rests on both the specificity of the antisera used, and on the identification of the molecule(s) whose recognized determinants are responsible for the staining patterns observed in situ. The specificity of the three antisera used in this study is validated by the ability of each to (*a*) recognize adult cat plasma FN on antigen-transfer analyses; (*b*) recognize known FN-positive compartments, i.e., growing blood vessels, the pia mater, and the choroid plexus (Schachner et al., 1978; Pateau et al., 1980*a*; Minier et al., 1981; Hatten et al., 1982; Jones et al., 1982; Hynes et al., 1986); and (*c*) show susceptibility to blocking by 23 nM (10 μg/ml) of FN on antigen transfers and in tissue sections. In addition, the nearly identical immunostaining patterns amongst all three antisera on transfers and in tissue sections are consistent with the interpretation that each antisera is recognizing the same antigen, i.e., FN, the antigen against which each antiserum was raised. However, we cannot be absolutely certain of the identity of the recognized antigen in the subplate without both cDNA in situ hybridiza-

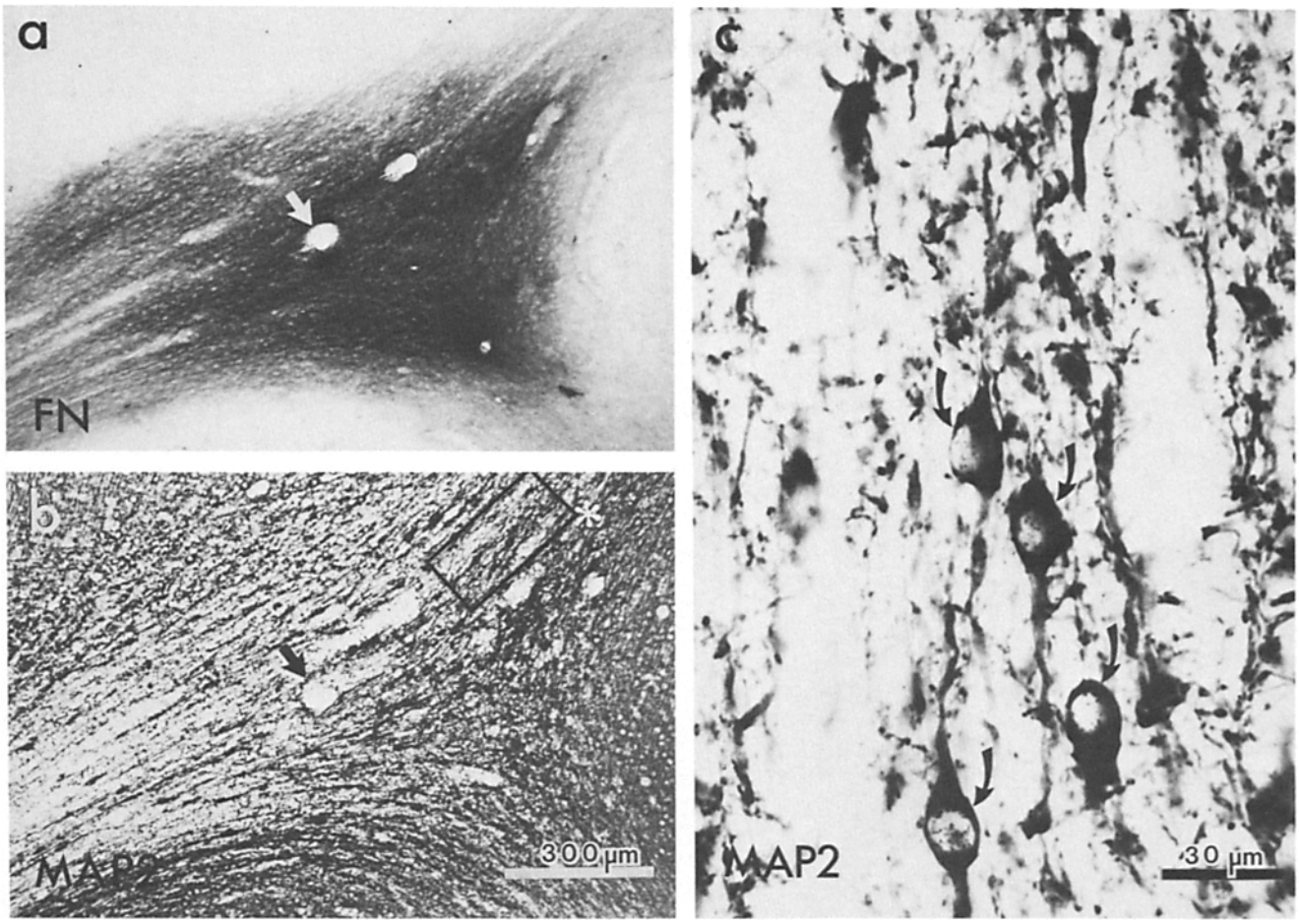

Figure 9. Subplate neurons coincide postnatally with islands of FN-like immunostaining. (*a*) An enlargement of the FN-immunostained region enclosed in the rectangle of Fig. 6 *g* at P28. (*b*) An adjacent section (cf. arrows pointing to same blood vessel) immunostained with MAP2 to show that many subplate neurons are still present in this part of the maturing white matter. (Note that at this age, the neurons of the cerebral cortex are also MAP2 immunoreactive). That these MAP2-labeled cells are subplate neurons can be shown by autoradiography after prior [^{3}H]thymidine administration at their birthdate on E27. The area enclosed by the rectangle in *b* is enlarged in *c* (*asterisk* in *upper right corner*) to reveal the presence of autoradiographically labeled subplate neurons (curved arrows; note silver grains over their nuclei).

tion and specific FN mRNA evidence. Because of this uncertainty, we have retained the term FN-like to refer to the results shown on blots and in tissue sections. In the following discussion (below) the term FN (instead of FN-like) will be used, but the true identity of the observed molecule(s) awaits further study.

A variety of observations suggest that the antisera recognizes a cellular form of FNs in fetal cat brain. Cell surface FNs are known to have a higher molecular mass than plasma FN (Hynes, 1976; Yamada and Kennedy, 1979; Hynes and Yamada, 1982; Hynes, 1985), to show cross-reactivity with antisera against plasma FN (Ruoslahti and Vaheri, 1974; Hynes and Yamada, 1982), and to exhibit varied migration depending on electrophoretic conditions (Hynes, 1976; Yamada and Kennedy, 1979), all of which were observed in the present study. The existence of the 200–250-kD broad band is also consistent with the known heterogeneity of cellular FN subunits that arise through variations in posttranscriptional mRNA splicing and/or differences in glycosylation (Paul et al., 1986).

Several cellular compartments within brain probably contribute to the broad band observed in antigen transfer analyses. While we cannot exclude the possibility that the entire broad band is due only to the blood vessels, pia mater, and choroid plexus, such an interpretation does not take into account our findings that (*a*) a similar band is recognized in synaptosome preparations from which these cellular elements have been at least partially eliminated, and (*b*) the subplate and marginal zones are immunostained in tissue sections. Thus, several cellular compartments within the developing brain probably contain FNs, a suggestion consistent with the observation that depletion of FN immunostaining in the subplate by means of neurotoxins leaves the blood vessel staining intact. We cannot determine whether each compartment contributes a separate component to the 200–250-kD broad band, or whether instead the same heterogeneous molecular form is made by all cellular elements. Evidence favoring the possibility that the forms associated with each cellular element differ comes from studies of cultured endothelial cells that appear to synthesize FNs with molecular masses of <250 kD (Jaffe and Mosher, 1978; Macarak et al., 1978; Birdwell et al., 1978; Sage et al., 1979; Vlodavsky

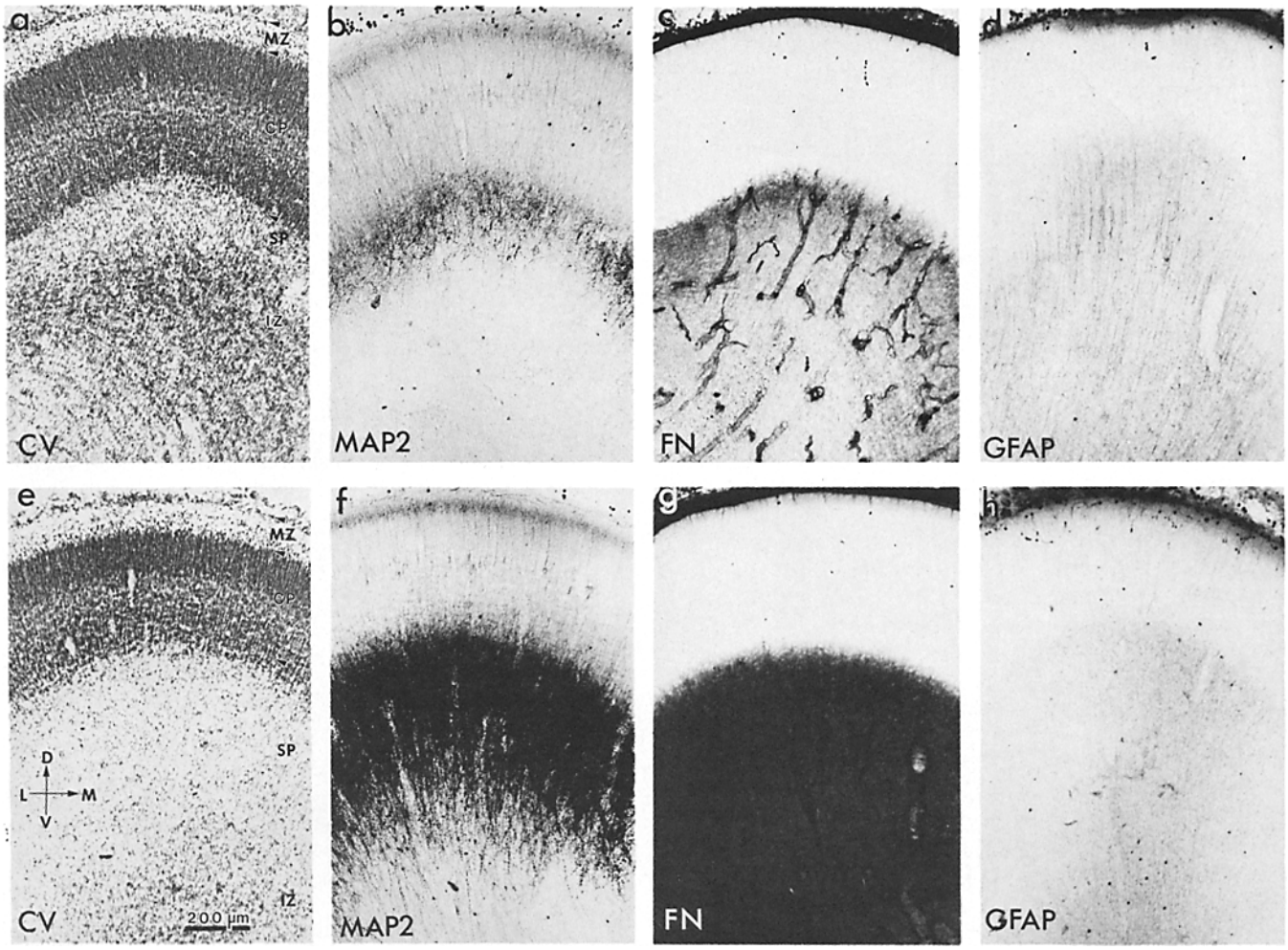

Figure 10. The elimination of subplate neurons is correlated with a decrease in FN-like immunostaining. At E45, neocortical injections of the neurotoxin kainic acid were made into one hemisphere to delete subplate neurons (*a–d*); vehicle solution (0.9% sterile saline) was injected in the other hemisphere (*e–h*). The consequences of the injections were examined at E52. Adjacent coronal sections were stained with cresyl violet (*CV*), or with antibodies against FN, MAP2, or GFAP. In cresyl violet–stained sections, the marginal zone (*MZ*) and cortical plate (*CP*) appear essentially normal, but there is an increase in cell density within the subplate (*SP*) and intermediate zone (*IZ*) on the lesioned side (cf. *a* and *e*). Within the lesioned hemisphere, MAP2 immunostaining is greatly reduced (cf. *b* and *f*), indicating a loss of subplate neurons. Adjacent sections (within 400 μm) show that while blood vessel staining is present in both hemispheres, FN-like immunostaining of the subplate is drastically reduced on the side of the neurotoxin lesion (*c*). At the same time, immunostaining for GFAP is increased in the lesioned hemisphere (cf. *d* and *h*). Orientation of each section is with pial surface towards the top of the page as indicated in *e*: *D*, dorsal; *M*, medial; *V*, ventral; and *L*, lateral.

et al., 1979). Whatever the case, the fact that all three antisera recognize one broad band in antigen transfer analyses of whole brain preparations argues that the immunostaining seen in tissue sections is due to FN rather than to the presence of different molecules sharing the same determinants. Thus, our results suggest that a molecule similar or identical to FN is present in the fetal cat telencephalon and is associated not only with plasma, blood vessels, pia mater, and choroid plexus but also with elements of the subplate and marginal zones.

Comparisons with Previous Studies

FN immunoreactivity has been reported in the subplate and marginal zone of the developing fetal mouse cerebral cortex (Stewart and Pearlman, 1987) and in one study of the mouse cerebellum (Hatten et al., 1982; but see Hynes et al., 1986). While our own results cannot address the discrepancy that exists between the two studies on cerebellum, they are consistent with the recent study of mouse cerebral cortex. It is noteworthy that mouse cortical immunostaining can only be detected for a brief 7-d period, whereas in the cat, it is present for over 2 mo. This difference underscores the point that the failure to find FN immunostaining in a species with a rapid developmental time table may be due to its very brief expression. While we did not look for FNs in cat cerebellum, we did find thalamic and midbrain immunostaining in this study, suggesting that the presence of FNs in the central nervous system is a more generalized phenomenon, at least in cat.

Our finding that FN immunoreactivity is present in the developing central nervous system is not in agreement with the results of most other previous studies. However, there are

many methodological explanations for this discrepancy. First, some studies did not concentrate on the developing neocortex, instead examining other central nervous system regions (Schachner et al., 1978; Minier et al., 1981; Jones et al., 1982; Hynes et al., 1986). Second, as mentioned above, many of the reports studied animals in which the pace of development is considerably faster than that in the cat (Schachner et al., 1978; Minier et al., 1981; Hynes et al., 1986), making the detection of developmentally relevant FN critically dependent on choosing the appropriate age, as well as the appropriate location. Further, significant differences exist in the histological techniques used in our study and those used previously, such as fixation, tissue processing, and immunohistochemistry, and the period of primary antiserum incubation, which in our study was 48–72 times longer than in others (Schachner et al., 1978; Minier et al., 1981; Jones et al., 1982). We can demonstrate FN immunostaining in vibratome and cryostat sections, with or without Triton X-100 (but more robust staining was found in vibratome sections with Triton), suggesting that one of the more important variables is probably the period of primary antisera incubation rather than tissue preparation.

It is also remarkable that we did not find blood vessels immunoreactive for FN in the adult cerebral cortex, in contrast to previous reports (Stenman and Vaheri, 1978; Schachner et al., 1978; Pateau et al., 1980*a*; Minier et al., 1981; Jones et al., 1982). This is unlikely to reflect overfixation, since fetal blood vessel staining is present in identically prepared tissue. The discrepancy may again be explained by methodological differences: most previous studies did not use an initial buffer perfusion to remove plasma FN from within blood vessels. Others examined brain tissue that may have undergone pathologic changes, prompting the neurosurgical resection from which it was obtained (Pateau et al., 1980*a*); injured blood vessels can respond by producing FN (Clark et al., 1982; Grinnel, 1984). Thus, we suggest that in the cat, blood vessels of the developing cerebral cortex do produce FNs, while the normal adult endothelium of the cerebral cortex does not produce FN or produces it at levels below immunohistochemical detection.

What Cellular Components Synthesize FN?

A major finding of this study is that the subplate neurons are correlated temporally and spatially with the presence of FN immunoreactivity. The results in Fig. 7 suggest that the lower subplate neurons may be more closely associated with FN than those of the upper subplate, since the greatest FN staining is located in the lower subplate. This implies that FN-mediated development involves the participation of only part of the subplate neuron population. Such population heterogeneity has previously been indicated by the different neuropeptide immunoreactivities of the upper vs. lower subplate neurons (somatostatin vs. neuropeptide Y respectively; Chun et al., 1987). However, both upper and lower subplate neurons appear to direct many of their cell processes (probably dendrites) into the lower subplate (Figs. 8 and 10) so that it is possible that not only the lower subplate neurons but also those of the upper subplate are associated closely with FN.

The loss of FN immunoreactivity after elimination of subplate neurons raises the possibility that the subplate neurons themselves may be one source of FN. However, until it can be proven that subplate cells synthesize FN, one must consider the many other potential sources for FN production, including plasma (Mosesson and Umfleet, 1970; reviewed by Hynes and Yamada, 1982), CSF (Kuusela et al., 1978), the choroid plexus, leptomeninges and blood vessels, astrocytes (Kavinsky and Garber, 1979; Price and Hynes, 1985; but contrast with Raff et al., 1979; Kennedy et al., 1980; Pateau et al., 1980*b*; Stieg et al., 1980; Jones et al., 1982), and macrophages (reviewed by Hynes, 1981; Hynes and Yamada, 1982). We note simply that while each of the cellular elements listed above certainly could synthesize the observed FN molecule and supply it to the subplate and marginal zones, none appears to show as strong a temporal and spatial correlation with FN immunostaining as do the subplate neurons themselves.

Another possibility is that subplate (and marginal zone) neurons could regulate, if not synthesize, FNs by interacting with FN-synthesizing cells such as glia. This is perhaps a more reasonable alternative, since there are no known examples indicating that neurons can synthesize FNs. For instance, the elimination of subplate neurons by the neurotoxin injections might alter glial expression of FNs. It is unlikely that neurotoxins eliminate the glia or macrophages, since inspection of the lesion sites indicate that in fact many macrophage-like cells have invaded the area, and GFAP immunostaining shows that the radial glial cells are also still present. These observations are consistent with previous studies indicating that glia are not eliminated by exposure to these neurotoxins, though they can become "reactive" in response to neuronal destruction (Coyle et al., 1981). Thus, it may be that when subplate neurons are eliminated by neurotoxin treatments, the cellular interactions required for FN expression are interrupted and FN levels in the subplate fall.

Possible Roles of FN in Cerebral Cortical Development

The results of this study show that FNs or similar molecules are present in the subplate and marginal zones, but are not detectable within the cortical plate. This differential distribution must be considered in thinking about the roles that such molecules might play during the development of the cerebral cortex. Of the possible roles for this extracellular matrix component, FNs may act to promote cell migration and/or neurite elongation, functions they are known to subserve in other systems (Hynes and Yamada, 1982; Hatten et al., 1982; Rogers et al., 1983; Hynes 1985; Thiery and Duband, 1986). The studies of Rakic (1972, 1977) have shown that neurons generated at the ventricular surface must migrate long distances to take up their positions within the cortical plate. Moreover, during development, many axonal projection systems must grow to or from the cerebral cortex (thalamocortical: Anker and Cragg, 1974; Rakic, 1977; Lund and Mustari, 1977; Wise and Jones, 1978; Crandall and Caviness, 1984; Shatz and Luskin, 1986; corticothalamic: Shatz and Rakic, 1981; callosal: Wise and Jones, 1978; Innocenti, 1981; monaminergic: Lidov et al., 1978). Our results indicate that FNs are certainly found in zones where these developmental events occur, namely the subplate and marginal zones. It must be noted, however, that both cell migration and axon elongation also occur within the cortical plate. Thus, FNs need not mediate these interactions within the cortical plate itself.

The location of FNs are nevertheless appropriate to mediate cell migration and/or axon elongation within the subplate

(and marginal zone). There is no direct experimental evidence demonstrating that FNs mediate these functions in the telencephalon. However, when we decreased FN levels in the subplate by making kainic acid lesions, a hypercellularity resulted (see Fig. 10 *a*). This increase in cell density could be due to the fact that the region has been invaded by glia in response to damage caused by the lesion (Coyle et al., 1978; Murabe et al., 1981). Another explanation for the hypercellularity, which does not exclude the first, is that cells normally migrating to the cortical plate cannot traverse the lesioned area because FN levels have been decreased. Preliminary counts of spindle-like cells thought to be migrating neurons have revealed a twofold increase in number (lesion vs. control) and this could indicate that cell migration within the subplate may be, in part, FN-mediated.

We have so far considered cellular interactions common to both the cortical plate and the subplate and have suggested that FNs may subserve these interactions in one area but not in the other. Another possibility is that FNs function in interactions restricted and unique to the subplate. As mentioned in the introduction, the subplate is not only a zone through which cells migrate and axons grow, but is also a complex synaptic neuropil at a time when the cortical plate is still in the early stages of differentiation. Elements that participate in this neuropil include the subplate neurons, known to receive synaptic contacts, (Chun et al., 1987) and the ingrowing axonal systems, known to wait for several weeks before invading the cortical plate (Rakic, 1977; Lund and Mustari, 1977; Wise and Jones, 1978; Innocenti, 1981; Crandall and Caviness, 1984; Shatz and Luskin, 1986). These considerations raise the possibility that axons prefer to remain within the FN-rich, cell-poor subplate, and it is only when FN levels begin to fall that the extracellular milieu of the cell-dense cortical plate becomes more preferable for axon elongation. Thus, as has been suggested for other systems and species, ingrowing mammalian cortical axons may have a hierarchy of substrate preferences (Berlot and Goodman, 1984; Tomaselli et al., 1986). The spatiotemporal regulation of substrate hierarchy could dictate whether axons wait within the subplate, or grow into the cortical plate.

Among other developing axonal systems known to wait in the subplate are the cortical association and callosal systems that connect regions of the cerebral cortex (Wise and Jones, 1978; Innocenti, 1981; Olavarria and Van Sluyters, 1985). Axons belonging to both of these systems are known to undergo rather extensive remodeling through the elimination of transient collaterals, some of which occurs during the waiting period (Innocenti and Caminiti, 1980; Innocenti, 1981; O'Leary et al., 1981; Ivy and Killackey, 1982; reviewed by Stanfield, 1984). The mechanism for this elimination is not known but it has been suggested that macrophage-like microglia may be capable of phagocytosing the unwanted transient collaterals (Ling, 1976; Innocenti et al., 1983*a*, *b*; Killackey, 1984). These glial cells are transitory, and have been observed in many parts of the brain, including the developing cerebral white matter (subplate) of at least several species, including mouse (Perry et al., 1985), rat (Ling, 1976, 1977; Valentino and Jones, 1981), and man (Kershman, 1939; Choi, 1981). In the postnatal cat brain, these glial cells are located in the same regions (Innocenti et al., 1983*a*, *b*) as the islands of FN immunostaining reported here.

The spatiotemporal association between the microglia and FN immunostaining suggests other functions for FNs, at least during neonatal life: recruiting microglia and promoting phagocytosis. It is possible that proteolytic fragments of FNs produced in the subplate during the neonatal period of collateral retraction and subplate neuron disappearance attract microglia in a manner similar to that known to occur for blood monocytes (Norris et al., 1982). This suggestion is not unreasonable, since in lineage the microglia are thought to derive from blood monocytes (Valentino and Jones, 1981; Perry et al., 1985; Esiri and Mcgee, 1986). It is also known that FNs and fragments can serve as opsonins in stimulating phagocytosis by macrophages (Gudewicz et al., 1980; Czop et al., 1985). Thus, elements of the subplate associated with FNs such as the subplate neurons or the unwanted axon collaterals could be preferentially removed by microglial phagocytosis.

We have suggested possible roles for FNs during cerebral cortical development. While such roles are speculative, the fact remains that FNs appear to be localized within a special embryonic zone in which coexist cellular interactions characteristic of the mature nervous system, as well as those associated with the developing nervous system (neurons and synapses on the one hand, versus migrating neurons, growing axons, and radial glia on the other). The extracellular milieu established by FNs (or similar molecules) thus correlates spatially and temporally with these interactions, and may be critical in allowing them to occur within the subplate (and marginal zone). From previous related studies, the subplate is likely also to contain extracellular matrix molecules known as glycosaminoglycans (i.e., hyaluronidase-sensitive anionic binding sites; Nakanishi, 1983), which could maintain extracellular spaces (Bondareff and Pysh, 1968; Sumi, 1969; Cragg, 1975, 1979; Nakanishi, 1983) through which migration and elongation are facilitated. Further, the presence of synapses in the subplate suggests that there may be other matrix molecules important to the formation and maintenance of synaptic sites, as is thought to occur at the neuromuscular junction (Fallon et al., 1985; Wallace et al., 1985). Thus, in the formation of the mammalian cerebral cortex, a variety of extracellular matrix molecules, one of which appears to be FN, could mediate the cellular interactions unique to the subplate and marginal zones.

We wish to thank Dr. L. F. Reichardt and Dr. C. S. Goodman for their helpful criticisms of the manuscript; Drs. R. Vallee, W. Theurkauf, and F. Luca for their gift of MAP2 antibodies; and Dr. L. Eng for his gift of GFAP antibody. We thank Mark Nakamura for assistance with fetal surgeries and histology, and Cecele Thomas for word processing.

This research was supported by National Institutes of Health grant EY02858 to C. J. Shatz and by National Institutes of Health training grant GM07365 to J. J. M. Chun.

Received for publication 16 June 1987, and in revised form 28 October 1987.

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