The Earliest-Generated Neurons of the Cat Cerebral Cortex: Characterization by MAP2 and Neurotransmitter Immunohistochemistry During Fetal Life

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The earliest-generated neurons of the cat cerebral cortex have been studied here during development using a combination of ³H-thymidine birthdating with immunohistochemistry for the neuron-specific protein MAP2 or for several neuropeptides/transmitters. These neurons are the first postmitotic cells of the cortex, with birthdates during the 1-week period preceding the genesis of cells of the adult cerebral cortex (Luskin and Shatz, 1985a; Chun et al., 1987). However, they are transient and the majority disappear by adulthood (Luskin and Shatz, 1985a; Chun and Shatz, 1989). When autoradiographic birthdating is combined with MAP2 immunostaining during fetal life, the entire population of these early-generated neurons appears to be stained, resulting in labeled bands above and below the cortical plate. The band above the cortical plate (in the marginal zone) contains early-generated neurons with horizontal morphologies, while the thicker band beneath the cortical plate (within the intermediate zone) contains the somata of early-generated neurons and their elaborate processes that are frequently directed towards the ventricular surface. In view of the correspondence between the location of the early-generated neurons and the MAP2-immunostained band beneath the cortical plate, we suggest that this combined approach can be used to define accurately the subdivision of the intermediate zone known as the subplate.

The early-generated neurons are also immunoreactive for GABA, neuropeptide Y (NPY), somatostatin (SRIF), and cholecystikinin (CCK) during fetal life. While GABA, NPY, and SRIF immunostaining could be detected by embryonic day 50 (E50), that for CCK was not found until E60. Moreover, there is a relationship between neuropeptide immunoreactivity and location within the cerebral wall. The marginal-zone neurons are immunoreactive only for CCK. The subplate neurons are immunoreactive for CCK, SRIF, and NPY. Most of those immunoreactive for SRIF tend to be clustered within the upper part of the subplate, while those immunoreactive for NPY tend to be located more deeply. Cells immunoreac-

tive for GABA are more uniformly distributed throughout the cerebral wall.

These observations demonstrate directly that the marginal zone and subplate contain peptide- and GABA-immunoreactive neurons that belong to the earliest-generated cell population of the cerebral cortex. The presence of these early-generated neurons, which achieve a remarkable degree of maturity during fetal life, suggests that they perform an essential, yet transient, role in the development of the cerebral cortex.

Two critical events that occur during the development of the mammalian neocortex are the establishment of the 6 cortical layers and the coordinate attainment of the proper sets of connections between the cortex and other parts of the brain. For these to occur, both migrating neurons (Rakic, 1972, 1977) and ingrowing axons that are destined for the cortical plate (developing neocortex) must traverse significant distances through an intervening region: the intermediate zone (or future white matter; Boulder Committee, 1970). The intermediate zone also contains "waiting" axon: many ingrowing axonal systems do not immediately invade the cortical plate but rather accumulate below it for several days to weeks (Lund and Mustari, 1977; Rakic, 1977; Wise et al., 1977; Wise and Jones, 1978; Innocenti, 1981; Shatz and Luskin, 1986).

The intermediate zone also contains a population of the earliest-generated cells of the telencephalon. In the cat, these cells have discrete birthdates: they are generated between embryonic days 24 (E24) and E30, while the neurons of the adult cortex are only generated thereafter (Luskin and Shatz, 1985a). Initially forming a layer of cells in the cerebral wall, the population is split apart by later-born neurons of the cortical plate. Thus, cells of this early-generated population come to be positioned not only beneath the cortical plate in a part of the intermediate zone known as the subplate, but also above the cortical plate in the marginal zone (future layer 1; Rickmann et al., 1977; Raedler and Raedler, 1978; Luskin and Shatz, 1985a). Both the marginal-zone and the subplate-cell populations are transient: although present in large numbers during fetal and early postnatal life, the majority vanish by 2–3 months after birth (Luskin and Shatz, 1985a; for a review, see Shatz et al., 1988).

Developmental studies relying on Nissl staining, Golgi impregnations, or electron microscopy have also identified neurons in the marginal and intermediate zones (Marginal zone: Cajal, 1891; Retzius, 1891; Astrom, 1967; Stensaa, 1967a–d; Sas and Sanides, 1970; Baron and Gallego, 1971; Marin-Padilla, 1971, 1972; Konig et al., 1975, 1981; Raedler and Sievers, 1976;

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Bradford et al., 1977; Raedler and Raedler, 1978; Edmunds and Parnavelas, 1982; Marin-Padilla and Marin-Padilla, 1982; Parnavelas and Edmunds, 1983; Intermediate zone: Kolliker, 1896; Cajal, 1911; Lorente de No, 1933; Godina, 1951; Astrom, 1967; Stensaas, 1967a-d; Marin-Padilla, 1971, 1972, 1978; Rickmann et al., 1977; Raedler and Raedler, 1978; Raedler et al., 1980; Rickmann and Wolff, 1981). Cells within the intermediate zone are also immunoreactive for neurotransmitters as reported for neuropeptide Y (NPY; Foster and Schultzberg, 1984; Woodhams et al., 1985), somatostatin (SRIF; Cavanagh and Parnavelas, 1988; Naus et al., 1988), GABA or its synthesizing enzyme glutamic acid decarboxylase (GABA or GAD; Wolff et al., 1984; Lauder et al., 1986), and postnatal cholecystikinin (CCK; Meyer and Wahle, 1988).

Here we examine whether the early-generated and transient marginal-zone and subplate neurons are those observed by Golgi impregnations, electron microscopy, or neurotransmitter immunoreactivity. Immunostaining for the neuron-specific protein MAP2 (microtubule associated protein 2; Matus et al., 1981; Vallee, 1982; De Camilli et al., 1984; see Discussion), NPY, SRIF, CCK, or GABA was combined with ^3H -thymidine autoradiographic birthdating to definitively prove the identity of subplate neurons studied using these different approaches. Moreover, these techniques have permitted us to examine the morphological appearance and quantitative distribution of the early-generated population within the developing fetal cat cerebral cortex. A brief report of some of these results has appeared previously (Chun et al., 1987).

Materials and Methods

Twenty-five cat brains between the ages of embryonic day (E) 46 and 65 (birth) plus one postnatal day (P) 7 and 1 adult brain analyzed for MAP2 were examined using immunohistochemistry with and without ^3H -thymidine autoradiography. Table 1 summarizes the procedures used at each age. Fetal ages were determined by timed breedings as described previously (Luskin and Shatz, 1985a).

Surgical procedure. Surgical techniques and procedures have been described elsewhere (Luskin and Shatz, 1985a; Chun and Shatz, 1988a, b); an abbreviated version is included. Animals were first anesthetized with halothane (1–2% in O_2) or intramuscular injection of ketamine (20 mg/kg) and acepromazine (0.2 mg/kg), followed by halothane (0.5–2% in O_2). Ventilation and heart rate were monitored continuously to ensure effective anesthesia. Animals also received an i.v. drip of 5% dextrose/saline. Postoperative care included analgesics and 24 hr/day monitoring in our veterinary intensive care unit.

Fetal birthdate labeling was made through intrauterine injection of ^3H -thymidine (NET 027Z, 76.1 Ci/mmol, DuPont, Wilmington, DE; 500–750 μCi /fetus) between the ages of E26 and E28 in order to specifically label the early-generated population of subplate and marginal-zone neurons (Luskin and Shatz, 1985a; Chun et al., 1987).

Tissue preparation for histology. Fetuses were anesthetized by transplacental halothane, and postnatal animals were anesthetized using a combination of ketamine (20 mg/kg plus acepromazine 0.2 mg/kg) and nembutal (50 mg/kg). Anesthetized animals were then perfused using a peristaltic pump at flow rates ranging from 3.5–10 ml/min for the youngest to oldest ages (E65). Following an initial wash with Ringer's solution (124 mM NaCl, 5 mM KCl, 1.15 mM KH_2PO_4 , 1.15 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mM CaCl_2 , 25 mM NaHCO_3 , 10 mM glucose; pH 7.4) at 25°C for 5 min, animals were perfused with fresh 4% paraformaldehyde in 0.1 M monobasic sodium phosphate buffer, pH 7.4 (fixative), for 30 min at 25°C. Brains were removed and placed into circulating, fresh fixative for 12–18 hr at 4°C. Brains sectioned by cryostat were transferred from fixative into circulating buffer containing PBS (20 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4) with 6% sucrose and 0.025% NaN_3 for 18–24 hr at 4°C. Rapid tissue freezing was accomplished by immersion into dry ice-cooled liquid CCl_2F_2 . Brains were either sectioned immediately at 8–10 μm or stored at -60°C and processed within 48 hr. Sections through the telencephalon were cut in the coronal plane

Table 1. Experimental age and analysis performed

Age	No. of animals	^3H -thymidine	MAP2	GABA	Pep-tides
E46	1	+	+		
E48 blot	1				
E50	2	+	+	+	+
E50	2		+	+	+
E52	2		+		
E54	1	+	+	+	+
E56 blot	1				
E56	3	+	+	+	+
E56	1		+	+	+
E58	2	+	+	+	+
E58	3		+	+	+
E60	2		+	+	+
E65	2		+	+	+
P7	1	+	+	+	+
Adult blot	1				

with an emphasis on neocortex at the level of the lateral geniculate nucleus. Mounted sections were stored at -20°C and processed for immunohistochemistry within 4 hr.

Brains sectioned by Vibratome were fixed identically, then transferred into buffer without sucrose. After 18–24 hr, brains were embedded in 30% albumin plus 0.5% gelatin cross-linked with 5% (v/v) of 50% glutaraldehyde and cut at 80 μm (fetal material) in the coronal plane. Sections were stored in PBS plus 0.025% sodium azide at 4°C and then processed for immunohistochemistry. (For further details, see Chun and Shatz, 1988a, b.)

Immunohistochemistry and autoradiography. Immunohistochemical reagents employed in this study are summarized in Table 2. Sections were first preincubated for 1 hr at 25°C in “incubation buffer” containing PBS, 0.02% sodium azide (NaN_3), 2.5% BSA (Sigma, fraction V), and 0.3% Triton X-100 (Sigma), filtered with a 0.22 μm Millex-GV membrane (Millipore, Bedford, MA) just prior to use. The incubation buffer was then drained away, and fresh buffer was applied to the sections with the addition of the primary antiserum/antibody. Blocking controls were carried out identically except for the addition of the appropriate blocking reagent to the buffer containing the primary antibody. After incubation for 15–24 hr, sections were washed (in PBS without NaN_3 , 4 changes over 30 min), and bound antibody was localized using an avidin-biotin-complex system (ABC; Vector Labs, Burlingame, CA) as follows: the sections were incubated with biotinylated secondary antibody (60 min), washed in PBS (4 changes over 30 min), incubated in ABC (horseradish peroxidase or HRP) solution (40–60 min), and then washed in 50 mM Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5; 4 changes over 30 min). The HRP reaction commenced with the addition of 0.5 mg/ml 3,3'-diaminobenzidine (DAB; Sigma) with 0.0075% H_2O_2 and was carried out for 10–15 min. The reacted sections were washed in TBS (20 min with 2 changes), followed by 3 washes in distilled water, dehydration in graded ethanols, and immersion in 100% xylene for 8 hr. Sections were then either coverslipped in Permount (Fisher Scientific, Fair Lawn, NJ) and observed or rehydrated and dipped in 0.4% osmium tetroxide in distilled water for 30 sec to enhance the HRP reaction product. Sections were dehydrated, coverslipped, and observed. Those destined for ^3H -thymidine autoradiography were osmicated as above, then washed in distilled water, dried, dipped in emulsion, exposed, and developed as described previously (Luskin and Shatz, 1985a). Cells with greater than 20% of the maximum number of silvergrains over their nuclei were considered labeled.

Figure 1 illustrates representative immunostained sections and some of their appropriate controls for MAP2 (Fig. 1, A–C), GABA (Fig. 1, D, E), and NPY (Fig. 1, F, G). Specific immunostaining was not produced by normal rabbit serum (polyclonal antibodies) or isotype-matched controls (for MAP2). Further, all neuropeptide or GABA immunoreactivity was blocked by the simultaneous incubation of antisera with the appropriate neuropeptide or GABA, respectively, and only the respective reagent was capable of blocking immunostaining.

Figure 1. Examples of immuno-stained sections from the fetal neocortex and adjacent control sections are shown for MAP2 (*A–C*), GABA (*D, E*), and NPY (*F, G*). Since both a mouse monoclonal antibody and rabbit polyclonal antiserum were used against MAP2 (both giving qualitatively identical results), the MAP2-immunostained section (*A*) is compared to the mouse isotype-matched (*B*) and the normal rabbit serum controls (*C*). Similarly, the GABA-immunostained section (*D*; Materials and Methods for explanation of vertical line) contrasts with the blocking control in which 80 μ M GABA was present during the primary antibody incubation. All neuropeptides could be blocked by their respective control peptides (not shown) and, in addition, NPY (*F*) could be blocked by a 15 nM solution of the true TRQRY amide (*G*) but not by related peptides (see Table 2). *A–E* are from an E50 neocortex; *F* and *G* from an E54 neocortex. Pial surface is towards the top of each section. The 300 μ m scale bar in *A* pertains to *A–E*; 30 μ m bar in *F* refers to *F* and *G*.

Table 2. Immunohistochemical reagents, dilutions, and sources

Reagent	Dilution	Source
NPY	1:1000	Dr. C. Evans, Stanford University
SRIF	1:1000	Immuno Nuclear Corporation, Stillwater, MN
CCK	1:1000	Immuno Nuclear Corporation
GABA	1:2000	Immuno Nuclear Corporation
MAP2	1:2500	Drs. W. Theurkauf and R. Vallee; Worcester Foundation, Shrewsbury, MA
Mouse-anti-MAP2 monoclonal (MAP2-3)	1:30	Drs. F. Luca and R. Vallee
Mouse IgG1 and IgG2b controls	1:30	Dr. I. Weismann, Stanford University
Normal rabbit sera	1:1000	Various sources
NPY, CCK, SRIF peptides	50 nM	Peninsula Labs, Belmont, CA
TRQRYamide, YPMRFamide, YRPRYamide	15 nM	Dr. C. Evans
GABA	80 μ M	Sigma Chemical Company, St. Louis, MO

Western blot analyses for MAP2. Animals prepared for Western blot analyses first were perfused with Ringer's solution to remove blood components. The brains were then rapidly removed from the cranium and processed using a procedure modified from Berkowitz et al. (1977). Twenty ml of brain tissue (including all of the telencephalon) were homogenized in 60 ml of buffer (0.1 M Pipes-NaOH, 2 mM EGTA, 1 mM MgSO₄, 4 M glycerol, pH 6.9 at 4°C) with a motor-driven teflon-glass homogenizer. The homogenate was centrifuged for 15 min at 10,200 g using a Beckman JS13 rotor. The pellet was discarded and the supernatant recentrifuged for 70 min at 142,800 g using a Beckman Ti50 rotor. The resulting new supernatant was combined with 5× boiling sample buffer (5× buffer contains 10% SDS, 5 mM DTT, 50% glycerol, 50 mM Tris-HCl, pH 8, plus bromophenyl blue) to yield a 1× solution. Solubilized samples were stored at -80°C or immediately electrophoresed on a 4–15% gradient polyacrylamide gel using SDS-PAGE (Laemmli, 1970) along with molecular weight standards (Sigma, St. Louis, MO). After electrophoresis, the gel lanes were either cut out and stained with Coomassie blue or transferred electrophoretically onto nitrocellulose membrane (Towbin et al., 1979; Bittner et al., 1980). Following transfer, the nitrocellulose was incubated at room temperature with gentle agitation in 5% BSA and 10 mM TBS plus 0.1% Triton X-100 and 0.02% NaN₃ for 8–12 hr, followed by addition of the primary antiserum or normal rabbit serum controls (1:20,000) for 12–24 hr. The bound antibody was visualized using the following schedule: the membrane was removed from the primary antiserum and washed with 5 changes of 10 mM TBS over a 50-min period. Using a Vectastain ABC kit (Vector Labs, Burlingame, CA), the membrane was exposed to the secondary antibody diluted in 10 mM TBS for 2 hr, washed as above, then similarly exposed to the ABC reagent for 1 hr and again washed. The HRP reaction was carried out using a solution of 17% methanol, 0.5% 4-chloro-1-naphthol and 0.15% H₂O₂ diluted into 10 mM TBS and reacted for 1 min at room temperature. The membrane was then washed in distilled water, dried, and stored in the dark. The presence of MAP2 in both the adult and the prenatal cat is shown in the Western blots of Figure 2.

Measurements. The areas of the subplate, the cortical plate, or the marginal zone were determined by making camera lucida drawings of at least 4 MAP2-immunostained sections in the following manner. First,

Figure 2. Adult-like MAP2 is present in fetal brain by antigen transfer analyses (Western blots). Reducing conditions were used in non-quantitative transfers of SDS-PAGE (4–15% gradient gel) to reveal the characteristic 300 MW doublet of MAP2 in adult (lanes 1–3) and at E48 (lane 4). No other major bands were observed. These bands were absent from control blots.

the base of the subplate (farthest from the pial surface) was determined. A conservative value was obtained by designating the base of the subplate as that region where MAP2-immunoreactive processes become very sparse (see Fig. 6). The top border of the subplate (i.e., just below the cortical plate) was established either by using ³H-thymidine labeling to mark the location of the most superficial of the early-generated subplate cells (Luskin and Shatz, 1985a; see Fig. 7) or in conjunction with MAP2 immunohistochemistry (Chun et al., 1987; see Fig. 6). The area of the subplate extending from beneath the cingulate gyrus to beneath the ectosylvian gyrus was measured. Area measurements of the cortical plate or marginal zone were made in a similar fashion so that the length of cortex included in the measurements was equal to that determined for the subplate. The resulting areas were then digitized and calculated using a Sigma-Scan measurement system (Jandel Scientific, Sausalito, CA).

Counts of cells immunostained by MAP2, NPY, SRIF, or CCK at ages E50, E56, and E60 were made in similar regions at each age within the medial bank of the lateral gyrus and within the ectosylvian gyrus (1 animal at each age was analyzed). To accomplish this, a line was drawn from the pial surface to the ventricular surface as in Figure 1D. The number of immunostained cells was counted (at ×312) within a window 20 μ m deep and 400 μ m wide moved in adjacent steps from the pial surface to the ventricular surface along the line. Both regions of cortex were counted in this fashion in each of 5 sections. The data were com-

Figure 3. MAP2-immunoreactive layer is present within the intermediate zone throughout the fetal telencephalon. An 80- μ m-thick coronal Vibratome section cut in the coronal plane was immunostained for MAP2 at E52. One-half of the brain is shown. A dark MAP2-immunoreactive layer can be seen below and parallel to the less immunostained cortical plate (CP). The marginal zone (MZ) is also stained. The heavily stained layer extends well into the intermediate zone (IZ). Overlying the ventricle (V) is the ventricular zone (VZ), which also exhibits faint MAP2-immunoreactivity. Non-neocortical structures such as the hippocampal formation (H) and the lateral geniculate nucleus (L) are also labeled by MAP2 immunostaining. The region within the rectangle is enlarged in Figure 6. D, dorsal; M, medial.

binned into bins of 60 μ m (combining 3 consecutive 20 μ m windows). This method of counting was repeated for each of the immunostained sections of interest, and the resulting data were plotted as histograms (see Figs. 12, 13).

Results

The results are presented in 3 parts. First, the pattern of MAP2 immunostaining is shown, along with its relationship to the subplate and marginal zones within fetal regions of the neocortex. The identity and morphology of the MAP2-immunostained elements within the subplate and marginal zone are also examined. Next, the GABA-, NPY-, SRIF-, and CCK-immunoreactive cells within the subplate and marginal zone are shown to belong to the early-generated population, and their morphologies and distributions are examined. Finally, changes in the subplate with development are examined.

Relation of MAP2 immunoreactivity to early-generated cells

We first used the neuron-specific marker MAP2 to examine the organization of the fetal neocortex. An example of a section

immunostained for MAP2 at E52 is shown in Figure 3. Most of the major embryonic zones as previously defined (Boulder Committee, 1970) are indicated, including the outermost marginal zone (MZ: future cortical layer 1), the subjacent cortical plate (CP: future cortical layers 2–6), the intermediate zone (IZ: future white matter), and the ventricular zone (VZ: future ependymal zone). The heaviest stain within the neocortex is located within the marginal zone and the more superficial part of the intermediate zone, and staining extends rather uniformly throughout the entire hemisphere. This pattern is observed in all regions of the brain examined (from the frontal lobe to the posterior pole). Some immunostaining is also just visible in the deep intermediate zone and the ventricular zone as well as within the cortical plate.

What are the MAP2-immunoreactive elements of the marginal zone and subplate? At least some, if not most, are the earliest-generated cells of the neocortex along with their processes. This point is demonstrated by combining 3 H-thymidine autoradiography following an injection at E27 with MAP2 immunohistochemistry at E56, as shown in Figure 4A: every 3 H-thymidine-labeled cell is also MAP2 immunostained. In fact, it

Figure 4. MAP2 immunostaining reveals a variety of subplate neuron morphologies. E56 brain previously labeled with ^3H -thymidine at E28 was immunostained with MAP2. *A*, Examples of autoradiographically labeled subplate neurons and their MAP2 morphologies are shown from a cryostat section (straight arrows: silver grains over nuclei; curved arrows: MAP2-immunoreactive processes). *B–D*, Morphologies of subplate neurons at E56 from Vibratome sections. A major process is often directed towards the ventricle as indicated (curved arrows); ventricle towards the bottom of the page. Note that other processes extend from various portions of the subplate neuron in many directions (open arrows). Scale bar for *B–D* is shown in *D*.

was rare to find ^3H -thymidine-labeled cells not also labeled by MAP2 in these double-label experiments, suggesting that many, if not all, of the early-generated cells residing in the subplate are neurons (see also Chun and Shatz, 1988a). Further, Figure 4, *B–D* shows that many MAP2-stained cells have a major dendrite extending towards the ventricular zone as well as many other dendritic processes. Note that many of the processes are beaded and that MAP2-immunoreactive processes virtually fill the field of each micrograph. These observations confirm our previous demonstration that many, if not all, of the early-generated cells are likely to be neurons (Chun et al., 1987). Moreover, the MAP2 immunostaining seen in the upper region of the intermediate zone in Figure 3 is associated with the somata and elaborate processes of the subplate neurons.

MAP2 immunostaining within the marginal zone is also directly associated with the early-generated cells, as shown in Figure 5*A*, where combined ^3H -thymidine birthdating at E28 and MAP2 immunohistochemistry at E52 are combined. The MAP2-immunoreactive processes are often horizontally di-

rected (Fig. 5, *B, C*). As in the subplate, many processes within the marginal zone are also beaded.

The MAP2-immunoreactive subplate and marginal zone thus appear to reflect the presence of the early-generated subplate and marginal-zone neurons themselves. Further, the elaborate processes of these neurons contribute to the complexity observed throughout both regions. In view of this conclusion, it seems reasonable to suggest that the lower border of the subplate can be defined as the region of the intermediate zone in which MAP2 immunostaining becomes rather sparse. This point is illustrated in Figure 6, which compares the relationship between MAP2 immunostaining and the histological organization of the cerebral wall as revealed in a cresyl-violet-stained section. We shall therefore refer to the thick band of MAP2 immunostaining beneath the cortical plate as the subplate.

On the other hand, MAP2 immunostaining alone (particularly at older fetal ages) cannot be used to determine accurately the upper border between the subplate and the cortical plate, because neurons belonging to cortical layers 5 and 6 become im-

Figure 5. MAP2 immunostaining reveals a range of marginal-zone neuron morphologies. *A*, Autoradiographically labeled MAP2-immunoreactive marginal-zone neuron is shown for a brain immunostained for MAP2 at E56 after being labeled with ^3H -thymidine at E28 (straight arrow: silver grains; curved arrows: MAP2). *B*, *C*, Horizontal processes (curved arrows) of these neurons from an E52 brain. Some processes make more extreme curves within the marginal zone (small arrows in *C*). Pial surface towards the top of the page.

munoreactive as they mature. The border can be determined with accuracy, however, by using ^3H -thymidine birthdating of the subplate neurons at E28–30 (when the most superficial subplate neurons are generated; see Luskin and Shatz, 1985a). For example, Figure 7 shows adjacent cryostat sections processed for MAP2 immunohistochemistry (Fig. 7*A*) and autoradiography (Fig. 7*B*). Note that the zone of ^3H -thymidine-labeled cells coincides with a region of MAP2 immunostaining that is in transition between the orderly staining of pyramidal cells at the base of layer 6 and the more complex pattern of staining within deeper regions of the subplate.

Relationship between GABA- and neuropeptide-immunoreactive cells and the early-generated neurons

To further characterize the subplate and marginal-zone neurons and to learn more about their detailed morphology we immunostained sections from the fetal brain with antibodies against NPY, SRIF, CCK, and GABA. This choice of antibodies was motivated by studies of the adult cerebral cortex indicating that part of the small population of neurons known to be present in the white matter is immunoreactive for some of these neuropeptides (SRIF: Bennett-Clarke et al., 1980; Finley et al., 1981; Sorensen, 1982; Hendry et al., 1984a; Schmechel et al., 1984; Somogyi et al., 1984; Laemle and Feldman, 1985; NPY: Adrian

et al., 1983; Allen et al., 1983; Hendry et al., 1984a; Chan-Palay et al., 1985; CCK: Innis et al., 1979; Hendry et al., 1983; Somogyi et al., 1984; GABA or GAD: Ribak, 1978; Hendrickson et al., 1981; Schmechel et al., 1984; Somogyi et al., 1984; Wahle et al., 1987) and by the results of our preliminary studies suggesting that some of the early-generated neurons are immunoreactive for these particular transmitters (Chun et al., 1987).

Figure 8 shows that subplate cells labeled at E27 with ^3H -thymidine can also be immunoreactive for GABA (Fig. 8, *A–C*; marginal-zone neurons can also be labeled), NPY (Fig. 8, *D–F*), SRIF (Fig. 8, *G–I*), or CCK (Fig. 8, *J, K*) when stained in later fetal (or neonatal for CCK) life. In addition to the double-labeled cells present in the cryostat sections shown in Figure 8, each experiment also shows that not all cells generated at the same time can be immunostained with a given neuropeptide antibody (e.g., Fig. 8, *E, G*, arrows). The converse was also true: not all immunostained cells were ^3H -thymidine labeled. This observation is consistent with the fact that ^3H -thymidine is available for only about 2 hr after delivery, whereas the entire population of subplate and marginal-zone neurons are generated over a 1-week period between E24 and E30 (Luskin and Shatz, 1985a, b). Thus, only a small fraction of the entire population will be autoradiographically labeled following a single injection at E27. As discussed further below, CCK-immunoreactive cells

Figure 6. Relationship of MAP2 immunostaining to the histological organization of the cerebral wall at E52. *A*, Enlargement of a region similar to that shown in Figure 3 (rectangle). *B*, Adjacent section stained with cresyl violet to show the location of cell bodies. The lines divide the section into the conventional fetal zones: the marginal zone (*MZ*; future layer 1), the cortical plate (*CP*; future cortical layers 2–6), the intermediate zone (*IZ*; future white matter), and the cell germinal region called the ventricular zone (*VZ*; future ependyma). At this age, MAP2 prominently stains the marginal zone, a few pyramidal neurons in the cortical plate, and a large region beneath the cortical plate that extends through a good portion of the intermediate zone: we have called this entire region of dense MAP2 immunoreactivity the subplate (*SP*). Thus, the subplate occupies approximately the upper half of the intermediate zone. See text for further details.

Figure 7. Upper border of the subplate as defined by ^3H -thymidine birthdating of subplate neurons. Subplate neurons birthdated at E28 are the most superficial members of this transient population (Luskin and Shatz, 1985a). They can be identified autoradiographically at later dates (E56 in *B*; silver grains over nuclei appear white in this darkfield photograph). When compared to an adjacent section of brain immunostained for MAP2 (*A*), the boundary between upper subplate (SP_U) and the later-generated neurons of cortical layer 6 can be accurately determined. *CP*, Cortical plate; *SP*, subplate.

were not observed until the last week of gestation, when they are located predominantly within the marginal zone.

The morphology of the immunostained subplate neurons can be more fully appreciated in Vibratome sections as shown in Figure 9. Two common features are the presence of thick processes—probably dendrites—often directed towards the ventricular surface (cf. Fig. 4), and fine, long processes, often directed towards the pial surface. NPY-immunoreactive cells frequently have such a process, probably an axon, that loops towards the pial surface (Fig. 9, *D*, *E*).

The morphologies revealed by NPY and SRIF immunostaining can be seen more clearly in camera lucida drawings of fetal neurons such as those shown in Figure 10. The morphologies of NPY-immunoreactive neurons are shown at E54 in Figure 10*A* (the long process extends from the subplate to the marginal zone), and those of SRIF-immunoreactive neurons are shown at E56 in Figure 10*B*. These examples illustrate the elaborate and varied appearance of subplate neurons. Thus, the results presented above demonstrate that subplate neurons as identified by ^3H -thymidine birthdating can achieve a high degree of morphological maturity prior to birth and can be immunoreactive for GABA, NPY, SRIF, and CCK, whereas the marginal-zone neurons can be immunoreactive for CCK and GABA. We also have found that at least some of the SRIF-immunoreactive neurons within the subplate are also immunostained by GABA (J. Chun and C. Shatz, unpublished observations), consistent with the colocalization of this neuropeptide with GABA as reported in adult cells of the white matter (Hendry et al., 1984b; Somogyi et al., 1984).

Variation and developmental changes in the subplate

The immunohistochemical observations described above suggest that the subplate (and also the marginal zone) is an elaborate neuropil consisting primarily of the somata and a dense network of processes belonging to the early-generated subplate neurons, some of which are also GABA- and neuropeptide-immunoreactive. We were interested in examining whether there is any systemic relationship between the apparent transmitter phenotype of immunostained neurons and their location within the marginal zone or subplate during fetal life, particularly since we had previously noted the existence of such a relationship in the early postnatal cerebral cortex (Chun et al., 1987). To address this question, 3 consecutive sections through the medial wall of the lateral gyrus (future visual cortex) at E54 were immunostained for GABA, NPY, or SRIF (CCK-immunoreactive neurons are absent at this time; see Figs. 12 and 13), and the positions of all immunoreactive cells were plotted in camera lucida drawings. The 3 resulting drawings were superimposed as shown in Figure 11. (At this age, there were so many GABA-immunoreactive cells that only those present in the wedge illustrated have been drawn.) While GABA-immunoreactive cells do not appear to be clustered preferentially from pial to ventricular surface, SRIF-immunoreactive neurons are grouped predominantly within the upper subplate. NPY-immunoreactive neurons, in contrast, tend to be situated more deeply.

To confirm our impression that there is a relationship between particular peptide-immunoreactive neurons in the fetal neocortex and their laminar positions, we plotted the positions of

Figure 8. Early-generated neurons are also immunoreactive for GABA and various neuropeptides. Autoradiographically identified subplate neurons are immunoreactive for GABA (A–C), NPY (D–F), SRIF (G–I), and CCK (J, K; L, One marginal zone neuron is also shown). Some cell processes can be discerned in this cryostat-sectioned material (arrowheads). Examples of ^3H -thymidine-labeled subplate neurons not immunoreactive for GABA or the neuropeptides examined were present in all experiments (e.g., straight arrows in E and G). Subplate neurons were birthdated with ^3H -thymidine at E27 and examined at E56 (A–C, G–I), E54 (D–F) and postnatal day 7 (J–L). Pial surface towards top of page.

Table 3. Total numbers of cells, by age and immunoreactivity^a

Age	Visual cortex				Temporal cortex			
	GABA	NPY	SRIF	CCK	GABA	NPY	SRIF	CCK
E50	2001	46	408		1867	40	623	
E56	1076	20	162		1338	25	205	
E60	1413	110	220	19	1066	145	502	29

^a See Figures 12 and 13.

over 11,300 immunostained cells from the pial to the ventricular surface at E50, E56, and E60. Both the lateral gyrus (visual cortex; Fig. 12) and the ectosylvian gyrus (temporal lobe; Fig. 13; see Materials and Methods) were examined. The histograms of Figures 12 and 13 show the distribution of immunoreactive cells as a function of depth from the pial surface. The length of each bar in the histograms corresponds to the percentage of cells showing a particular transmitter or peptide immunoreactivity at each 60 μ m step in depth (total numbers are shown in Table 3). At each age, and within both cortical areas, inspection of the histograms confirms the impression that GABA-, NPY-, SRIF-, and CCK-immunoreactive cells are not randomly distributed. First, the majority of the NPY- or SRIF-immunoreactive cells are not located in the cortical plate. Rather, especially at E50 and E56, cells tend to cluster in such a way that more of the NPY-immunoreactive cells are found deeper in the subplate, whereas the SRIF-immunoreactive cells are more concentrated at the border between the subplate and the cortical plate. Of the neuropeptides examined, only CCK-immunoreactive cells were found in the marginal zone, and only by E60. These cells also populate the deep subplate. However, the distribution of GABA-immunoreactive cells is more uniform, showing only a slight tendency to concentrate nearer the pial surface. In addition, a small percentage of cells with immunoreactivity for GABA, NPY, and SRIF is present surprisingly deep in the intermediate zone near and even within the ventricular zone. At least some, if not all, of these cells are also part of the subplate neuron population because they can be labeled by both ³H-thymidine birthdating at E26–28 and immunohistochemistry for MAP2, as well as for the neuropeptides and GABA.

A final issue is whether the dimensions of the subplate, as defined in this study using MAP2 immunohistochemistry, change during the fetal period studied here. Such a change is implied by Figures 12 and 13. To assess quantitatively the change in the dimensions of the subplate relative to the cortical plate, a ratio of the areas of the 2 zones was calculated (see Materials and Methods). The results are shown graphically in Figure 14, in which the ratio of subplate to cortical plate area is plotted against the embryonic age (days). A value of 1.0 means that the structures have equal areas and therefore equal thicknesses. It should be noted that the increase in the ratio between E46 and E56 is due to an increase in the thickness (from pial to ventricular surface) of the subplate, since the cortical plate does not become thinner (it actually becomes thicker) during this time.

The subsequent decrease in the ratio is due both to the continued growth of the cortical plate and to a less pronounced decrease in the thickness of the subplate. An analysis of the changes in the size of the marginal zone is difficult because the adult layer 1 is in many ways similar to the marginal zone, except for a loss of the marginal-zone neurons. Nevertheless, it is notable that the ratio of the marginal zone to the cortical plate area varies between 0.3 and 0.5 (i.e., equals 30–50% of the cortical plate area) during the developmental period shown in Figure 14.

The results of Figures 11–14 demonstrate that the subplate is not a homogeneous layer but rather is stratified with respect to cells expressing neuropeptide (and to a lesser extent, GABA) immunoreactivities. This phenomenon is present at several developmental ages in at least 2 distinct cortical regions. Further, the subplate increases in size between E46 and E56 but then decreases perceptibly by birth (E65), as documented by the change in the ratio of subplate to cortical plate area.

Discussion

The results of this study show that the two fetal zones containing the earliest postmitotic cells of the cerebral cortex are also rich in immunoreactivity for MAP2 (a neuron-specific protein) and for a variety of neuropeptides and GABA during development. As discussed below, we believe that, particularly at the earliest ages studied (E46–E56), most, if not all, of the MAP2 immunostaining in the subplate is directly associated with the early-generated cells. This conclusion is in agreement with the results of a previous study in which ³H-thymidine labeling in conjunction with MAP2 immunohistochemistry was used as part of the proof that the early-generated cells residing in the subplate are neurons (Chun et al., 1987). The present results demonstrating that there are distinct and extensive bands of immunostaining for MAP2 above and below the cortical plate throughout the neocortex during development suggest that the regions containing the early-generated population of neurons are much more elaborate and extensive than previously supposed.

The combined use of ³H-thymidine autoradiography with GABA and neuropeptide immunohistochemistry has also permitted us to show conclusively that some, if not all, of the cells located within the subplate and marginal zone that are immunoreactive for GABA, NPY, SRIF, or CCK are the early-generated neurons, and that cells with different transmitter phenotypes tend to be located at different depths within the

Figure 9. Examples of fetal subplate neuron morphologies revealed by GABA and neuropeptide immunostaining. GABA (A–C, at E56), NPY (D–F, at E54), SRIF (G–I, at E56), and CCK (J, K; L is from the marginal zone; E60). Multiple processes emanating from the cells can be seen in these Vibratome sections (*small arrows*). Some are directed towards the pial surface (towards top of page) and some branch (e.g., H). NPY morphologies are remarkable for the looping axons that are commonly present. Note that many of the processes are also beaded (cf. Fig. 4).

Figure 10. Camera lucida drawings of subplate neurons immunoreactive for NPY (at E54) and SRIF (at E56). Examples are shown from subplate beneath the lateral gyrus, visual cortex (V), or the ectosylvian gyrus of the temporal lobe (T). One NPY immunoreactive cell with a process that traversed the cortical plate from the subplate to the marginal zone is illustrated; arrowheads mark continuation of process. Calibration, 10 μ m; nearest pial surface is towards the top of page.

Figure 12. Differential location of immunoreactive cells from developing visual cortex at 3 different ages, as revealed by histograms. Distance in micrometers from the pial surface (Y-axis) versus the percentage of cells displaying each type of immunoreactivities is plotted. Note that the majority of the NPY-immunoreactive neurons reside deeper in the subplate (SP) than do the SRIF-immunoreactive cells, which are predominantly within the upper subplate. GABA-immunoreactive cells are found throughout the telencephalic wall from pial to ventricular surface. CCK-immunoreactive cells are at their highest percentage in the marginal zone (MZ; actual percentage, 31%; off scale on graph) and deeper within the subplate. Note the presence of a few immunoreactive cells within the ventricular zone (VZ). CP, Cortical plate. See Table 3 for more information.

Technical considerations

The validity of our results relies on several important methodological considerations. The first is that MAP2 is in fact neuron specific. Evidence for this point is extensive within both the adult (Matus et al., 1981; Miller et al., 1982; Vallee, 1982; Caceres et al., 1983, 1984; Bernhardt and Matus, 1984; De Camilli et al., 1984) and the developing (Bernhardt and Matus, 1982; Bernhardt et al., 1985; Crandall et al., 1986) nervous system, in which MAP2 immunostaining has been shown to be confined to the dendrites and somata of neurons. That MAP2 was recognized in this study is supported by the presence of a MAP2-like molecule in Western blots, an identical immunostaining pattern produced by a polyclonal and a monoclonal antibody against MAP2, the absence of immunostaining from control sections, and the neuron-like morphologies of the immunostained cells. Because the vast majority of cells labeled by

Figure 13. Differential location of immunoreactive cells from temporal cortex at 3 different ages. Note similarities with the analysis of visual cortex in Figure 12.

injections of ^3H -thymidine before E30 were also MAP2-immunoreactive (see also Chun et al., 1987; Chun and Shatz, 1988a), it is highly likely that the majority of early-generated cells located in the subplate and marginal zone are neurons.

A second issue is the specificity of immunostaining for the neuropeptides and GABA. Although we cannot be certain that the antibodies actually recognize these transmitters in tissue section, the results of both normal serum and blocking controls suggest that the employed antisera are specific for their respective molecules. The morphologies of the immunostained cells also suggest that neurons are being recognized, a suggestion consistent with the fact that they can be double labeled with ^3H -thymidine. However, although the morphology of the peptide-immunostained cells was distinctly neuronal, some of the GABA-immunoreactive cells might have been glia in view of their very small somata. These cells therefore were not included in the quantitative analysis of Figures 12 and 13.

A final point concerns the use of ^3H -thymidine to uniquely label the early-generated population without labeling neurons of layers 2–6. We administered ^3H -thymidine by making a single intrauterine injection between E26 and E28. These ages are well within the period during which only subplate and marginal-zone neurons are generated (Luskin and Shatz, 1985a, b). Moreover, ^3H -thymidine is only available for uptake during approximately 2 hr after the injection (Hickey et al., 1983), thereby

assuring even greater time resolution. For these reasons we believe that the vast majority of ^3H -thymidine-labeled cells studied here belong to the early-generated population.

Organization of the subplate

The boundaries of the subplate layer during development have not been clearly defined, leading to discrepancies in its exact location (cf. Kostovic and Molliver, 1974; McConnell, 1988). Moreover, as discussed more fully in the next section, the subplate neurons themselves have frequently been considered as part of the developing cortical plate (Astrom, 1967; Molliver and Van der Loos, 1970; Marin-Padilla, 1971, 1972; Derer, 1974) or, alternatively, as a distinct layer that becomes layer 6b or 7 in the adult (Sugita, 1917; Konig et al., 1975; Rickmann et al., 1977; Marin-Padilla, 1978; Raedler and Raedler, 1978; Raedler et al., 1980). We believe that the results presented here and elsewhere (Kostovic and Molliver, 1974; Rakic, 1977; Kostovic and Rakic, 1980, 1984; Luskin and Shatz, 1985a; Chun and Shatz, 1989) favor the interpretation that the subplate and the subplate neurons are distinct from the cortical plate and, further, that the subplate is a transient structure, with only a remnant surviving into adulthood as the interstitial neurons of the white matter (Kostovic and Rakic, 1980; Chun and Shatz, 1989).

We have presented evidence that the distribution of MAP2 immunostaining can be used to define the subplate layer. The layer includes, at its superficial border, the neurons of the upper subplate (defined by ^3H -thymidine labeling of the most superficial subplate neurons; Fig. 4 and Luskin and Shatz, 1985a). The deep border varies with age, but because the majority of early-generated subplate neurons and their processes are confined to the zone of heavy MAP2 immunoreactivity, it is reasonable to propose that this deep border coincides with the region of abrupt decrease in MAP2 immunostaining. This is not to say that there are no subplate neurons located more deeply; on the contrary, we have shown here that some are present even in the ventricular zone, but they are few in number.

A related issue is whether the MAP2 immunostaining within the subplate reflects only the presence of subplate neurons and their processes or whether some immunostaining is also associated with the distal dendrites of layer 6 neurons or even more superficial layers. We think this is unlikely both in view of the morphologic evidence presented here and because elimination of subplate neurons by neurotoxins greatly reduces MAP2 immunostaining of the subplate while the cortical plate remains intact (Chun and Shatz, 1988a). These considerations indicate that the subplate as defined using a combination of MAP2 immunohistochemistry and ^3H -thymidine autoradiography is a structure in its own right consisting of the transient subplate neurons. It is distinct from the cortical plate.

Previously described fetal cortical neurons are early-generated

The unambiguous location and horizontal morphologies of some of the marginal-zone neurons seen here using MAP2, CCK, and GABA immunostaining strongly suggest that these neurons are identical with the well-known Retzius-Cajal cell of Golgi studies (Ramón y Cajal, 1891; Retzius, 1891; Astrom, 1967; Stensaas, 1967a-d; Sas and Sanides, 1970; Baron and Gallego, 1971; Marin-Padilla, 1971, 1972; Marin-Padilla and Marin-Padilla, 1982; Konig et al., 1975, 1981; Raedler and Sievers, 1976; Bradford et al., 1977; Raedler and Raedler, 1978; Edmunds and Parnavelas, 1982; Parnavelas and Edmunds, 1983). The situation with

Figure 14. Quantitative description of the progressive change in the size of the subplate relative to the cortical plate. Ratio of subplate to cortical plate area was constructed at E46, E50, E52, E56, E58, E60, and E65 (birth). (See Materials and Methods for details.) Note that the 1.0 line marks the value at which the 2 areas are equal and that the ratio reaches its largest value at around E56. Because the cortical plate actually increases in thickness from E46 to E56, the increasing ratio reflects a marked increase in the thickness of the subplate. This trend reverses itself after E56, so that the ratio at birth is approximately 50% of the peak value.

respect to the subplate neurons is less clear because many previous morphological studies (Kolliker, 1896; Cajal, 1911; Lorente de No, 1933; Godina, 1951; Astrom, 1967; Stensaas, 1967a-d; Marin-Padilla, 1971, 1972) were not able to make the critical distinction between layer 6 of the cortical plate and the subplate. As we have shown here and in previous studies (Luskin and Shatz, 1985a; Chun et al., 1987; Chun and Shatz, 1988a, b), to do so requires the use of ^3H -thymidine autoradiography to mark unambiguously the zone of early-generated cells that we know does not persist into adult life (see Luskin and Shatz, 1985a, b; Chun and Shatz, 1989). Thus, many earlier studies assumed that neurons situated within or at the top of the intermediate zone were part of the cortical plate (layer 6) rather than the transient subplate. For example, in the particularly beautiful Golgi studies of the developing fetal neocortex, Astrom (1967, in sheep), Stensaas (1967a-d, in rabbit), Marin-Padilla (1971, 1972, in cat), and Lund et al. (1977, in monkey) described cells similar in morphology to those observed here. We suggest that at least some of the cells observed in these Golgi studies, including the stellate and Martinotti cells of Astrom (1967) and the Martinotti and pyramid-like cells of Marin-Padilla (1972), are in fact early-generated subplate neurons, most of which disappear by adulthood (Chun et al., 1987; Chun and Shatz, 1989). Golgi-impregnated cells with horizontal processes located within the fetal white matter (Stensaas, 1967c, d; Rickmann et al., 1977, in rat), are also likely to be subplate neurons. However, as mentioned above, proof that these Golgi-impregnated cells are subplate neurons requires the combined use of ^3H -thymidine birthdating, a particularly critical requisite during development because the Golgi technique is not specific for neurons and does not provide any information about the time of neurogenesis.

Previous immunohistochemical studies of developing cortex have also described neurons with morphologies and locations similar to those described here for NPY (Foster and Schultzberg, 1984; Woodhams et al., 1985), SRIF (Cavanagh and Parna-

velas, 1988; Naus et al., 1988, who have further identified mRNA for SRIF), GAD or GABA (Wolff et al., 1984; Lauder et al., 1986; Wahle et al., 1987; see also Chronwall and Wolff, 1980), and early postnatal NPY and CCK (Wahle and Meyer, 1987; Meyer and Wahle, 1988). Our combined morphological and ^3H -thymidine evidence shows that fetal white matter cells identified in the above studies are, at least in part, the early-generated subplate and marginal-zone neurons.

Role of the subplate during development

It has been suggested that the subplate is an ontogenetic remnant of neocortical evolution (Marin-Padilla, 1971). That is, during development the mammalian neocortex begins as a primordial plexiform layer resembling the adult amphibian, then reptilian cortex, and then through subsequent development, the mammalian organization emerges. While this is certainly a reasonable description, perhaps it is more informative to view the subplate as a structure that promotes a set of cellular and molecular interactions that are required for the formation of the adult 6-layered cerebral cortex. If so, it is necessary to consider the nature of such interactions (for a review, see Shatz et al., 1988).

One such interaction is that the subplate neurons could serve as temporary targets for ingrowing afferent systems. It is now well known that many ingrowing axonal systems (e.g., thalamocortical, callosal) wait within the subplate for days to weeks, depending on species before invading the cortical plate (Lund and Mustari, 1977; Rakic, 1977; Wise et al., 1977; Wise and Jones, 1978; Innocenti, 1981; Crandall and Caviness, 1984; Kostovic and Rakic, 1984; Shatz and Luskin, 1986). For example, in the cat's visual system geniculocortical axons appear to arrive within the subplate even before the birth of their principal targets, the neurons of cortical layer 4 (Shatz and Luskin, 1986). While these geniculocortical axons grow into the cortical plate by birth, callosal axons continue to wait in the subplate for some time thereafter (Wise and Jones, 1976; Innocenti, 1981; McConnell and Shatz, 1988), consistent with the fact that the subplate itself persists into postnatal life. (see Chun and Shatz, 1989). Moreover, it may also be significant that the decrease in relative thickness of the subplate seen here after E56 correlates well with the time that the geniculocortical axons appear to leave the subplate and invade the cortical plate (Shatz and Luskin, 1986). Marginal-zone neurons may play a similar role, because thalamocortical afferents are also present there in development (Lund and Mustari, 1977; LeVay et al., 1978; Parnavelas and Chatzissavidou, 1981; Kato et al., 1984; Shatz and Luskin, 1986).

The cellular interactions responsible for the waiting period are unknown, but one possibility is that the presence of subplate neurons creates a unique extracellular environment that somehow promotes the waiting of axons rather than growth into the cortical plate. It appears that the subplate (and marginal zone), but not the cortical plate, contains a fibronectin-like molecule during development (Stewart and Pearlman, 1987; Chun and Shatz, 1988a). Moreover, the expression of this molecule may be dependent on the presence of subplate neurons since eliminating them by neurotoxin treatment decreases fibronectin immunostaining, and the immunostaining normally disappears during development with the disappearance of the subplate neurons (Chun and Shatz, 1988a). These observations suggest that the subplate contains information that allows specific extracellular interactions necessary for cortical development.

Subplate neurons could also participate in a transient synaptic

network during development. The results of many previous ultrastructural studies (Kostovic et al., 1973; Molliver et al., 1973; Kostovic and Molliver, 1974; Cragg, 1975; Konig et al., 1975; Kristt and Molliver, 1976; Wolff, 1976; Blue and Parnavelas, 1983) have shown that the first synapses during development are formed within the subplate and the marginal zones. Immunohistochemistry for synaptic vesicle antigens has further shown that the entire subplate is a transient synaptic zone during fetal life (Chun and Shatz, 1988b). The subplate neurons themselves appear to be the recipients of at least some of the synapses, as demonstrated by EM studies of HRP-labeled (Chun et al., 1987) or ultrastructurally identified (Valverde and Facal-Valverde, 1988) subplate neurons, and subplate neurons can make local or interhemispheric connections (Chun et al., 1987; J. J. M. Chun and C. J. Shatz, unpublished observations). While there is no conclusive proof, reasonable candidates for at least some of the presynaptic terminals include the waiting axons and the local and distant projections of the subplate neurons. Thus, all the elements required for a synaptic network are present, raising the possibility that it is functioning during fetal life. Support for this view comes from the known presence of at least one molecule that participates in presynaptic transmitter release (synapsin I; Chun and Shatz, 1988b). Moreover, evoked field potential or single unit recordings in neonatal cats (Purpura, 1961a, b; Purpura et al., 1965; Huttenlocher, 1967) and fetal sheep (Bernhard et al., 1959, 1967; Molliver, 1967; Meyerson and Persson, 1969) have suggested indirectly that the developing white matter is a site of synaptic interactions.

The fact that subplate neurons appear to contain neuropeptides and GABA suggests several other developmental roles in addition to those noted above. For example, there are now several studies in which neuropeptides or other transmitters have been implicated in controlling neurite outgrowth and growth cone motility in the invertebrate nervous system (Bullock, 1987; Haydon et al., 1987), and it is conceivable that the peptides and/or GABA could have similar effects within the subplate and marginal zone. If so, then the observation here that there is a tangential organization of early-generated peptide-immunoreactive neurons within the fetal subplate and marginal zone could confer a measure of specificity on the interactions between axons and their proposed targets within the subplate: different sets of waiting axons could interact with different peptide-immunoreactive subpopulations of the early-generated neurons. Another possibility is that peptide immunoreactivities are correlated with the specific sets of connections, either distant or local circuit, made by subplate neurons. Regardless of how the subplate neurons exert their effect, whether it is through their interactions via the extracellular matrix, their transmitters, or their connections, it is likely that these special neurons play an instructive role in the formation of the mammalian cerebral cortex.

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