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Title of Dissertation: "Contributions of Early Versus Later-Generated Cortical Layers to the Development of Laminar patterns in Ferret Somatosensory Cortex"

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"CONTRIBUTIONS OF EARLY VERSUS LATER-GENERATED CORTICAL LAYERS TO THE DEVELOPMENT OF LAMINAR PATTERNS OF FERRET SOMATOSENSORY CORTEX"

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Abstract

Title of Dissertation: Contributions of early versus later-generated cortical layers to the development of laminar patterns of ferret Somatosensory Cortex

Stephen Charles Noctor, Doctor of Philosophy, 1998

Thesis directed by: Sharon L. Juliano, Ph.D.
Professor of Neuroscience, and Anatomy & Cell Biology

The inside-out sequence of generation and formation of the cortical layers is well documented and has been observed in many species. While it is recognized that the early-generated components of cerebral cortex are essential for the formation of important architectural structures, the contributions of later generated cortical layers have not been completely addressed. This study examines the role of individual cortical layers in the development of the cortical laminae by disrupting the formation of specific cortical layers with the antimitotic methylazoxymethanol (MAM). Chapter 1 introduces the ferret as a model for studying key aspects of cortical development, and presents findings from birthdating studies that determined the timing of final mitosis and migration for cortical neurons in each layer of the ferret somatosensory cortex. The findings from these experiments were used to target the formation of specific cortical layers through timed *in utero* injections of MAM. Chapter 2 presents findings that

layer 4 formation is disrupted after *in utero* injections of MAM on embryonic day 33 (E33). Treatment with MAM on E33 resulted in a greatly reduced thickness of layer 4, and reduced density of neurons within the remaining layer 4. Neuronal genesis resumed after E33 MAM treatment; neurons generated after the treatment migrated to appropriate locations and the overall laminar patterns of somatosensory cortex were preserved. Chapter 3 reports findings from experiments that further explore the ability of cortical cells to migrate in E33 MAM-treated cortex. Cortical precursor cells obtained from the ventricular zone of normal ferrets were transplanted into organotypic slices prepared from both layer 4-disrupted and normal ferret neonates. The transplanted VZ cells tended to migrate close to the cortical sites they would normally populate according to their date of birth. Chapter 4 presents results from studies that disrupted the formation of earlier generated layers of cortex with MAM treatment on embryonic day 24 (E24) or 28 (E28). In contrast to the E33 MAM treatment, E24 treatment severely disrupted formation of the cortical laminae. The cortical plate was much thinner compared to normals, and contained ectopic groupings of cells within the cortex. The disrupted cortical laminae were accompanied by distorted radial glial cells, and misplaced Cajal-Retzius cells. Further analysis suggests that interference with early development of neocortex leads to premature differentiation of radial glial cells into astrocytes, as demonstrated by the presence of glial fibrillary acidic protein (GFAP). A discussion comparing the relative contributions of early versus later-generated layers to the development of cortical lamination follows in Chapter 5.

CONTRIBUTIONS OF EARLY VERSUS LATER-GENERATED CORTICAL LAYERS TO THE DEVELOPMENT OF LAMINAR PATTERNS OF FERRET SOMATOSENSORY CORTEX

by

Stephen Charles Noctor

Dissertation submitted to the Faculty of the Program
in the Neurosciences Graduate Program of the
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To my mother and father for demonstrating day in and day out the hard work and dedication that are required to contribute and be productive.

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foundation for obtaining quality histological and immunhistochemical data. Nate Scholnicoff will always be remembered for his technical support, magic, and clowning skills.

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CHAPTER 1. NORMAL LAMINAR DEVELOPMENT OF FERRET SOMATOSENSORY CORTEX

Introduction

Cortical development follows a similar sequence in many mammals. During gestation, the first cortical neurons go through final mitosis in the neuroepithelium surrounding the lateral ventricles, called the ventricular zone (VZ), and migrate outward to the pial surface forming the first structure in cortex called the preplate. The preplate is composed of two populations of cells, the marginal zone (future layer 1) closest to the pial surface, and the deeper subplate. Both components of the preplate are thought to be composed largely of a transient population of neurons that die as the animal matures. Cortical neurons are then generated in an "inside-out" sequence in the ventricular zone: neurons that populate the deepest layers go through final mitosis and migrate into cortex, before the more superficial layers. As the cortical neurons migrate into cortex, they insinuate themselves between the components of the preplate, splitting the marginal zone and subplate. As neurons split the preplate, they form the cortical plate. At the end of neurogenesis, the cortical plate is composed of the future layers 2 through 6. Layers 5 and 6 differentiate and are the first to become recognizable, leaving a dense band of cells between the marginal zone and layers 5/6 that will form the future layers 2 through 4. In this manuscript, layers 2 through 6 will be termed the cortical plate, and the dense band of cells that forms layers 2 through 4 will be termed the dense cortical plate. The timing of these events varies in each species, from a number of days to a period spanning weeks or months. The series of experiments to be described in this thesis study cortical development in the ferret somatosensory cortex.

The ferret is becoming an important animal model for studying development, and many recent studies focus on the developing ferret to understand mechanisms of maturation in the neocortex. Ferrets are especially good models for understanding development of the neocortex, since they have a relatively short period of gestation (41 days), yet a protracted period of cortical neogenesis that continues after birth (Jackson et al., 1989). The combination of a relatively short gestation with a protracted period of cortical development is most useful since it can allow for more precise identification of key events in cortical development.

Although the clearly described inside-out pattern of development in cortical neogenesis has been known for many years, details about the emergence of layers in the cerebral cortex are not available for many species (Bayer and Altman, 1991). To more clearly understand mechanisms contributing to development of the cerebral cortex, it is necessary to provide more detail regarding the date of birth of neurons residing in various regions of the cerebral cortex. A detailed study of cortical neogenesis of ferret visual cortex (areas 17 and 18) was completed by Jackson and colleagues (1989). Although this study provides a good basis for understanding the relative timing of formation of layers in ferret neocortex, it does not answer specific questions about cortical areas other than visual.

In the past several years, a number of studies suggest that different sensory regions of neocortex may also differ in their developmental time line and in response to deprivation. For example, application of tetrodotoxin (TTX) or

N-methyl-D-aspartate (NMDA) antagonists to either the eye or visual cortex prevents formation of ocular dominance columns (Stryker and Harris, 1986; Reiter et al., 1986). Similar applications to peripheral or central sites in the rodent somatosensory system do not inhibit formation of cellular aggregates in the barrel system (Hendersen et al., 1992; Chiaia et al., 1992; Chiaia et al., 1994). A partial explanation for the differences induced by pharmacologic blockade of activity in the two systems is the relative state of maturation in each cortical site. That is, the somatosensory system appears to mature before the visual system, thereby influencing the ability to display subsequent plastic changes in the neocortex. The details of cortical development have rarely been compared in different sensory regions, especially in a more highly developed mammal like the ferret. The current study was designed to determine the date of birth for neurons that populate each cortical layer in ferret somatosensory cortex. Data are presented indicating that the somatosensory cortex develops substantially earlier than the dates reported for ferret visual cortex.

MATERIALS AND METHODS

Pregnant or neonatal ferrets were injected with 5-bromo-2'-deoxyuridine (BrdU). BrdU has proven over the past several years to be a useful alternative to [^{3}H] thymidine for assessing the birthdates of neurons. BrdU, an analogue of thymidine, becomes incorporated into the nucleus during cell division, and can be subsequently localized immunohistochemically; it thereby eliminates the need for radioactivity and autoradiography (Miller and Nowakowski, 1988; Gratzner, 1982; Nowakowski et al., 1989). A total of 19 pregnant ferrets were injected with

BrdU or [^{3}H] thymidine, and 89 of their offspring are included in this study. Timed pregnant ferrets were obtained from Marshall Farms, New Rose, NY. The day of conception is considered to be embryonic day 0 (E0) and the day of birth (consistently E41 or 42) considered postnatal day 0 (P0). Sixteen ferret kits were injected with BrdU and/or [^{3}H] thymidine postnatally. In these cases, the ferret kit received an I.P. injection of either BrdU (150 mg/k in 0.15 ml) or [^{3}H] thymidine (500 µCi in 0.15 ml). At selected time points, ferrets were killed and their somatosensory cortex examined for distribution of cells that incorporated BrdU into their nucleus (BrdU+) while dividing.

Two methods of injection were used: I.V. or I.P. Both methods resulted in good uptake of the thymidine analogue and resulting label. Animals that received IV injections were anesthetized with halothane (2-3% through a nose cone). A small incision was made over the external jugular vein, and a catheter inserted. The BrdU was injected (50 - 75 mg/kg, in saline with 0.007N NaOH) in a volume of 5 ml. Ferrets that received I.P. injections were also anesthetized with halothane and the same dilution of BrdU injected. To compare possible differences in uptake between BrdU and [^{3}H] thymidine, 3 animals received [^{3}H] thymidine injections IV (4 mCi in 4cc of saline) at time points that corresponded with selected BrdU injections (see Table 1); 3 pregnant jills and 2 postnatal kits (P1) were injected with both BrdU and [^{3}H] thymidine. After different survival times (see Table 1) ferrets were given an overdose of pentobarbital Na (I.P., 60 mg/kg) and perfused through the heart with saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer, followed by 10% sucrose in the

same buffer. The brain was removed and sunk in the 10% sucrose buffer, followed by 20% sucrose buffer at 4°C. Each brain was then blocked and frozen in isopentane and kept at -70°C until cut in a cryostat. Sections were cut at 40 µm thickness in the coronal plane and collected on subbed slides. Alternate series of sections were saved for staining with cresyl violet for visualization of Nissl substance, immunohistochemistry for BrdU, or for autoradiography (if [^{3}H] thymidine was injected). The Nissl-stained sections were used to assess the locations of cortical layers.

The reaction for visualizing BrdU involved an adaptation of the procedure used by Miller and Nowakowski (1988). The sections were incubated in 95% EtOH and 5% acetic acid for 30 minutes at room temperature, and then in 2N HCl with 1 mg/ml pepsin for 1 hour at 37ºC. This was followed by 1 rinse for 3 minutes with phosphate buffered saline (PBS) at pH 8.5. The sections were then incubated overnight in the primary antibody (anti-BrdU, Becton Dickson, San Jose, CA, concentration 1:20) in PBS (pH 7.4) with 0.05% Tween 20 at 4ºC. After this, sections were rinsed in PBS, pH 7.4, for 3 x 5 minutes and incubated in the secondary biotinylated antibody (concentration 1:100) in PBS with 1.5% normal horse serum. This was followed by 3 rinses in PBS at pH 7.4 for 5 minutes each. Sections were then incubated in ABC (avidin biotin complex, Elite standard Vectastain® kit, Vector Laboratories, Burlingame, CA) for 1 hour and rinsed in PBS pH 7.4 three times, for 5 minutes each. The sections were then placed in diaminobenzidine (40 mg/ml) for 8 minutes, followed by a final rinse in PBS pH 7.4. for 5 minutes.

The sections used for visualizing [^{3}H] thymidine were dipped in Kodak NTB-2 emulsion and stored in the dark at 4°C for 4-6 weeks. After development in Kodak D-19, the sections were fixed and subsequently stained for appreciation of Nissl substance with cresyl violet. Cells were considered heavily labeled with [^{3}H] thymidine if they contained at least five times as many silver grains as those observed in background (i.e., unlabeled) regions.

The labeled cells were viewed on a light microscope and their locations drawn by using a drawing tube. The cells incorporating BrdU were easy to view, in almost all instances they were clearly and darkly stained. For the vast majority of cases, the BrdU was injected days to months before the animal was killed, allowing enough time for the BrdU to be further diluted in non-neuronal cells that might have taken up the thymidine analogue. Any lightly or ambiguously labeled cells were excluded from analysis. Adjacent sections stained for Nissl substance were used to identify cortical architecture. The photomicrographs shown here were digitized and enhanced slightly to increase contrast using Adobe Photoshop®.

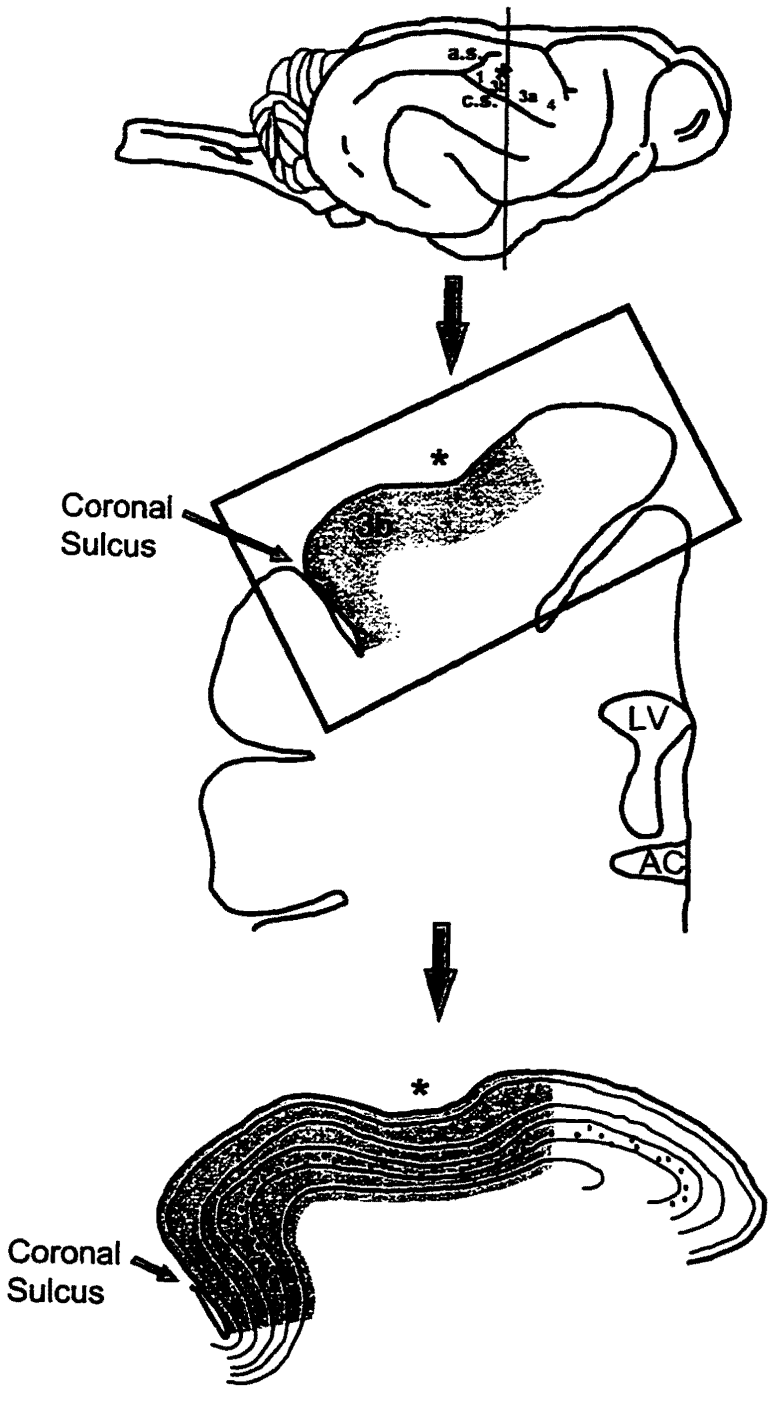
A quantitative analysis was conducted on representative sections at different ages. A given section was digitized using Image Pro Plus® (Media Cybernetics, Silver Spring, MD) and the optical density of 5 representative BrdU-labeled cells determined on each section to be analyzed. These cells were chosen by eye and always fell into a limited range of density values. A threshold value was set at the mean optical density value of the five labeled cells; this value always included clearly labeled cells but excluded lightly or ambiguously labeled cells. The

thresholded cells were counted in a 500μm wide band of cortex and assigned to bins according to their laminar position. After the counting, the file was transferred to MS-Excel® and a histogram created.

Results

Since the development of the neocortex proceeds in a well known lateral-to-medial and rostral-to-caudal progression, analysis was focused on a limited area of the somatosensory cortex. The forelimb region of primary somatosensory cortex is located in a region that can be reliably found using a number of cytoarchitectural and morphologic criteria (Juliano et al., 1996; McLaughlin et al., 1998) While cortical architecture is not mature in young ferrets, the position of the forelimb can be recognized grossly by the location of the post cruciate dimple, and in coronal tissue sections by the dimple, the placement of the anterior commissure and the coronal sulcus (Figure 1). In neonates, the same area was located using relationships between major structures such as the thalamus, basal ganglia, third ventricle, and anterior commissure. As the animal matures, delineation of the different cytoarchitectural fields becomes evident (Figure 2; Juliano et al., 1996; McLaughlin et al., 1995, 1996). At younger ages (postnatal day 1; P1 to P7) the cortical plate could be delineated, which consisted of the poorly differentiated layers 5 and 6, and the forming layers 2-4. Layer 1 was also present as well as a distinct subplate (Figure 2). By P14 each cortical layer could be distinguished, although the cytoarchitecture in somatosensory cortex was not mature until P28.

Figure 1. Schematic dorsolateral view of a ferret brain (top panel); the plane of section is indicated by a vertical line and the postcentral dimple denoted by an asterisk. The cytoarchitectural fields in the region of interest that can be viewed from the surface are indicated (areas 4, 3a, 3b, 1). The appearance of a coronal section through the marked plane is shown in the middle panel. The region of interest is shaded in the middle and bottom panels. The bottom panel indicates a typical region in a section used for analysis in the adult. Relationships between major structures such as the thalamus, third ventricle, and anterior commissure were used to locate the same area in neonatal ferrets.


a.s.
c.s.
3a
4
*
Coronal
Sulcus
3b
LV
AC
*
Coronal
Sulcus

Through comparison of the BrdU label with adjacent sections stained for Nissl substance, each labeled cell could be assigned a laminar position. Injections were made in pregnant ferrets or postnatal animals at time points ranging from E22 to P7 (see Table 1).

Injections on E22-E26.

BrdU incorporation after injections at this age labels cells in the subplate (Figures 2, 3, and 4). Labeled cells are clearly observed in young animals from P1-P7 in the subplate; they are diminished by P14, and by P28, only a few labeled cells are seen in the white matter of mature cortex (Figures 3 and 5). These observations confirm earlier reports that many subplate cells do not survive into adulthood (Luskin and Shatz, 1985; Allendoerfer and Shatz, 1994). Cells labeled after injection at E22 are positioned slightly deeper within the subplate than cells labeled after injection at E26 (Figure 3).

Injections on E28-E30.

Injections of BrdU on these days result in labeled cells located in the deeper layers. After E28 injections, the labeled cells are distributed in the immature layers 5 and 6 on P1, with the strongest concentration located in the deepest part of the cortex, near the junction with the subplate (Figure 6). E30 injections also result in BrdU+ cells in deep portions of immature cortex, although shifted slightly superficial compared with cells labeled after E28 injections (Figure 6). Injections at both times result in labeled cells throughout the thickness of layers 5 and 6, although even at P1, a distinct stratification of label is evident, with injections at E28 resulting in an overall deeper pattern of label than injections at

Table 1

Dates of BrdU and [^{3}H] thymidine injection and subsequent dates of analysis.

Dates of injection	**Dates of Analysis**				
	P1	P7	P14	P28	P64
E22	●(1)	●(1)			●(1)
E26	●(1) ■(1)	●(1) ■(1)	●(1) ■(1)	●(1) ■(1)	●(1) ■(1)
E28	●(4)		●(3)	●(3)	
E30	●(2)	●(1)	●(1)	●(1)	●(1)
E33	●(4)	●(2)	●(3)	●(3)	
E34	●(3) ■(1)		●(2) ■(1)	●(3) ■(1)	●(2) ■(1)
E35	●(2)		●(1)	●(2)	
E38	●(2) ■(1)	●(1) ■(1)	●(2)	●(2) ■(1)	●(1)
P1	●(2) ■(2)	●(1)		●(2)	●(2)
P2				●(1)	
P4		●(1)	●(1)	●(2)	
P7				●(1)	

● refers to dates of analysis of BrdU injections

■ refers to dates of analysis of [^{3}H] thymidine injection

Each symbol represents a date of analysis. The number of animals analyzed at that age is included in parentheses. A total of 19 pregnant ferrets were injected; 89 kits used for analysis.

Figure 2. Photomicrographs of adjacent sections either stained for Nissl substance (on the left) or immunohistochemical visualization of bromodeoxyuridine (BrdU). The localization of BrdU+ cells to cortical layers is shown in the adjacent Nissl-stained sections. Two examples are illustrated, one in which the BrdU was injected into the pregnant dam on embryonic day (E22) and labeled cells appear in the subplate on postnatal day 1 (P1; upper panels) and another in which BrdU was injected into a ferret kit on P2, which produced label in the cytoarchitecturally mature cortex in upper layer 2 on P28 (bottom panels). Cortical layers are indicated with numbers (CP, cortical plate; SP, subplate) Scale bar = 100 μm for the upper panels and 250 μm for the lower panels.

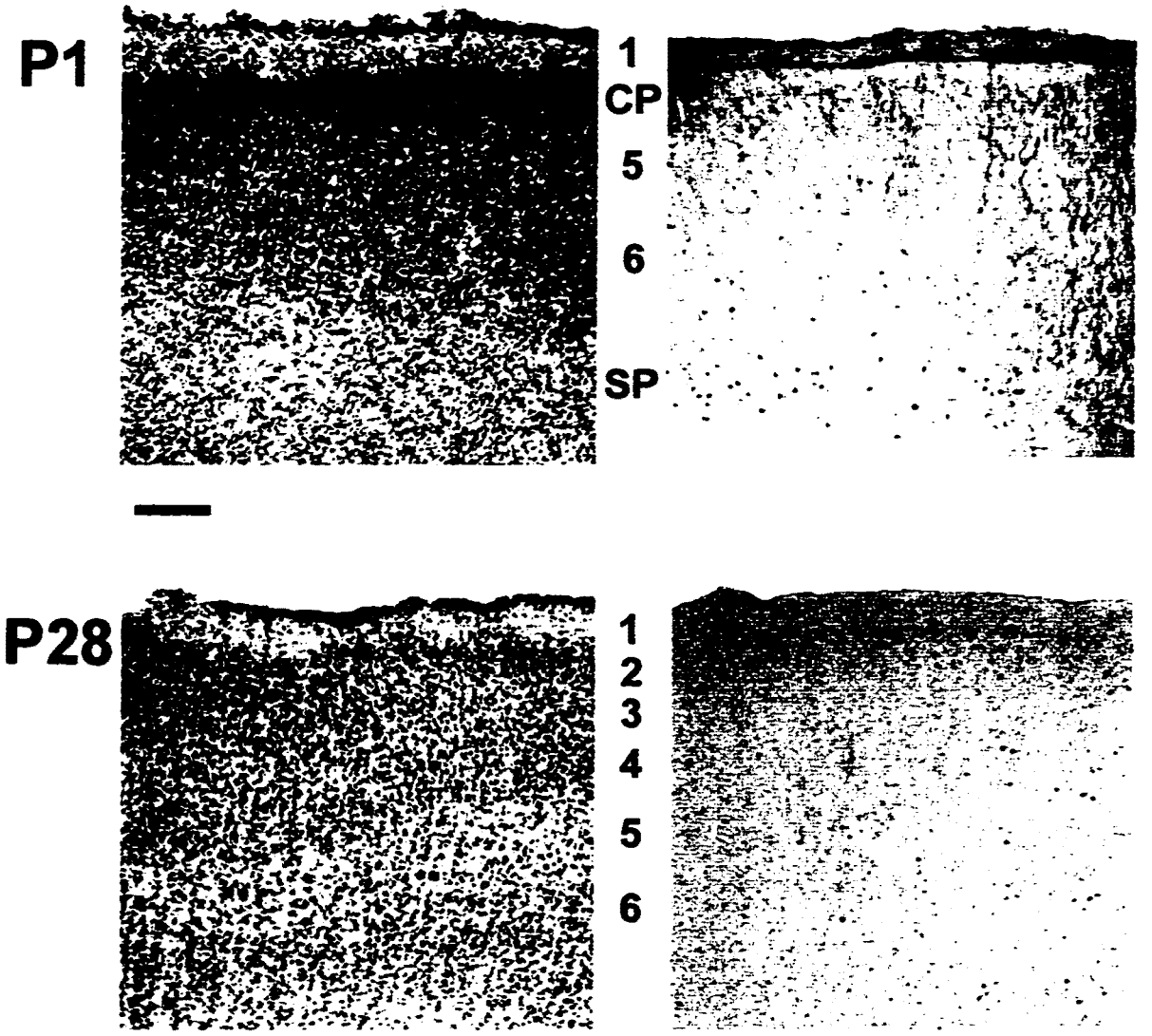

P1
1
CP
5
6
SP
P28
1
2
3
4
5
6

Figure 3. Drawings of the positions of BrdU+ cells after injections on E22 or E26. Illustrated are labeled cells observed on P1 (left panels) or in the mature cortex (right panels). Injections on either of these days results in labeled cells in the subplate region on P1 (left panels). By P28, however, only a few labeled cells remain, which are roughly concentrated near the remnants of the subplate region. The labeled cells are in a more superficial position after E26 injections, compared with E22 injections. Both scale bars = 1 mm; the bar on the left pertains to the P1 drawings, while the bar on the right pertains to the drawings of P28 sections. VZ, ventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; WM, white matter. Other cortical layers are indicated with numbers.

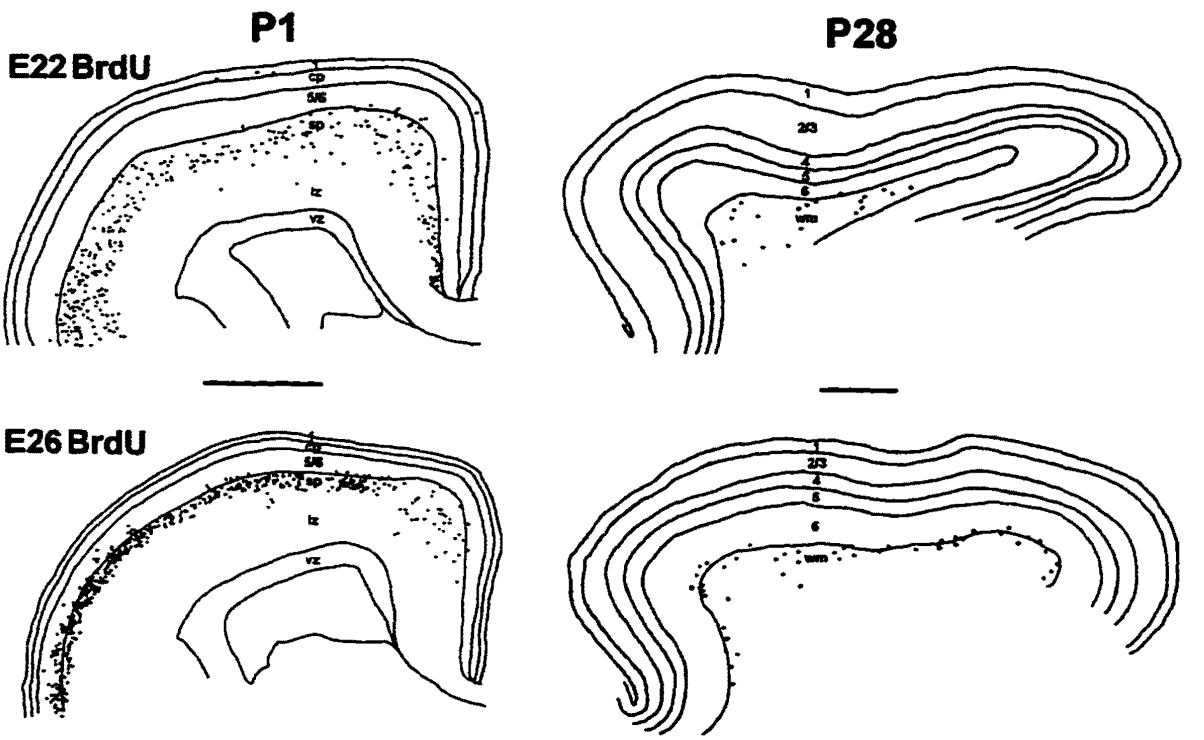

P1
P28
E22 BrdU
E26 BrdU
cp
5/6
sp
iz
vz
1
2/3
4
5
6
wm

Figure 4. Examples of BrdU immunoreactivity on P1 of two different animals, one (on the left) with labeled cells in the subplate (after BrdU injection on E22), and one with BrdU+ cells in the cortical plate (after BrdU injection on E33). CP (cortical plate); SP (subplate). Scale bar = 100 μm.

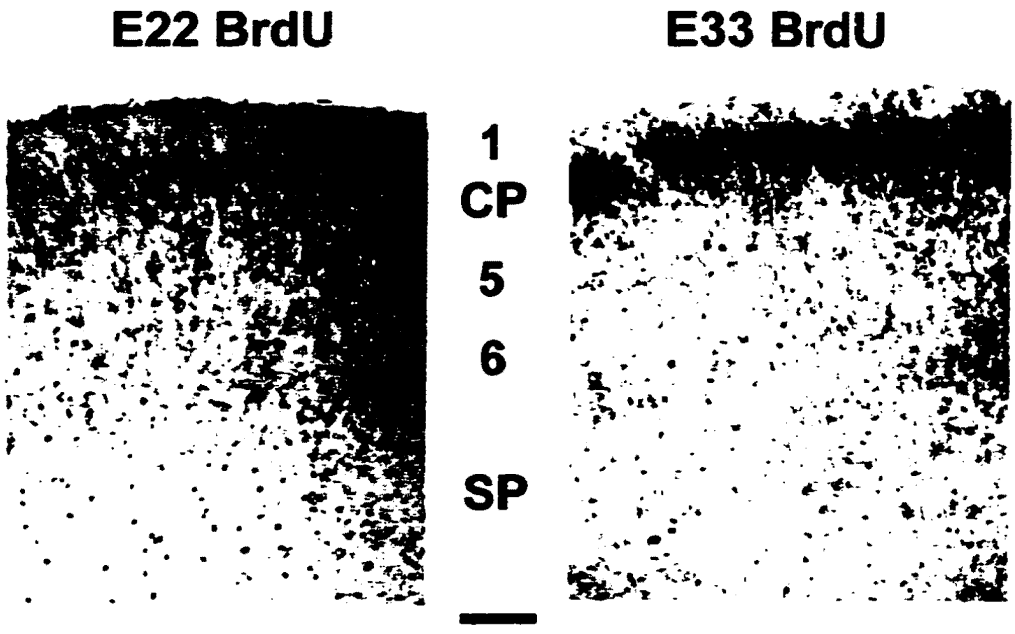
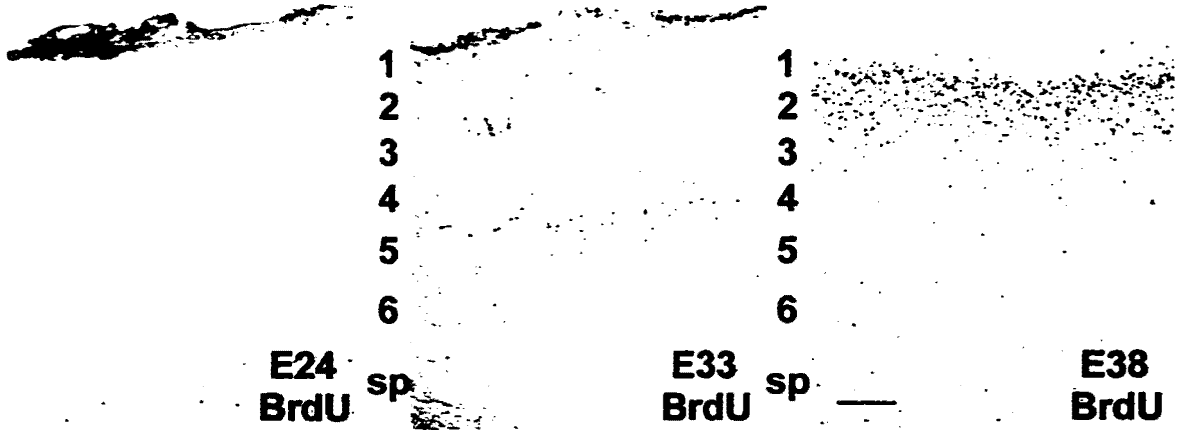

E22 BrdU
E33 BrdU
1
CP
5
6
SP

Figure 5. Examples of BrdU+ cells found in mature cortex (P28 or older) after *in utero* injections on different days: E24, E33, or E38. E24 injections (left panel) label subplate cells, as a result few immunoreactive cells are seen in mature cortex, although a few BrdU+ cells can be seen deep in the white matter. Injections on E33 result in labeled cells in layer 4 in the adult (middle panel). E38 injections resulted in label found predominantly in layer 2 in the mature animal (right panel), although BrdU+ cells can also be seen in layer 3, Scale bar = 200 μm.


1
2
3
4
5
6
E24
BrdU
sp
1
2
3
4
5
6
E33
BrdU
sp
E38
BrdU

E30. In mature cortex, the label is more clearly stratified and E28 BrdU injections label cells found in layer 6, while E30 injections result in layer 5 BrdU+ cells.

Injections at E33-E35.

In neonates (P1-3), BrdU injections on E33-35 produce labeled cells throughout the immature cortical plate above layer 5 (Figure 4). Even among BrdU+ cells resulting from injections at ages E33, E34, or E35, a slight stratification of labeled cells exists, with more cells located deeper in the cortical plate after E33 injections, and a greater number of cells found superficially after E34 injections (Figure 7). By P28, in the cytoarchitecturally mature somatosensory cortex, the majority of labeled cells produced from injections at E33-35 occurred in layer 4. At P28, the label was also slightly stratified, in that injections at E33 resulted in labeled cells found in slightly deeper portions of layer 4, and in upper layer 5, and injections at E35 caused BrdU+ cells in more superficial parts of layer 4 and in lower layer 3. Injections at E33-34 generated labeled cells almost totally confined to layer 4 (Figures 5 and 7).

Injections at E38-P1.

Injections at E38-P1 caused label in more superficial portions of cortex. After E38 injections, the BrdU+ cells were less stratified on P1 than after any of the previous injection times. Many BrdU+ cells were observed in all parts of the cortical plate and in the intermediate zone. These cells were presumed to be newly generated neurons migrating to their appropriate site in the cortex and were observed throughout the thickness of the immature cortical plate. By maturity, E38 injections clearly identified cells located in layer 2 (Figures 5 and 8).

Figure 6. Drawings of the positions of BrdU+ cells after injections on E28 or E30. Illustrated are labeled cells observed on P1 (left panels) or in mature cortex (right panels). Injections on E28 result in labeled cells located near the base of the forming cortical layers (i.e., presumptive layer 6) on P1, and are found in layer 6 in the mature cortex (top right panel). The E30 injection results in labeled cells shifted somewhat more superficially in the cortex (i.e., presumptive layer 5) and are found in layer 5 in adult cortex (bottom right panel). Scale bar = 1 mm. VZ, ventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; WM, white matter.

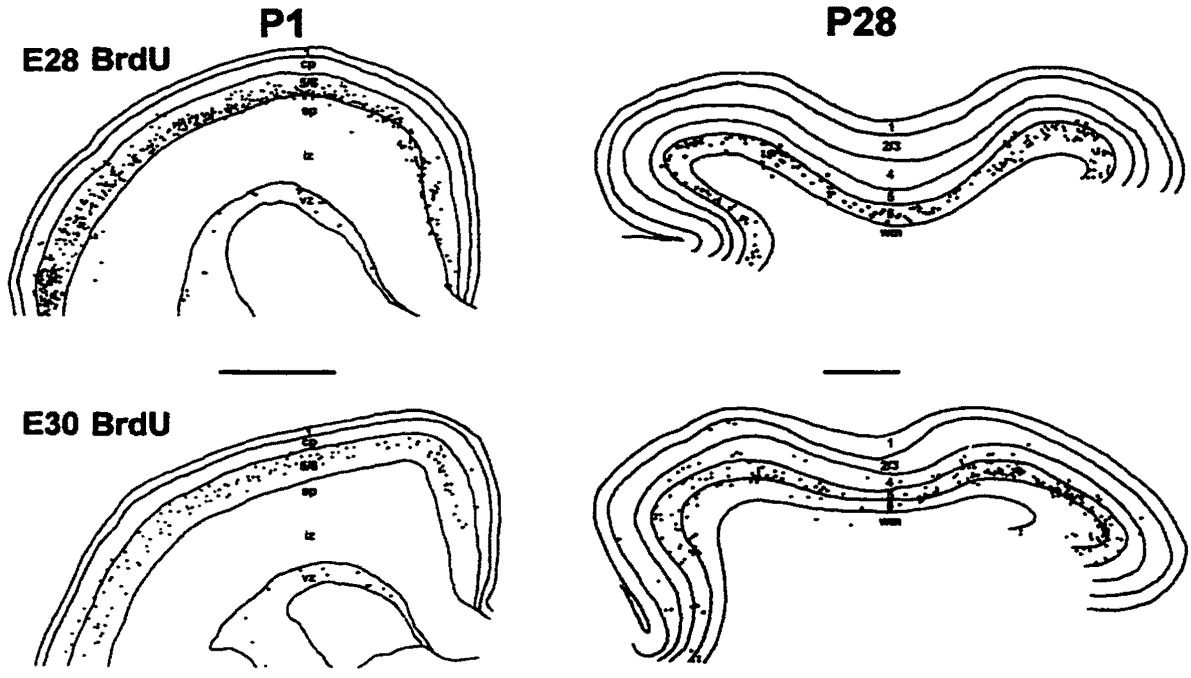

P1
P28
E28 BrdU
E30 BrdU

Figure 7. Drawings of the positions of BrdU+ cells after injections on E33 or E34. Illustrated are labeled cells observed on P1 (left panels) or in mature cortex (right panels). Injections on E33 result in labeled cells in the differentiating cortical plate on P1, and they are found in layer 4 in the mature cortex (top right panel). The E34 injection results in labeled cells shifted somewhat more superficially in the cortical plate and are found in layer 4 in adult cortex (bottom right panel). The bar on the left applies to the P1 drawings, while the bar on the right applies to the P28 drawings. Scale bar =1 mm. VZ, ventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; WM, white matter.

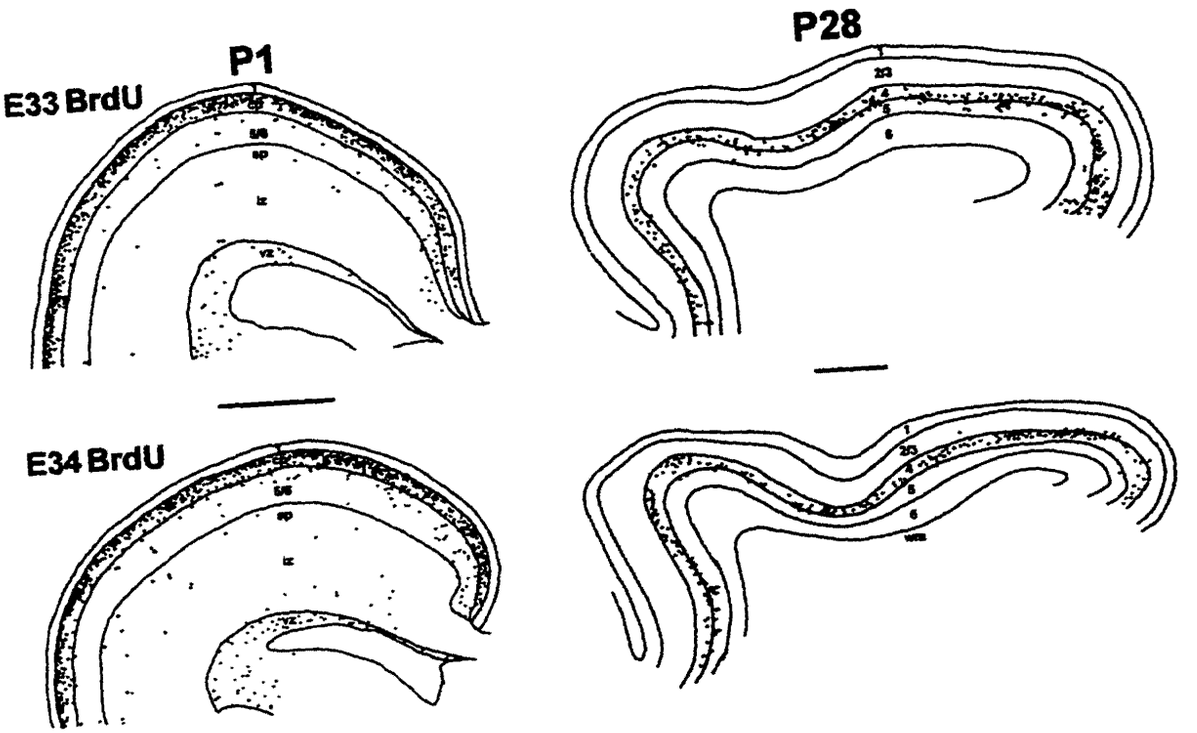

P1
E33 BrdU
E34 BrdU
P28

Figure 9 also illustrates images of cells labeled by injection of ^{3}H-thymidine on E38, indicating that the distributions of labeled cells resulting from this method and by BrdU injection were similar. By convention, cells incorporating ^{3}H-thymidine, as evidenced by silver grains occurring at least 5 times above background, were also distributed in all layers of the cortical plate (Luskin and Shatz, 1985).

After BrdU injections on P1, labeled cells were observed several days later, on P7, but these BrdU+ cells concentrated in the ventricular zone and the intermediate zone, very few labeled cells occurred in the cortex, indicating that neurons incorporating BrdU on P1 have not yet migrated to the cortex (Figure 8). By P28, however, very few BrdU+ cells were found in the somatosensory cortex, in comparison with the label observed after injections at other ages, although sparse label could be seen in upper layer 2.

Injections on P2-7.

We examined the somatosensory cortex of cytoarchitecturally mature animals (on P28) after BrdU injections into kits on postnatal days 2-7; a number of animals were assessed at intervening time points as well (see Table 1). BrdU injections on P2 led to a small amount of labeled cells in the upper part of layer 2, near the layer 2 - layer 1 border in adults (Figures 2, 10, and 11). Injections after this date, including postnatal days 4 or 7, did not label cells with a distinct laminar pattern, but only resulted in a few scattered darkly stained neurons within the somatosensory cortex (Figures 10,11).

Figure 8. Drawings of the positions of BrdU+ cells after injections on E38 or P1. Illustrated are labeled cells observed on P1 (upper left panel) or in mature cortex (right panels). Injections on E38 result in labeled cells located throughout the VZ, the IZ, and the cortex on P1; BrdU+ cells are found in layer 2 in the mature cortex (top right panel). The P1 injection results in labeled cells shifted somewhat more deeply towards the VZ on P7 and they are found in layer 2 in adult cortex (bottom right panel). In the examples on the left, the BrdU+ cells were still migrating toward the cortex on either P1 or P7 and had not yet arrived to their cortical destinations. In the case of the P1 injection evaluated on P7 (bottom left), the labeled cells were located closer to the VZ, since they had less time to travel to their destination. After injections on P1 and evaluations on P28, the BrdU+ cells appeared quite superficially within layer 2 of the cortex and were few in number. The scale bars = 1 mm; the bar on the left corresponds to the P1 and P7 drawings, the bar on the right corresponds to the drawings on the right. VZ, ventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; WM, white matter.

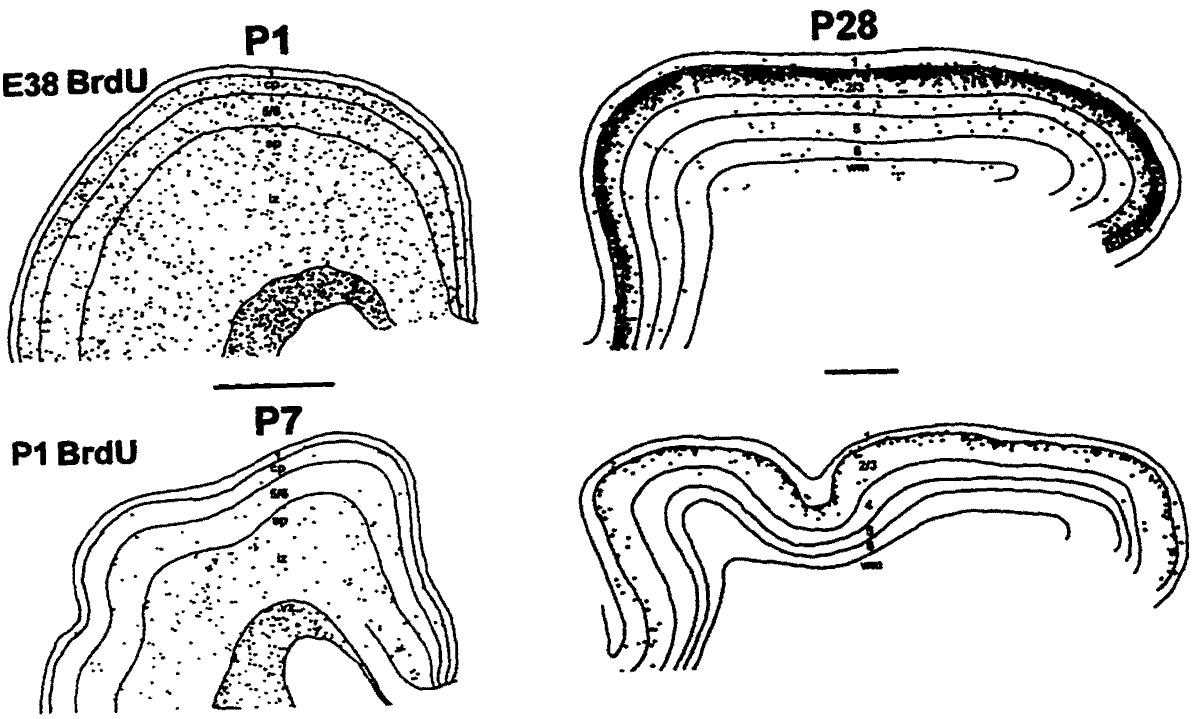
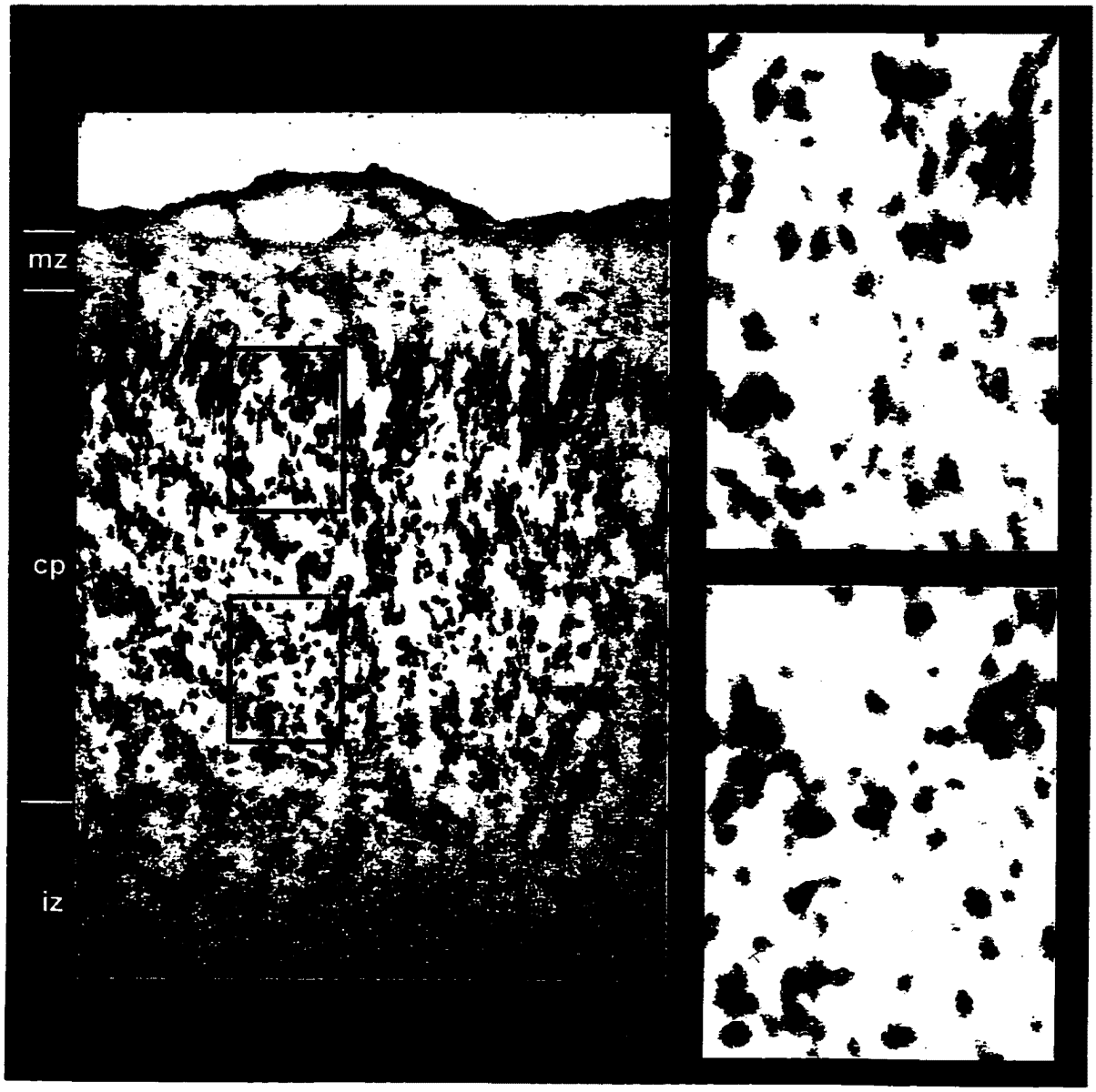

P1
E38 BrdU
P28
P7
P1 BrdU

Figure 9. An example of ^{3}H-thymidine label on P1 after injection on E38; the section is Nissl counterstained. The boxed areas are shown at higher power on the right for visualization of silver grains overlying cells incorporating ^{3}H-thymidine. This figure demonstrates that after E38 injections, labeled cells are present throughout the full thickness of the cortical plate. Scale bar = 60 μm for the left image and 30 μm for the photomicrographs on the right. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone.


mz
cp
iz

Previous investigations assessing birthdates of neurons populating ferret visual cortex concluded that visual cortical neurons were born up to two or more weeks after birth (Jackson et al., 1988). Since in these experiments, the somatosensory cortical neurons completed their generation by two days of postnatal life, several time points were assessed to determine the presence of BrdU+ cells in visual cortex (conducted on the same animals used for somatosensory cortex assessment). Neurons populating the visual cortex were generated for at least one week after only occasional scattered BrdU+ cells were observed in somatosensory cortex. Examples can be seen in Figures 10 and 11, in which the ferret kit received an injection of BrdU at P7, resulting in labeled cells in layer 2 of primary visual cortex. Injections at the same age resulted in no above background BrdU label in the somatosensory cortex.

Quantitative analysis.

Histograms of the distribution of labeled cells were calculated for each animal (see Methods). In Figures 12 and 13, the number of labeled cells are indicated as a percent of the total cells, and plotted as a function of their distance from the pia. The representative histograms indicate that for most cortical layers, the distribution of labeled cells in postnatal animals are roughly located in sites appropriate to their adult locations in the cortical plate or subplate. (The exceptions to this are the results of injections on or after E38, which label cells that ultimately reside in layer 2, see below.) Figure 12 illustrates that the early generated cells (E22 and E26) belonging to the subplate are present at birth in an appropriate site, although somewhat less focused than the later born layers.

Figure 10. Drawings of the positions of BrdU+ neurons after injection of BrdU on P4 (top panels) or P7 (bottom panels). After injection on P4, there are few labeled cells in the cortical plate by P7 (upper left panel), on P28 very few BrdU+ cells are found in the cortex, aside from a few scattered BrdU+ cells. Injections on P7 also result in very few BrdU+ cells in mature cortex (bottom left), whereas in primary visual cortex many labeled cells can be seen on P28 in layer 2-3 after a P7 BrdU injection (bottom right panel). VZ, ventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; WM, white matter. Scale bars = 1 mm.

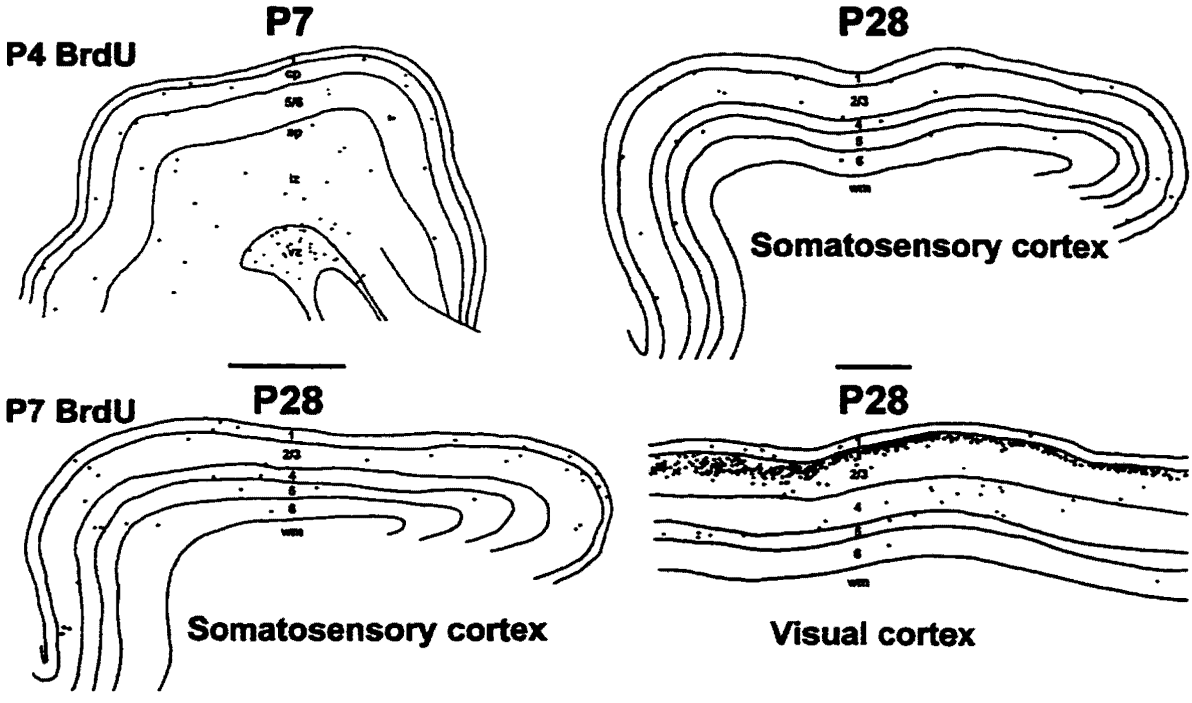
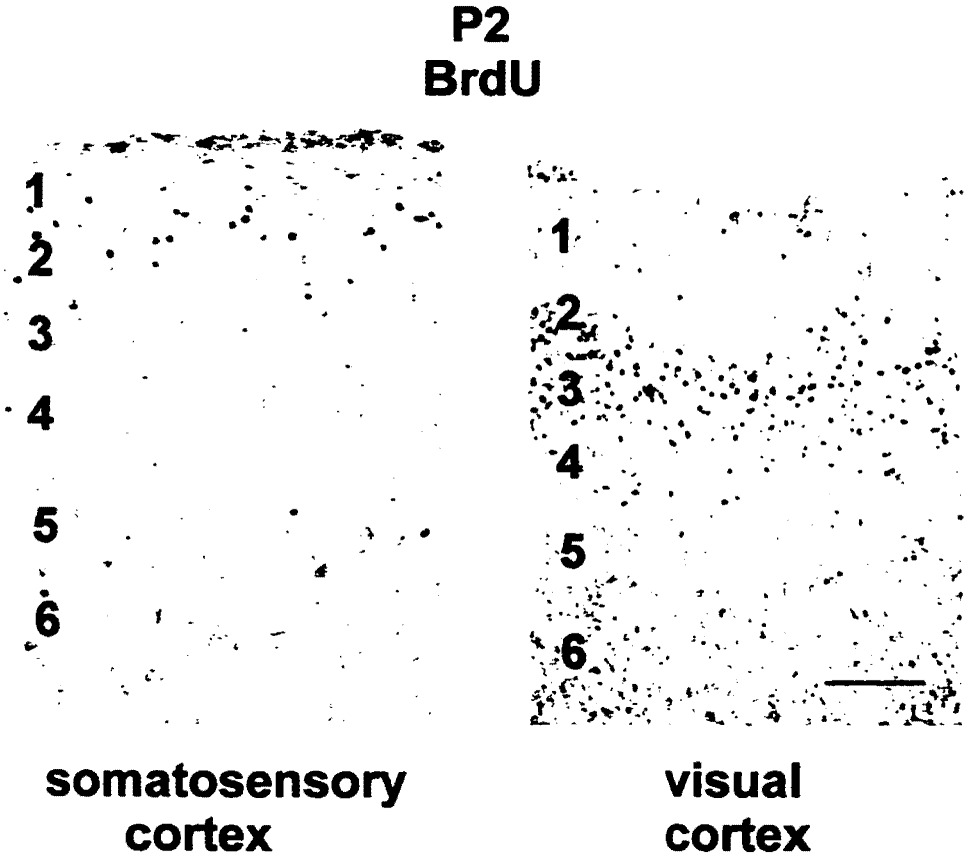

P7
P4 BrdU
P28
Somatosensory cortex
P28
P7 BrdU
Somatosensory cortex
P28
Visual cortex

Figure 11. Examples of BrdU+ cells in cytoarchitecturally mature somatosensory and visual cortices after BrdU injection on P2 (left panel, somatosensory cortex) or on P7 (right panel, visual cortex). Scale bar = 200 μm.


P2
BrdU
1
2
3
4
5
6
1
2
3
4
5
6
somatosensory cortex
visual cortex

No histograms are shown for adults after BrdU injections on days E22-26, since very few BrdU+ neurons remain into maturity. Layer 6 and layer 5 cells (E28 and E30; Figure 12) also reside in a roughly appropriate location at birth, although they are distributed through more bins than labeled cells found in the adult somatosensory cortex. For example, on P1 after E30 injections, no more than 30% of the total labeled cells are found in a single bin (Figure 12), while more than 65% of the total labeled cells born on E30 are located in specific bin on P28 (Figure 13). The cells belonging to the later-born layers 4 and 3 are also distinctly situated by P1 (Figure 12). Similar to the earlier generated layers the majority of labeled cells in mature cortex reside in fewer bins than in newborn cortex (E33 and E34; Figure 13). Fifty percent of BrdU+ cells generated on E34 are found in a single bin by P64, whereas only 40% of the BrdU+ cells born on the same date are in a single bin on P1. Cells belonging to layer 2, however, are not clearly positioned in a discrete cortical site by P1 (Figure 12). In this case, early after birth, the BrdU+ cells are still migrating to the cortex and occupy the intermediate zone and all layers of the cortical plate. When the cortex reaches cytoarchitectural and laminar maturity, the distributions of BrdU-labeled cells are more focused and clearly reside in a discrete cortical layer. A small number of neurons that populate the somatosensory cortex continue to be generated postnatally. Histograms made on P7 after P1 injections indicate that many BrdU+ cells disperse throughout the intermediate zone, which are presumably on their way to the cortex (Figure 12). When the cortex reaches its mature laminar pattern, BrdU+ cells occur predominantly in a single bin after P2 injections (Figure 13). Very few somatosensory cortical neurons are born after P2.

Figure 12. Histograms of the distribution of BrdU-labeled cells in neonatal ferrets plotted in relation to their depth from the pial surface. The distributions are normalized to reflect the total number of cells in each section. The plots shown here reflect the distribution of P1 neocortical cells that were generated from E22 (i.e., those belonging to the subplate, shown in upper left panel) to P1 (upper layer 2, shown in lower right panel). The BrdU+ cells are located in positions appropriate to their mature distribution by P1; the label shifts superficially after sequential injections on subsequent embryonic days. Injections on E38 and P1, however, label cells that are not focused in a specific bin, but are distributed throughout the intermediate zone and cortical plate. Presumably, these cells did not have time to migrate to their appropriate locations by the date of analysis. See text for details. The locations of labeled cells to CP, SP or IZ were determined by correlation with adjacent Nissl-stained sections and are indicated below each histogram. The mean number of cells per section is indicated at the upper right of each histogram. Each bin represents an increment of 50 μm as measured from the pia. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone.

Distribution of BrdU-Labeled Cells in Neonatal Ferrets After Different BrdU Treatment Dates

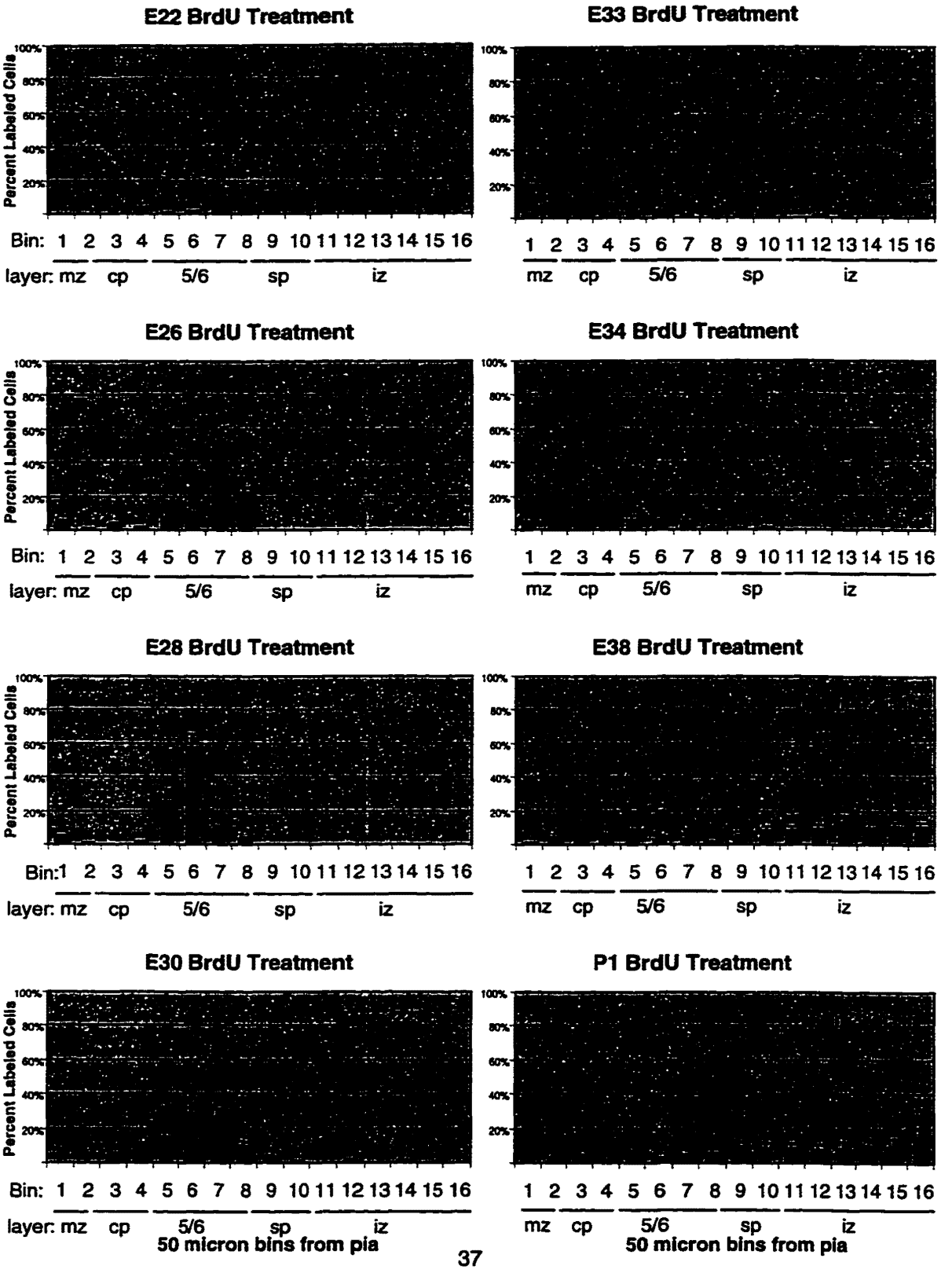
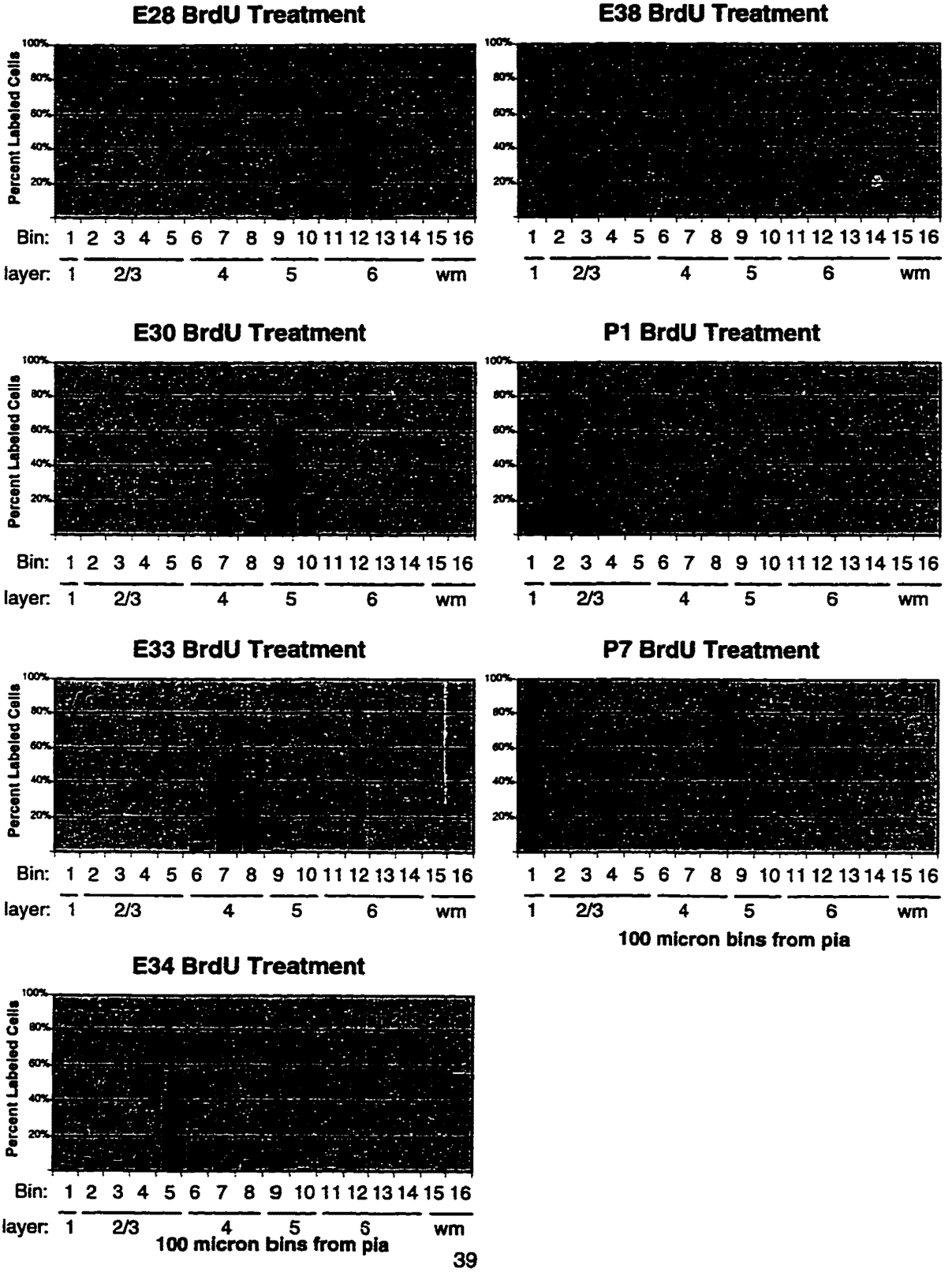


Figure 13. Histograms of the distribution of BrdU-labeled cells in cytoarchitecturally mature somatosensory cortex after BrdU injections on different embryonic days. Conventions are as for Figure 12. The distribution of BrdU+ cells is more focused in mature cortex (a greater percentage of cells in a single bin) than at P1. These histograms do not represent absolute numbers of cells, but normalized percentages per section. The layers in which the labeled cells are found are indicated along the x-axis of each histogram. The mean number of cells per section is indicated at the upper right of each histogram. The age of the animal at sacrifice is indicated at the upper left of each histogram. Each bin represents a 100μm increment as measured from the pia. wm, white matter.

Distribution of BrdU-Labeled Cells in Adult Ferrets After Different BrdU Treatment Dates



Discussion

Summary and formation of layers.

A summary of results can be seen in Figure 14. These findings indicate that the well-known inside-out development of cerebral cortex also occurs in the development of ferret somatosensory cortex. By birth, components of all layers have been generated. A few cells destined to populate the somatosensory cortex continue to be born postnatally, but they are not great in number or generated much after P2. Although components of each layer have already been produced, the layers are not distinctly formed at birth. At P1, the layers formed early during corticogenesis (layers 5 and 6) are relatively organized. The layers born later (layers 4,3,2) are not yet clearly formed on P1, although layers 4 and 3 have largely migrated into the cortex. Cells populating layer 2 continue migrating to their destination for several days after birth. The overall picture suggests that the distinct pattern of cortical layers emerges over a period of several weeks, leading to each lamina being clearly separate from its neighbor by 4 weeks of age. The majority of cells populating a given layer of somatosensory cortex are generated over a period of about 3 days, although neurons populating the subplate and layer 2 take somewhat longer. This is in contrast to neurons generated in ferret primary visual cortex (area 17) in which (several) neocortical layers are born over more protracted periods. Figure 15 compares developmental time lines for neurons generated in somatosensory versus visual cortex (Jackson et al., 1989; Johnson and Casagrande, 1993). The development

Figure 14. Summary diagram of where BrdU+ neurons are found on P1 and P28 in the somatosensory cortex after BrdU injections on different embryonic and early postnatal days. Many neurons generated on a specific day are roughly in their appropriate layers by P1, although the laminar arrangement is not as distinct as observed in the cytoarchitecturally mature somatosensory cortex.

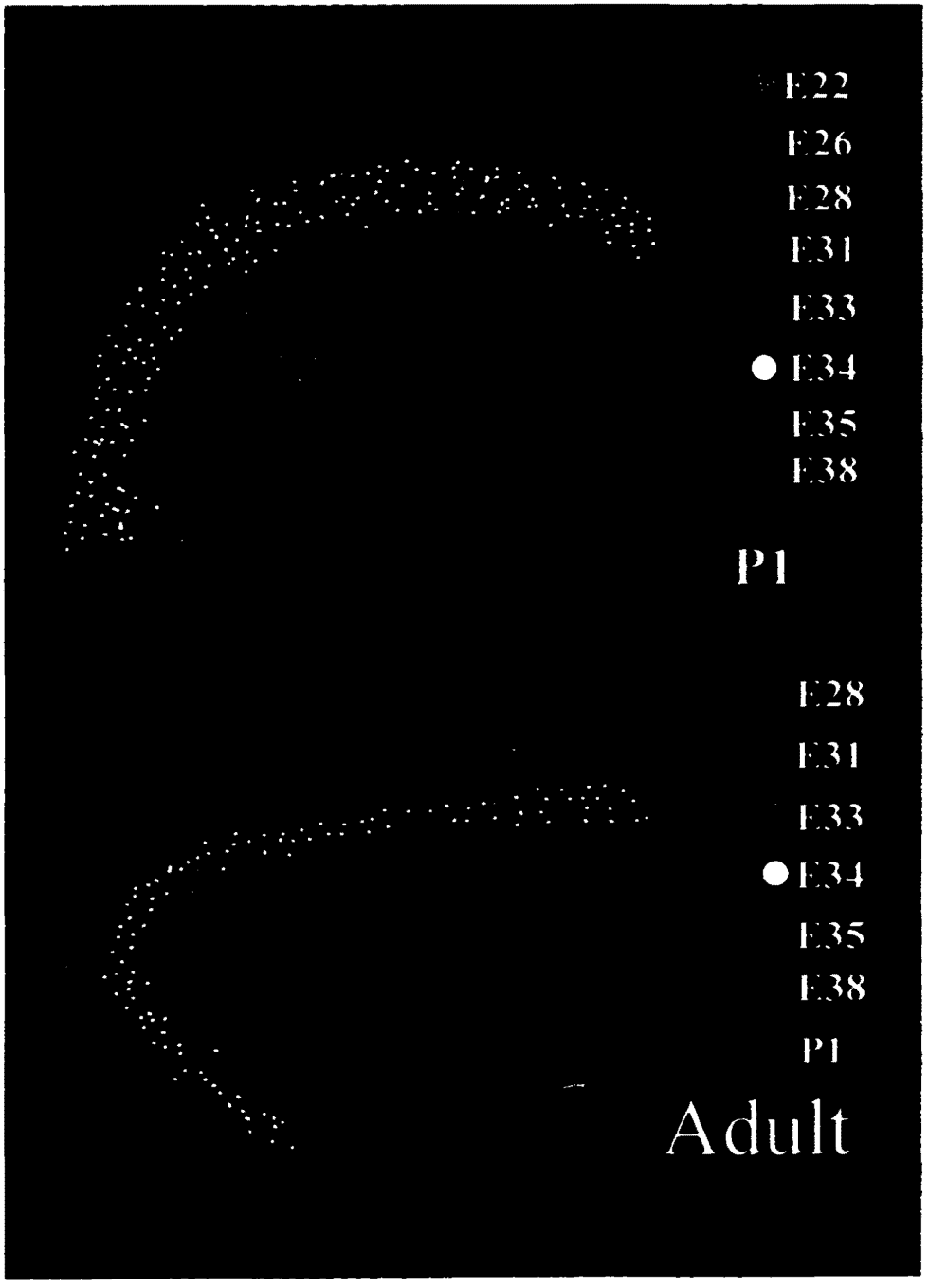
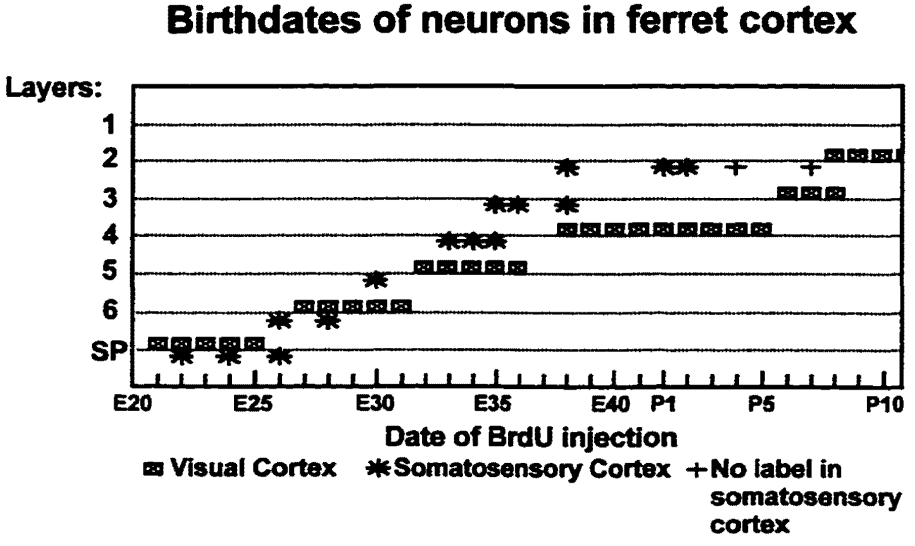

E22
E26
E28
E31
E33
E34
E35
E38
P1
E28
E31
E33
E34
E35
E38
P1
Adult

Figure 15. Graph representing the birthdates of neurons populating the somatosensory cortex versus visual cortex of the ferret. The neuronal birthdates are indicated on the x-axis, and the cortical layers are indicated on the y-axis. Dates for generation of neurons populating visual cortex were obtained from Jackson et al. (1988) and Johnson and Casagrande (1993). Although both the somatosensory and visual cortex begin generation of neurons at approximately similar dates, the visual cortex lags behind, especially upon birth of layer 4, which takes over 1 week for visual cortex, and approximately 3 days for somatosensory cortex. The subplate and layer 2 of the somatosensory cortex are generated over a period slightly longer than 3 days. Most neurons that populate the somatosensory cortex are born by P2, while neurons that reside in primary visual cortex continue to be generated for over a week postnatally.


Birthdates of neurons in ferret cortex
Layers:
1
2
3
4
5
6
SP
E20
E25
E30
E35
E40
P1
P5
P10
Date of BrdU injection
Visual Cortex
Somatosensory Cortex
No label in somatosensory cortex

of both cortical regions begins along similar time lines; the birth of neurons populating the somatosensory cortex proceeds somewhat earlier than visual cortex, as would be expected from the rostro-caudal gradient of development. The two patterns of cortical generation begin to diverge, however, during birth of layer 4 in the visual cortex. The generation of neurons in layer 4 of area 17 occurs over a substantially greater number of days than the birth of layer 4 in primary somatosensory cortex. This differential period of neuronal birth extends the period of neogenesis in the visual cortex, so that many cortical neurons are generated after the animal is born. In some ways this is not surprising, since layer 4 of area 17 is known to have more neurons than layer 4 of other cortical regions (e.g., Hendry, 1987).

The finding that the birthdates for neurons of somatosensory and visual cortex are different may in part reflect the different techniques used. The previous study evaluating birthdates of ferret visual cortex used ^{3}H-thymidine to determine when neurons in areas 17 and 18 are generated (Jackson et al., 1988), whereas the current study used BrdU-birthdating. In several animals of this study, ^{3}H-thymidine incorporation was used to determine when a cell was generated to supplement the BrdU analysis, and no differences were found in the distributions of labeled cells using either method. In 3 pregnant ferrets and 2 postnatal kits BrdU and ^{3}H-thymidine were injected together and confirmed that similar populations of cells incorporate both markers. In addition, Miller and Nowakowski (1989) report in their study using sections double-labeled with both ^{3}H-thymidine and BrdU, cells were either labeled with both markers or neither

marker, supplying strong evidence that both thymidine analogues label very similar, if not identical, populations of neurons. Other studies also report similar distributions of labeled cells using either marker (DeFazio et al., 1987). The method used in this paper (BrdU incorporation and immunohistochemical identification) yielded results comparable to those used in previous papers using ^{3}H-thymidine to determine the birthdates of cells in ferret visual cortex (Jackson et al., 1989; Johnson and Casagrande, 1993). Although this was not an exhaustive study comparing the BrdU label occurring in visual versus somatosensory cortex, the label distributed in area 17 was evaluated on several dates. For example, injections on P7 yielded no label in somatosensory cortex, while many labeled cells were observed in the visual cortex. The current results therefore confirm previous findings that neurons populating ferret visual cortex continue to be generated for considerable periods postnatally, while neurons in ferret somatosensory cortex undergo their last mitosis, for the most part, prenatally.

One surprising observation was the relative lack of BrdU+ cells in layer 1 after injections on any of the dates used in this study. There were occasional cells labeled after injections at all time points, but none of the selected dates resulted in a distinct distribution in layer 1. The most likely explanation is that cells populating layer 1 are generated earlier than the injection dates of this study. The marginal zone, comprised of layer 1 and the subplate, is known to be the earliest born component of the neocortex. The layers of cortex that develop subsequently insinuate themselves between the components of the preplate, the

subplate and layer 1 (Bayer and Altman, 1991). Evidence also exists supporting the idea that at least one component of the layer 1, the Cajal Retzius cells, are born before the subplate cells (see Bayer and Altman, 1991, for review). This may account for the lack of conspicuously labeled cells residing in layer 1.

Differences between visual and somatosensory development.

Although these findings globally confirm the well-known rostral to caudal development of the neocortex, they are somewhat surprising because of the magnitude of the timing difference between generation of the two populations of neurons. This is of interest because a number of other recent studies indicate that features of somatosensory cortex mature considerably earlier than visual cortex. In cats, thalamocortical and cortico-cortical terminations are relatively mature by the first week of life in the somatosensory cortex, and kittens posses a crude somatotopic arrangement on P1 (Sonty and Juliano, 1997; Juliano et al., 1996; Rubel, 1971; Wise et al., 1977). This contrasts with the architecture and connectional patterns of visual cortex in cats, which do not mature until about 8-10 weeks (LeVay et al., 1978). Functional properties in the visual cortex of cats and ferrets are reported to mature in conjunction with the architecture and connectional patterns (LeVay and Stryker, 1979; Nelson and Katz, 1995; Chapman and Stryker, 1992; 1993).

Other studies demonstrate that in the visual system, geniculocortical axons "wait" for protracted periods at the level of the subplate, before entering the cortex (Rakic, 1977; Lund and Mustari, 1977; Shatz and Luskin, 1986). During this waiting period, the thalamocortical projections are not topologically

organized, but rearrange more precisely as the cortex matures (Rakic, 1977; LeVay et al., 1978; LeVay and Stryker, 1979; Antonini and Stryker, 1993). In a rodent model, a waiting period does not appear to exist for thalamocortical projections into the somatosensory cortex, and topographic precision occurs early during development of thalamocortical axons (Catalano et al., 1991; Agmon et al., 1993; O'Leary et al., 1994; Schlagger and O'Leary, 1994). A similar precision occurs early in the development of thalamocortical projections in ferret somatosensory system (Juliano et al., 1996).

In addition, the development of visual cortex appears more susceptible to the effects of deprivation than comparable losses in the somatosensory system. Blockade or interference with afferent activity by peripheral or central application of TTX, or NMDA antagonists during cortical maturation, results in the failure of ocular dominance columns to form. These substances also interfere with plastic rearrangements that normally occur following monocular deprivation (Stryker and Harris, 1986; Reiter et al., 1986; Shatz and Stryker, 1988; Antonini and Stryker, 1993). In contrast, similar application of TTX or NMDA antagonists in the rodent somatosensory system, do not hinder the formation of the whisker-related patterns in the neocortex (Hendersen et al., 1992; Chiaia et al., 1992; Chiaia et al., 1994a). These substances also fail to block the normally occurring rearrangements of whisker-related patterns in the CNS after deprivation of input from a defined set of whiskers (Chiaia et al., 1994a&b).

These studies, in conjunction with the results presented here demonstrating a substantial timing difference in the generation of neurons populating the

somatosensory and visual cortices, suggest that tangible distinctions exist between the development of each area. The fact that somatosensory cortex is generated so early, compared to visual cortex, may encourage a more mature and rigid cortical architecture, which resists changes induced from the periphery, unless they occur very early during development. For example, to interfere with the normal segregation of retinogeniculate afferents in the visual system, it is necessary to block transmission early in the development of these fibers. The appropriate time to conduct a blockade varies with the species; in the case of cats, the appropriate time is before birth (Shatz and Stryker, 1988), while in ferrets, it is possible to block activity in this regard postnatally (Hahm et al., 1991). In the somatosensory system of hamsters, which are also altricial animals, experiments by Chiaia et al. (1994b) showed that thalamocortical fibers do not form a clear pattern until postnatal day 4. They found that blockade of activity by TTX using implants applied to the cortex failed to interfere either with development of whisker-related patterns in the somatosensory cortex, or with the plastic arrangements known to occur following sensory deprivation (Chiaia et al., 1994b). Applying substances that interfere with afferent activity very early during development, therefore, does not disrupt normal formation of the somatosensory architecture, while such treatments would dramatically interfere with the architecture of the visual system at a roughly similar time point in development. These differences suggest that distinct developmental mechanisms may distinguish between sensory cortical areas.

CHAPTER 2. DISRUPTING FORMATION OF LAYER FOUR WITH *IN UTERO* METHYLAZOXYMETHANOL TREATMENT

Introduction

Both the superficial and deep components of the preplate, i.e. the marginal zone and the subplate, have been recognized as important factors in further cortical development. Subplate neurons appear to be necessary for proper connections between the thalamus and cortex, and for the subsequent formation of cortical architecture (Ghosh et al., 1990; Ghosh and Shatz, 1992a,b; 1993). In the marginal zone, Cajal-Retzius neurons may regulate radial glial morphology and produce a stop signal for migrating cortical neurons (Frotscher, 1997; Hunter-Schaedle, 1997; Soriano et al., 1997). The role of later generated layers in formation of cortical structure is not as clearly defined. Each neocortical layer plays a distinct role in receiving, generating, and processing information either intrinsically or extrinsically. In primary sensory regions, layer 4 receives the primary projection from the thalamus. Information then transfers from layer 4 to the superficial and deep layers. For the cerebral cortex to process information correctly, connections between layer 4 and thalamus, and the other cortical laminae must be formed properly. Since layer 4 plays an integral role in sensory processing, I disrupted the formation of this layer to study how layer 4 contributes to the further development of the somatosensory cortex. The study examines the contribution of layer 4 neurons to the development of cerebral cortex by temporarily halting cortical neuronogenesis during the time layer 4 neurons are generated in the ventricular zone. Ferrets were treated on

embryonic day 33 with the anti-mitotic methylazoxymethanol acetate (MAM) to disrupt layer 4 formation. The treatment date was based on findings presented in Chapter 1.

Methylazoxymethanol has been successfully used by other groups to interfere with development of layer 4. Jones and colleagues disrupted formation of layer 4 in rats, and found that while the infragranular layers were intact, much of layer 2/3 was missing along with layer 4 (Jones et al., 1981; Floeter and Jones, 1984,1985; Yurkewicz et al., 1984). In these MAM-treated rats, nevertheless thalamic afferents entered cortex and these animals retained somatotopic maps. Woo and colleagues treated hamsters with MAM during layer 4 formation and found MAM effective in specifically reducing the number of layer 4 cells (Woo et al., 1996, Woo and Finlay, 1996). The current study used ferrets, in which generation of cortical neurons takes place over approximately 3 weeks, versus less than a week in rats and hamsters. More importantly, the generation of each neocortical layer occurs over a period of several days, so that appropriately timed injections of MAM can precisely target the generation of specific layers. The effects of the MAM treatment on ferret somatosensory cortex were measured by comparing numbers of cells per layer, cell packing density within each layer, and thickness of each layer in MAM-treated and normal adult ferrets.

MATERIALS AND METHODS

Pregnant ferrets obtained from Marshall Farms (New Rose, New York) were treated with single injections of methylazoxymethanol acetate (MAM) on embryonic day 33 (E33) to disrupt development of layer 4 in primary somatosensory cortex. Methylazoxymethanol selectively prevents the production of postmitotic neurons by stopping the synthesis of new DNA during S-phase at the time of the injection. MAM acts only on dividing cells, and *in vitro* studies have demonstrated that *in vitro* doses of MAM halted neuronal division but did not affect the morphology or generation of glial cell cultures (Cattaneo et al., 1993). Ongoing studies in our lab have determined that neuronal genesis recommences at eight hours after MAM treatment, while Evans and Jenkins (1976) found that MAM was active for 24 hours *in vivo*. Treatment dates were chosen based on the data in Chapter 1 that used bromodeoxyuridine (BrdU) as a thymidine analog to birthdate cortical neurons populating each layer of ferret somatosensory cortex (see Chapter 1, Noctor et al., 1997). Six of the pregnant ferrets received injections of BrdU 3 to 5 days after the MAM injection to test the viability and migration of cortical neurons after MAM treatment.

Cytoarchitecture of somatosensory cortex was examined in normal fetal ferrets at the time of the MAM treatment (E33), in normal and MAM-treated ferrets at birth P0-1), and in normal and MAM-treated ferrets 12 weeks old. Additional animals at each age were studied for immunoreactivity against BrdU.

Injections

A total of 38 normal and 44 MAM-treated ferrets were used in this study. Ten pregnant ferrets (Marshall Farms, New Rose, NY) were treated with single injections of MAM on E33 yielding almost 8 kits per litter. Ten adults, and 34 neonates from the MAM-treated litters were used in this study. Nine adults, and 29 neonates from normal litters were used as controls. The pregnant jills were anesthetized with 5% halothane and 0.5% N_2O and injected IP with 12mg/kg of MAM (Sigma) dissolved in sterile saline. Three to five days after MAM treatment, 6 of the 10 pregnant ferrets were treated with single IP injections of BrdU (65mg/kg dissolved in sterile saline with 0.007N NaOH, Sigma), as described above. Ferrets were closely monitored after injections to ensure proper health. After birth on E41, 34 MAM-treated, and 29 normal ferrets were used in experiments on postnatal day zero (P0), or P1. Three MAM-treated, and 3 normal ferrets were used at 12 weeks to study cytoarchitecture of mature ferret somatosensory cortex. Additional adult animals in each group were studied to support the findings.

Immunohistochemistry

Neonates were anesthetized with an overdose of pentobarbital Na (60mg/kg IP) then perfused through the heart with saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. Brains were removed, sunk in 20% sucrose for cryoprotection, frozen in isopentane at -35°C and stored at -80°C until cutting. Brain sections were cut on a cryostat at 30 µm and stained for identification of Nissl substance using cresyl violet. In animals that received

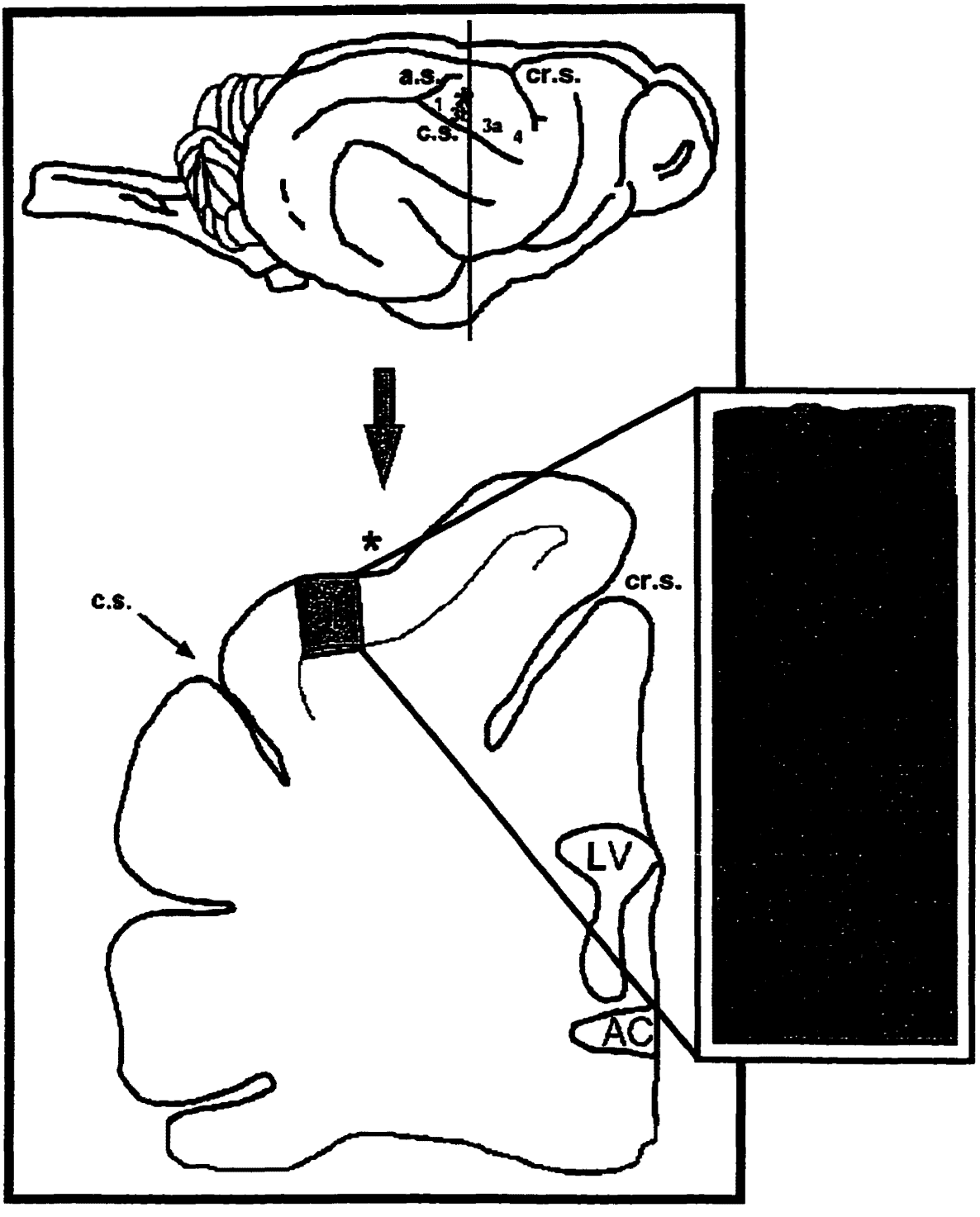
BrdU injections after MAM treatment, alternate sections were immunostained with antibodies directed against BrdU. The tissue was incubated for 30 minutes in 95% EtOH and 5% acetic acid followed by 1 hour in 2N HCl with 1mg/ml pepsin at 37°C. Sections were then rinsed with 0.1M PBS at pH 8.5 and incubated overnight at 4°C in the primary antibody (Anti-BrdU, Becton Dickson, 1:20) in PBS (pH 7.4) with 0.1% Triton X-100. The following day, standard procedures were followed for labeling the sections with secondary antibodies and peroxidase label using Vector kits (Vector Elite ABC kit, Vector).

Cytoarchitectural Analysis of Adult Somatosensory Cortex

To compare the cytoarchitecture of MAM-treated versus normal ferret somatosensory cortex 12 week old ferrets were anesthetized with an overdose of pentobarbital Na (60mg/Kg, IP) then perfused through the heart with saline followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. Brains were removed, coronally blocked and postfixed for at least 24 hours in the same fixative. Blocks of cortical tissue from 12 week old ferret brains extending from the cruciate sulcus to the fork of the ansate and coronal sulci were paraffin embedded and cut at 10 µm on a microtome. Sections were mounted on gelatin subbed slides then stained for Nissl substance. The forepaw representation in primary somatosensory cortex can be reliably identified by examining the relationships of gross structures such as the post cruciate dimple, the coronal and ansate sulci, and the anterior commissure in coronal sections (Juliano et al., 1996, McLaughlin et al., 1998). In addition, the cytoarchitectural characteristics of the tissue sections were examined starting rostrally at the medial border of the

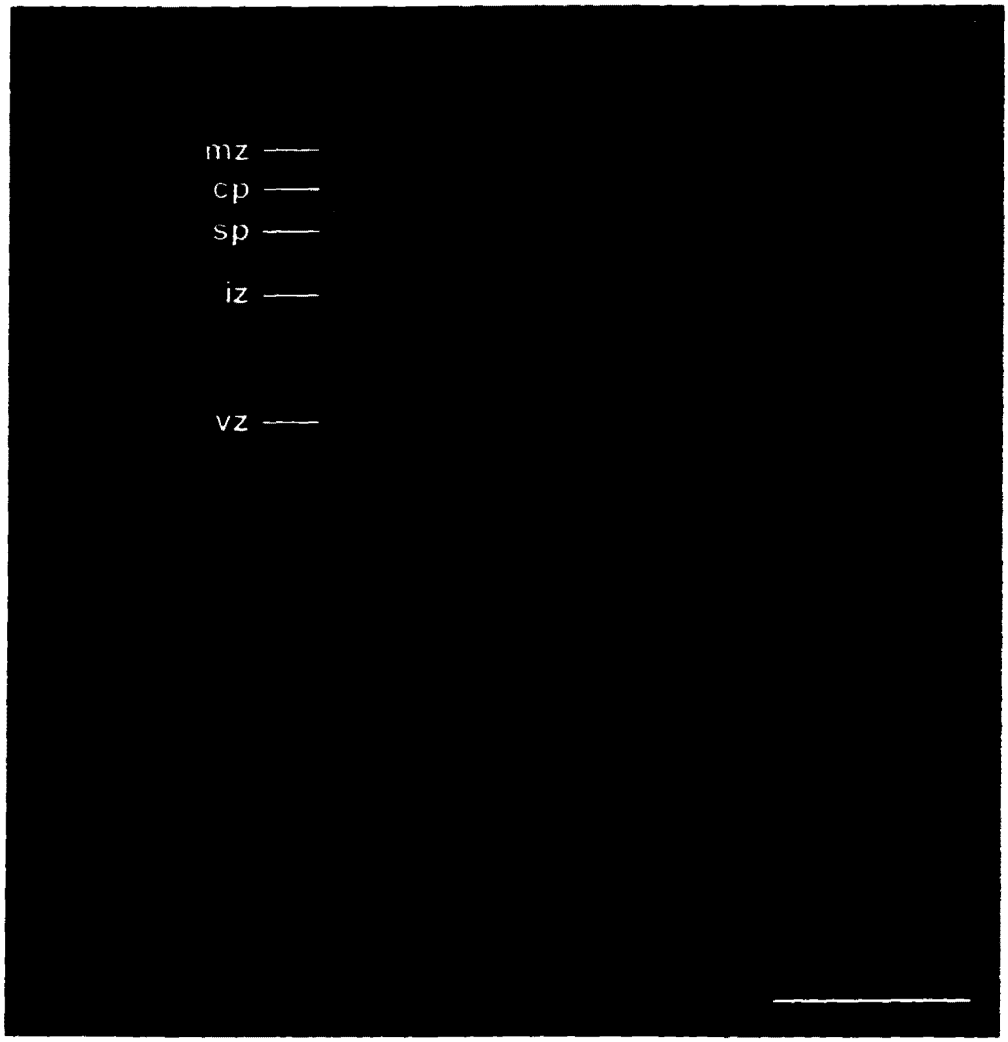
cruciate sulcus and progressing caudally to the intersection of the ansate and coronal sulci. Five equally spaced coronal sections were chosen from within area 3b of primary somatosensory cortex (approx 1 mm of tissue in the rostral to caudal plane). From these sections, a region of cortex lateral to the post cruciate dimple and medial to the coronal sulcus was digitally imaged extending from pia to white matter (Figure 16). Using the Image Pro Plus® program (Media Cybernetics, Silver Spring, Md), the thickness of cortex from pia to the bottom of layer six, and the thickness of each individual layer was measured. A grid of boxes 500 µm wide by 100 µm deep starting from the top of layer two and extending to the bottom of layer six was created for each section, and each box was assigned to a layer. The Nissl-stained cells were counted in each box of normal and MAM-treated animals. Only Nissl-stained cells containing a single nucleolus within the nucleus were included for analysis. Small, presumptive glial cells, and cells in which nucleoli were not present or could not be differentiated were excluded from analysis. To test if MAM treatment affected the size of cells, I measured the 2 dimensional surface area of each cell in which the nucleus contained a nucleolus. The data were imported into Microsoft Excel and t-tests were performed to compare thickness of cortex, thickness of the cortical layers, number of neurons within each box (as a measure of the packing density of neurons within a layer), and size of cells per box, between normal and E33 MAM-treated ferrets. Results from t-tests are included in Appendix A.

Figure 16. Primary somatosensory cortex was identified in coronal sections of normal and E33 MAM-treated adult ferrets. A region of cortex lateral to the post cruciate dimple (asterisk) and medial to the coronal sulcus (c.s.) was imaged in each section and a radial region of cortex 500 μm wide from the top of layer 2 to the white matter (shaded rectangle) analyzed. On the top is a lateral view of a ferret brain, the approximate positions of the cytoarchitectural areas are indicated. The vertical line indicates the placement of the region analyzed and is displayed in a coronal section below. The rectangular inset is an example of a digitized image taken from the selected region; the division of the selected region into a grid of boxes is illustrated. Cr. S., cruciate sulcus; A.S., ansate sulcus; C.S., coronal sulcus; L.V., lateral ventrical; A.C., anterior commissure; *, post cruciate dimple. Cortical areas denoted with numbers.



cr.s., cruciate sulcus; a.s., ansate sulcus;
c.s.; coronal sulcus; * - post cruciate dimple

Figure 17. Digitized image of a bisbenzimide-stained coronal section taken from a normal fetal ferret at age embryonic day 33. At this age the immature cortex consists of the marginal zone, a thin cortical plate and the subplate. Scale bar=500 µm. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; VZ, ventricular zone. Scale bar = 1 mm.


mz
cp
sp
iz
vz

RESULTS

Cytoarchitectural Analysis of Fetuses and Neonates

Ferrets are born after 41 days of gestation. On E33 in normal fetuses, the immature cortex consists of a marginal zone, a thin cortical plate, and the subplate (Figure 17). The cortical plate contains a dense homogeneous population of cells, individual cortical layers cannot be distinguished.

At birth, Nissl stained sections revealed very few differences between normal and E33 MAM-treated brains. After MAM treatment, the marginal zone, a dense cortical plate (layers 2-4), differentiating layers 5/6, and subplate are visible, and similar to normal neonatal ferrets (Figure 18). Neonatal cortex was slightly thinner after MAM treatment than in normals, but the difference was not significant (t-test). While components of layers 2-4 are in place in normals at birth, these layers have not fully formed and cortical neurons are still migrating to cortex, so the relatively normal appearance of E33 MAM-treated cortex was not too surprising (see Chapter 1, Noctor et al., 1997).

Qualitative BrdU Analysis.

Methylazoxymethanol-treated ferrets were injected with BrdU to test the viability and migration of cortical neurons after MAM treatment. Figure 19 is an image taken from an adult animal age P42 that was treated with MAM on E33, and subsequently treated with BrdU on E38, when layer 2 neurons are generated in the normal animal. BrdU positive neurons can be seen in layer 2, indicating that neurons continued to be born and migrate to appropriate locations in somatosensory cortex after MAM treatment.

Figure 18. Digitized images comparing Nissl-stained coronal sections from normal (A) and MAM-treated (B) neonatal ferrets. Although the MAM-treated cortex was slightly thinner, there are few observable differences between the two groups at this age. In each section, the marginal zone and cortical plate are present; layers 5 and 6 are beginning to differentiate. MZ, marginal zone; CP, cortical plate; SP, subplate. Scale bar = 250 μm.

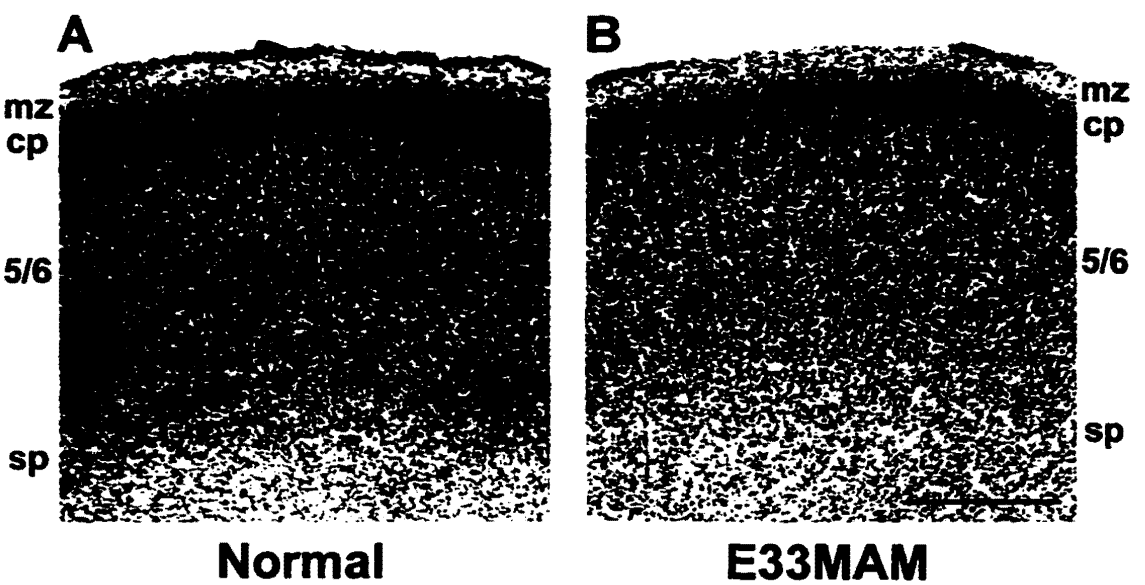
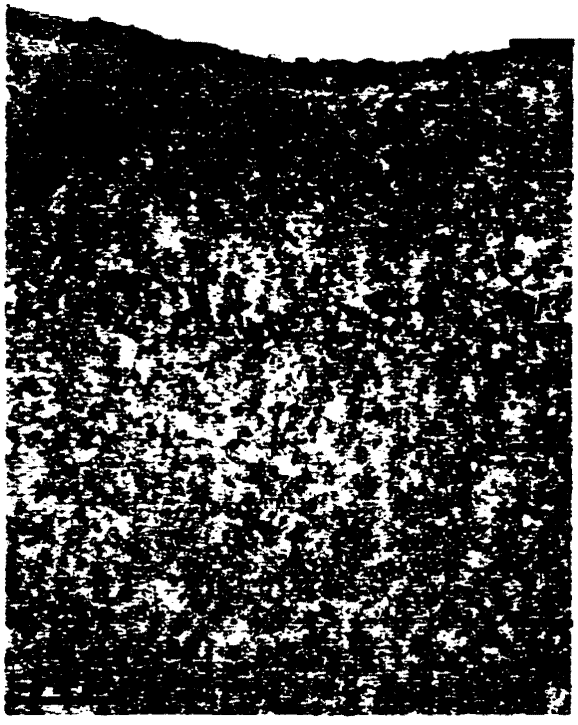

A
mz
cp
5/6
sp
Normal
B
mz
cp
5/6
sp
E33MAM

Figure 19. Photomicrograph of BrdU immunoreactivity in mature ferret cortex (P42) that received BrdU 5 days after E33 MAM treatment. The BrdU positive cells are located in an appropriate layer of somatosensory cortex, indicating that neurogenesis and migration resumed after the E33 MAM treatment. Scale bar = 100 µm.



Cytoarchitectural analysis of 12 week old ferrets.

Nissl-stained sections of primary somatosensory cortex were analyzed in E33 MAM-treated and normal ferrets at 3 months of age when the neocortex is cytoarchitecturally mature (Juliano et al., 1996). The total thickness of the neocortex, layers 1-6, was measured as well as the thickness of the individual layers. Neurons were counted and measured in each 100 μm bin and each bin assigned a layer from 2-6.

Figure 20 depicts an E33 MAM-treated cortex on the left, and a normal ferret cortex on the right. In the E33 MAM-treated somatosensory cortex, layers 1, 2, 3, 5 and 6 were clearly present, but layer 4 was greatly reduced in thickness; as a result, the infragranular layers were located closer to the pial surface. Treating ferrets with MAM on E33, therefore, disrupted layer 4 in somatosensory cortex. The histograms in Figure 21 demonstrate the cytoarchitectural differences between MAM-treated and normal adult ferret somatosensory cortex. Figure 21A demonstrates that E33 MAM-treated somatosensory cortex was significantly thinner than the normals, having a mean of 1153 μm versus 1472 μm ($p=0.03$, t-test). The histogram in Figure 21B shows this difference was proportionately the greatest in layer 4. Layer 4 was 69% thinner in E33 MAM-treated animals (83 μm thick compared to 268 μm in normals, $p<0.01$, t-test). The thickness of layer 3 was reduced 40% after E33 MAM treatment, with a mean thickness of 175 μm compared to 295 μm in the normals, $p<0.03$, t-test. The thickness of layers 1, 2, 5, or 6 were not altered by E33 MAM treatment (Figure 21B).

Figure 20. Digitized images of Nissl-stained sections of normal (right) and E33 MAM-treated (left) adult ferrets. In the E33 MAM-treated adult layer 4 is greatly reduced, yet the overall laminar patterns were preserved. Layers 1, 2/3, 5, and 6 are present and easily identifiable. WM, white matter. Scale bar=100 μm.

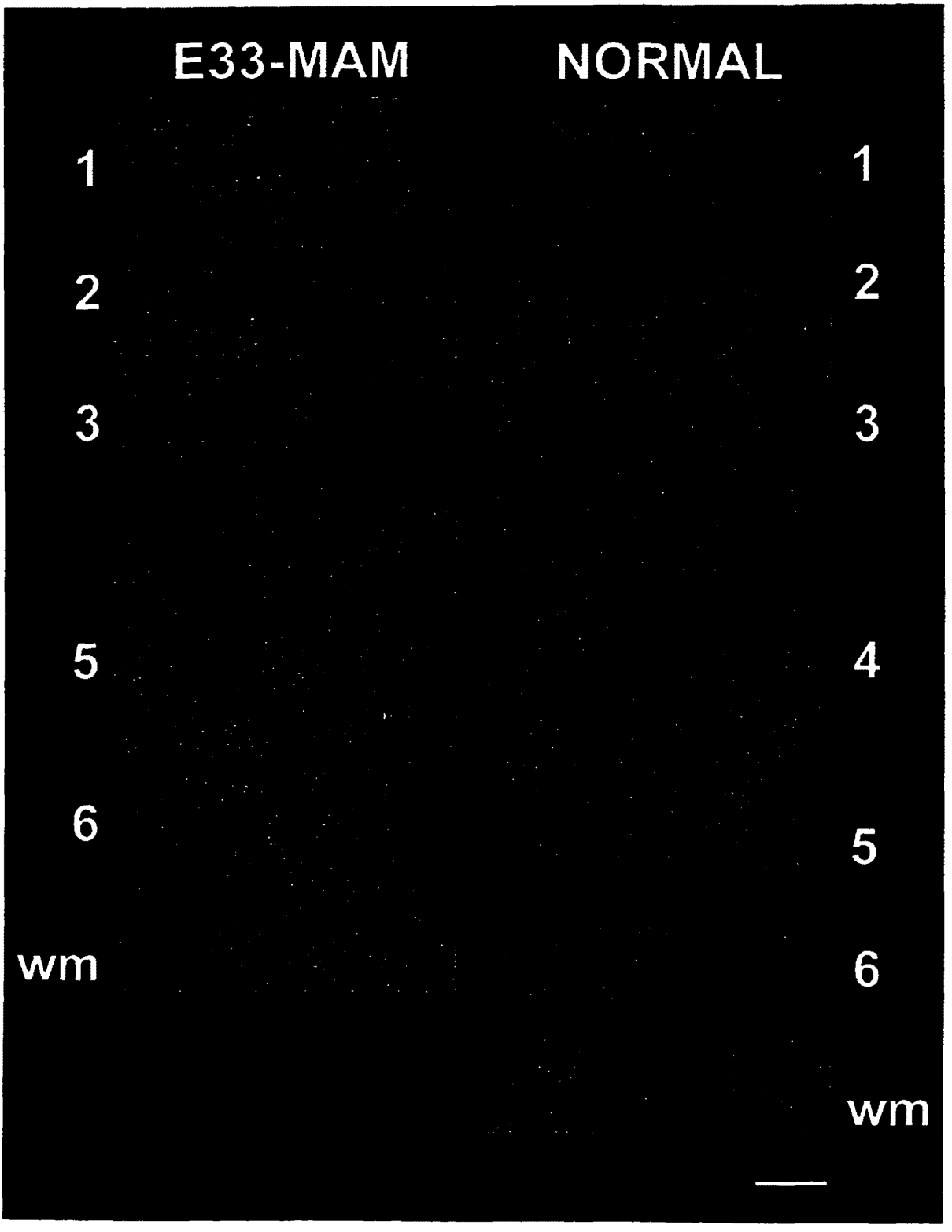
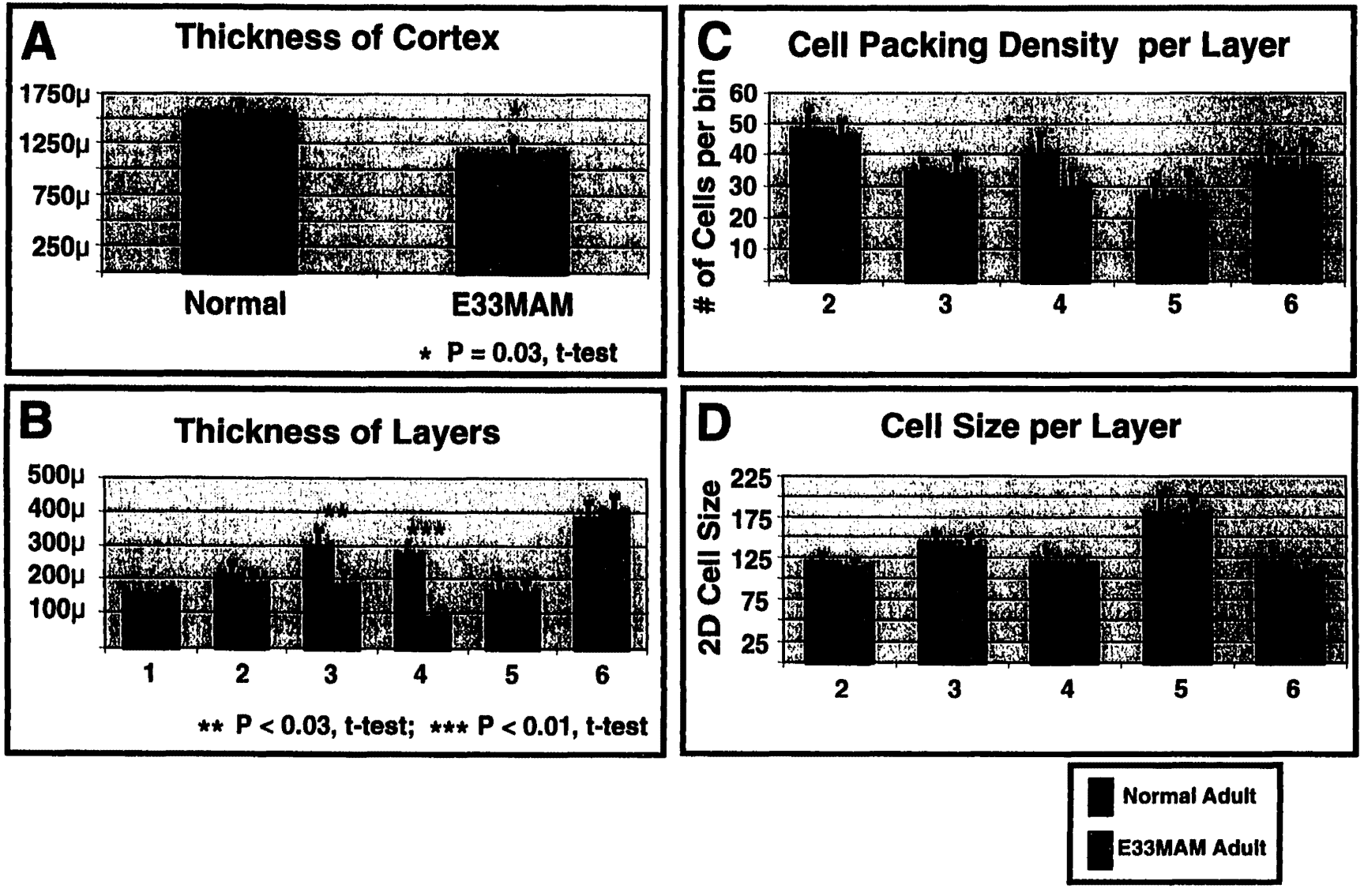

E33-MAM
NORMAL
1
2
3
5
6
wm
1
2
3
4
5
6
wm

Figure 21. Histograms comparing (A) cortical thickness, (B) layer thickness, (C) density of cells within boxes in each layer, and (D) cell size between normal (dark grey bars) and E33 MAM-treated (light grey bars) adult ferrets. Presented are average values obtained from 3 animals in each group. A) E33 MAM cortex was significantly thinner than normal cortex (asterisk, $p=0.03$). B) Layer 3 (double asterisk, $p<0.03$), and 4 (triple asterisk, $p<0.01$) were also significantly thinner in the MAM-treated animals compared to normals. C) Cell density was slightly lower in layer 4 but this difference was not significant ($p=0.07$, t-test). D) Cell size did not differ in any layer in the MAM-treated ferrets compared to normals. Error bars represent the standard deviation.

Effects of E33MAM Treatment



The number of neurons were counted within boxes in each layer to determine if MAM affected the packing density of remaining cells in each layer of adult neocortex. Figure 21C compares the number of neurons per box between normal and E33 MAM-treated ferrets. The density of neurons in layers 2, 3, 5, and 6 were not affected by the MAM treatment. Although the density of neurons within boxes in layer 4 was reduced, this difference was not significant ($p<0.07$, t-test).

The 2 dimensional surface area of all cells containing nucleoli was measured in each box to test if E33 MAM treatment altered the size of cells in each cortical layer. Cell surface area did not differ in any layer between the E33 MAM-treated and normal adult ferrets (Figure 21D).

Discussion

Summary and Use of MAM

Results from the current experiments indicate that methylazoxymethanol treatment specifically disrupts the generation of different populations of neocortical cells, after which corticogenesis resumes. In these experiments the thickness of layer 4 was reduced by nearly 70%, yet somatosensory cortex retained much of its characteristic appearance. Ferrets were treated with BrdU on varying dates after the MAM treatments, and the presence of BrdU positive neurons in appropriate layers after MAM treatment confirmed that generation and migration of neurons resumed after the MAM treatment. Furthermore, the neurons generated after MAM treatment were appropriate in morphology, size and density. This result is supported by the finding of Cattaneo et al. (1995) that

MAM transiently blocks neuroepithelial cell proliferation but does not interfere with cell survival. Cattaneo et al. (1995) also reported that MAM halts cell division in neuronal cell cultures, but does not affect morphology or generation of glial cell cultures.

MAM was first purified from the seeds of cycad plants, a staple in the diet on some South Pacific islands. It was later discovered that the seeds have toxic neurological effects when not properly prepared before ingestion. Since then, studies determined that MAM prohibits synthesis of DNA in dividing cells by methylating the 7 prime position of guanine in DNA and RNA forming 7-methyl-guanine (Matsumoto, 1966). The methylation of DNA and RNA inhibits the action of DNA and RNA polymerases resulting in decreased nucleic acid synthesis (Zedeck at al., 1970). The decrease in nucleic acid synthesis is accompanied by a drop in protein synthesis (Lundeen et al., 1971). Evans and Jenkins (1976) determined that the decrease in DNA and protein synthesis is followed by a decrease in cell division, which recovers within 24 hours. The short-term effect MAM has on cell division makes it possible to prevent specific populations of neurons from being generated through a single administration of MAM on an appropriate date.

Methylazoxymethanol has been used successfully by other groups to interfere with cortical development. These studies also disrupted development of layer 4, but using rats and hamsters, species in which cortical development is compressed into a relatively short period of time. Jones and colleagues found that MAM treatment timed to disrupt layer 4 generation in rats also drastically

reduced formation of the supragranular layers (Jones et al, 1981). The MAM treatment did not prevent the surviving layers from establishing normal efferent connections, nor did it prevent thalamic afferents from colonizing cortex and forming somatotopic maps (Floeter and Jones, 1984, 1985; Yurkewicz et al., 1984). Woo and colleagues found that MAM treatment in hamsters effectively eliminates layer 4 neurons from visual cortex and that layer 4 cells may be necessary for the survival of thalamic neurons projecting to the area with a disrupted layer 4 (Woo et al., 1996, Woo and Finlay, 1996). The development of cortex in ferrets occurs over a period of three weeks. In somatosensory cortex, with a few exceptions, each layer is born over a period of approximately 3 days. As a result, it was possible to focus MAM treatment to maximally interfere with the generation of a specific layer. In the rat and hamster models, several layers are born on a particular day of corticogenesis reducing the ability to focus on interrupting a particular layer. The current studies disrupted the generation of layer 4 neurons in the somatosensory cortex, while minimizing damage to the later-born supragranular neurons.

Specificity of MAM Treatment

This study examined the specificity of E33 MAM treatment to target the development of discrete populations of neocortical cells. Methylazoxymethanol remains active for a limited period of time. Ongoing studies in our lab indicate that the effects of MAM begin to diminish by 8 hours. Matsumoto et al. (1972) report that the effects of MAM last 24 hours in the ventricular zone of rats. Ferrets were treated with MAM on embryonic day 33 (E33) when layer 4 neurons

undergo final mitosis. At this time normal laminar development is immature and distinct layers cannot be identified (see Figure 17). Further laminar maturation occurring after E33 MAM treatment builds on the immature framework present on E33. After the E33 MAM treatment, cortical neogenesis resumed, cortical laminae were established, and somatosensory cortex acquired recognizable cytoarchitectural characteristics, despite the absence of a large percentage of layer four in somatosensory cortex. Layer 3 was also significantly thinner after the E33 MAM treatment. This is not surprising since a percentage of layer 3 precursor cells may have been affected by the MAM, presumably because they were mitotically active at the time of the treatment. Layer 2 was slightly thinner but this difference was not significant probably because a small number of layer 2 precursor cells were affected by the MAM treatment.

Given that after E33 MAM treatment the overall cortical structure and laminar pattern is similar to that in normal somatosensory cortex, what effect does the absence of layer 4 have on subsequent neocortical development and processing of stimuli? To address this question, ongoing studies in our lab are examining the effects of E33 MAM treatment on the formation of connections between thalamus and cortex in the ferret. Studies of both *in vivo* and *in vitro* projections from the thalamus to MAM-treated somatosensory cortex demonstrate wide spread terminations in the remaining layers (Palmer et al., 1996, 1997). In addition, receptor binding studies indicate that after E33 MAM treatment, several ligands retain their specific laminar pattern, including those specific for glutamate receptor subtypes (i.e. NMDA, kainate, and AMPA). The receptor binding

distribution for $GABA_A$ receptors, however, differed in the MAM-treated condition, compared to normal. In normal animals $GABA_A$ receptors showed a peak in layer 4, while after E33 MAM treatment, $GABA_A$ receptor binding showed no focal peak in a specific layer, but was distributed relatively equally in all layers, with a slight increase in layer 3 (Palmer et al., 1998). Expression of $GABA_A$ receptors appear to depend on thalamic innervation and thus likely represents the distribution of thalamic afferents after E33 MAM treatment.

CHAPTER 3. TRANSPLANTATION OF NORMAL VENTRICULAR ZONE CELLS INTO ORGANOTYPIC SLICE CULTURES PREPARED FROM E33 MAM-TREATED CORTEX.

Introduction

After E33 MAM treatment, the overall laminar pattern of ferret somatosensory cortex was maintained. Supragranular neurons migrated to appropriate locations, indicating that appropriate signals and mechanisms for neuronal migration were still intact after the E33 MAM treatment. To determine the potential for repair after E33 MAM treatment, cortical precursors obtained from the ventricular zone of normal ferrets were transplanted into organotypic cortical slice cultures prepared from normal and E33 MAM-treated neonates.

Cortical precursor cells migrate to both appropriate and inappropriate locations when transplanted into normal ferret visual cortex (McConnell 1985, 1988; McConnell and Kaznowski, 1991). Precursors destined for deep layers of cortex maintain their fate after transplantation, unless the cells go through s-phase of their last cell cycle in the ventricular zone of host animal, in which case the precursors adopt the fate of their host cohorts and migrate to superficial layers of cortex. The findings of McConnell and colleagues indicate that environmental factors within the ventricular zone alter laminar fate of cortical precursors at specific times of the cell cycle. Bohner et al. (1997) determined that precursor cells respond to short-range cues that may be present within the ventricular zone. If the precursor cells undergo their final cell cycle in the presence of their donor cohorts they maintained their laminar fate. Frantz and McConnell (1996) also found that the laminar fate of cortical precursors is

progressively restricted during cortical development therefore early generated cells can adopt the superficial fate of later generated cells, but not vice versa. The current set of experiments will test if transplanted cortical cells can replace the missing layer 4 cells in organotypic slice cultures prepared from E33 MAM-treated neonates.

Materials and Methods

Pregnant ferrets obtained from Marshall Farms (New Rose, New York) were treated with single injections of methylazoxymethanol acetate (MAM) on embryonic day 33 (E33) to disrupt development of layer four in primary somatosensory cortex. On the day of birth (E41), static interface organotypic slice cultures were prepared from the somatosensory cortex of MAM-treated and same-age normal ferret kits. After one day in culture, normal and MAM-treated slices were injected with cell suspensions of fluorescently-labeled cortical VZ cells obtained from the ventricular zone (VZ) of normal fetuses at different stages of development: embryonic day 33 (E33), postnatal day 1 (P1), or postnatal day 3 (P3). The E33 cells were chosen to match the population of cells disrupted by the E33 MAM treatment, P1 cells were chosen to match the age of the slices at the time of transplantation, and the population of cells normally migrating at that date. P3 cells were chosen as controls, since at this date neurogenesis is nearly complete in somatosensory cortex. The organotypic slices remained in culture from 3 to 18 days, allowing the transplanted VZ cells sufficient time to complete migration away from the injection site. The distance that transplanted cells traveled from the injection site was analyzed.

MAM Injections

Five pregnant ferrets (Marshall Farms, New Rose, NY) were treated with single injections of MAM on E33. Pregnant jills were anesthetized with 5% halothane and 0.5% N_2O and injected IP with 12mg/kg of MAM (Sigma) dissolved in sterile saline. The pregnant ferrets were closely monitored after injections to ensure proper health.

Organotypic Slice Preparation

On the day of birth, 12 E33 MAM-treated and 13 normal kits were anesthetized with pentobarbital sodium (50mg/Kg, IP) and when insensitive to pain, the brains were removed. All procedures were carried out in a sterile hood (AirTech, Anaheim, CA). Coronal sections 400 um thick were made from somatosensory cortex with a tissue chopper (Stoelting, Wood Dale, Ill), and the sections were placed on Falcon cell inserts in Corning culture trays. Slices were fed Minimum Essential Medium (GIBCO) with 25mM sodium bicarbonate (Sigma), 10% heat-inactivated horse serum (GIBCO), 10 µg/ml gentamycin (GIBCO), 2mM glutamine (GIBCO), 0.6% glucose (Sigma), and 0.00005% phenol red (GIBCO). The cultures trays were kept in a humidified incubator at 37°C with 95% O_2 and 5% CO_2.

Cell Suspension Preparation and Labeling

After one day in culture, suspensions of ventricular zone cells were injected into the slices. Cell suspensions were prepared from 29 ferret embryos at E33, 8 neonates at postnatal day 1 (P1), and 4 neonates on postnatal day 3 (P3). For preparation of ventricular zone cells from ferret embryos, five normal pregnant

jills were anesthetized with 2-3% isoflurane and 0.5% N_2O, and the embryos removed by caesarian section under sterile conditions. Jills were administered bupenorphine, 0.5 mg/Kg subcutaneous, and closely monitored after the operation to ensure proper recovery. The embryonic brains were removed and placed in chilled EBSS in the sterile hood where the remainder of procedures were carried out. For preparation of VZ cells from neonates, kits were anesthetized with Pentobarbital sodium as above, the brains were removed and placed in chilled EBSS. Coronal sections 400 µm thick were made from somatosensory cortex with a tissue chopper. The ventricular zone of neonatal and fetal brains was isolated from cortex under a dissecting microscope and placed in a petri dish with chilled EBSS. The pieces were minced with forceps and placed in 15 ml tube containing 5 ml EBSS with 1 mM L-cysteine, 0.5mM EDTA, 0.01%DNase (Boerhinger Mannheim), and 20U/ml papain (Worthington). Following 45 minutes on a shaker platform at 37°C, the tissue was triturated with a 10 ml pipet. Cells were spun down on setting #3 of a clinical centrifuge for 5 minutes, the supernatant aspirated and removed. Cells were resuspended in 2.5-3.0 ml EBSS with 1 mg/ml albumin and 1 mg/ml trypsin inhibitor from chicken egg white with 0.01% DNase. The suspension was triturated with fire-polished pipettes, and the supernatant layered over 5 ml EBSS with 10 mg/ml albumin and 10 mg/ml trypsin inhibitor from chicken egg white in a 15 ml centrifuge tube. Cells were spun down on setting #2 of a clinical centrifuge for 7 minutes, the pellet resuspended in culture medium, and the cells assayed for viability with a solution of acridine orange and ethidium bromide. Cell suspensions were

labeled with red or green microspheres (Molecular Probes), which were sterilized for 15 minutes at 70°C. The microspheres were diluted 1:100 in 1 ml calcium and magnesium free PBS, pH 7.4, with 0.6% glucose and 10% fetal bovine serum. The cell suspension was incubated with microspheres for 30 minutes at 37°C, gently mixing the vial every five minutes. After incubation with microspheres, the cell suspension was washed three times by centrifugation at 1000 rpm for 10 minutes, aspirating the supernatant and resuspending the pellet in incubation media. After the final spin, the pellet was resuspended in 10 µl organotypic culture media per brain. The cell suspensions were injected through glass pipettes 80-100 µm in diameter with an Eppendorf Microinjector. Injections were guided into the ventricular zone and white matter of the slices with a Leitz manipulator under a dissecting microscope.

Additional control injections of appropriate dilutions of microspheres in the culture media were placed into 4 of the slices to ensure that extraneous microspheres were not transported in the slices.

Labeling Slices with Dextran Injections

Six slices were injected with fluorescent-tagged dextrans to assess cortical structures in the organotypic cultures. Previous experiments in this lab demonstrated that dextran injections placed into acute slices of ferret somatosensory cortex taken from neonates specifically label radial glia and migrating neurons in the immature cortex (Juliano et al., 1996). Cultured slices to be injected were placed in a tissue chamber and perfused continuously with oxygenated aCSF (O_2:CO_2;95:5). Injections were guided with a surgical

microscope into the ventricular zone and white matter of each slice, and delivered Fluorescein dextran or Fluororuby (Molecular Probes) through glass pipettes 10-15 μm in diameter using iontophoresis for 3-4 minutes (3 μ amp, positive alternating current). Cultured slices remained in the tissue chamber 5-8 hours after the injections. The cultured slices were removed, placed in fixative (4% buffered paraformaldehyde with 10% sucrose) overnight and transferred to the same fixative with 20% sucrose for cryoprotection. After cutting at 40 μm on a freezing microtome the cultures were counterstained with bisbenzimide (0.0025% in 0.1M phosphate buffer) to reveal cytoarchitecture, and examined on a fluorescent microscope.

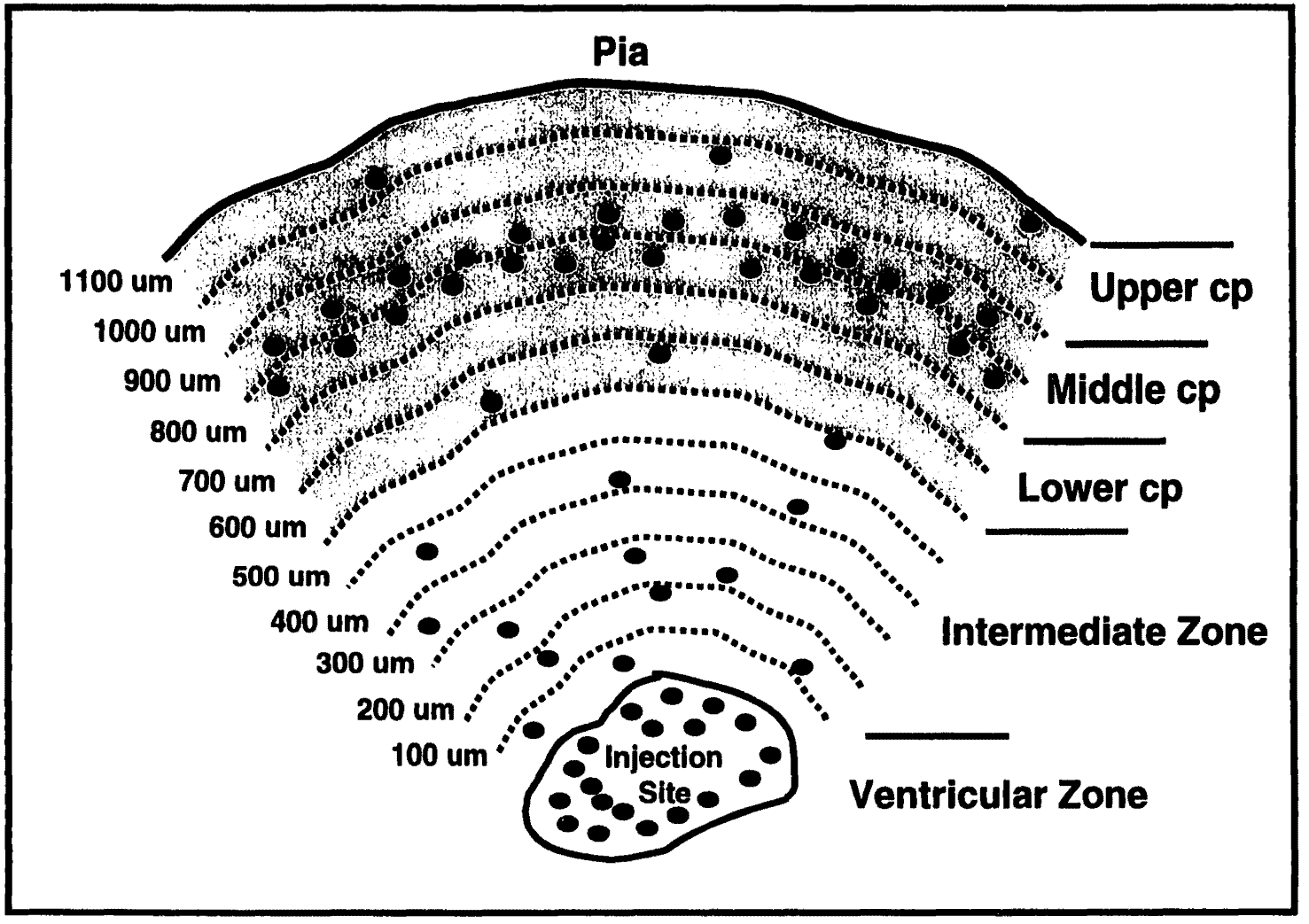
Immunohistochemistry

Several cultured slices from each treatment group were immunostained with MAP2 antibodies to determine phenotype of the transplanted cells. The organotypic cultures were removed and placed in fixative (4% paraformaldehyde in 0.1M phosphate buffer) for 30 minutes, and antibodies against MAP2 (Boehringer Mannheim, anti-MAP2) were applied to the sections after blocking with 10% normal horse serum, 0.2% gelatin plus 0.1% triton X-100. After a 12 hour incubation with the primary antibody (diluted 1:100 with 2% normal horse serum and 0.1% triton X), the sections were rinsed with 0.1 M PBS pH 7.4, and incubated in the secondary antibody (Vector Vectastain Elite ABC kit) for 1 hour. At this point, the resulting label was visualized using a Vector VIP peroxidase substrate kit. Control sections received no primary antibody.

Transplantation Analysis

The distance that transplanted cells traveled from the injection sites in each organotypic culture was measured using the Image Pro Plus® program (Media Cybernetics, Silver Spring, Md). Sections were counterstained with bisbenzimide (0.0025% in 0.1M phosphate buffer) to reveal cytoarchitecture. Whole slice cultures or sections cut at 40 µm on a freezing microtome were digitally imaged either with a confocal microscope (Bio-Rad MRC-600), or using a conventional fluorescent microscope. Image montages were constructed, and labeled cells identified with Adobe Photoshop®. In each cultured slice, injection sites were identified, and a grid of concentric bins 100 µm deep radiating out from the injection site to the top of the cortical plate was constructed in the Image Pro Plus® program. The bins encompassed all transplanted cells (Figure 22). The number of labeled cells were counted within the injection site, and in each of the 100 µm deep bins extending from the injection site to the pial surface. Since different numbers of cells migrated away from each injection site, non-parametric statistical analyses compared the percent of VZ cells that migrated into the cortical plate of the cultures from each injection site. The number of cells that migrated away from the injection sites was set to 100 percent. In addition, the relative position of VZ cells within the cortical plate (upper, middle, or lower cortical plate) was compared between groups. The upper cortical plate included bins within 200 µm from the pial surface. The middle cortical plate included bins within 200 to 400 µm from the pial surface, and the lower cortical plate included those bins within 400 to 600 µm from the pial surface (see Figure 22). For the

Figure 22. Illustration depicting analysis of the location of microsphere-labeled cells within the organotypic cultures. The injection site was identified, and concentric 100 μm deep bins were constructed extending from the injection to the pial surface. Only cells that migrated away from the injection site were included for analysis, this number was set to 100 percent. The number of cells located in the cortical plate was expressed as a percent of migrating cells. The percent of transplanted cells that migrated into the cortical plate was compared between groups. In addition, the relative position of transplanted cells (upper, middle, and lower cortical plate) was compared across groups.


Pia
1100 um
1000 um
900 um
800 um
700 um
600 um
500 um
400 um
300 um
200 um
100 um
Upper cp
Middle cp
Lower cp
Intermediate Zone
Injection Site
Ventricular Zone

first set of statistical analyses, the percent of E33, P1, and P3 VZ cells that migrated into the cortical plate of the organotypic cultures (normal and MAM-treated cultures combined) was compared across groups. For the second set of statistical analyses, the percent of each type of VZ cell (E33, P1, or P3) that reached the cortical plate was compared between normal and MAM-treated organotypic cultures. Results of the non-parametric statistical tests are included in Appendix B.

Results

Labeling cell suspensions with microspheres.

Microsphere-labeled cell suspensions obtained from the ventricular zone of normal ferret embryos and neonates were transplanted into organotypic slice cultures prepared from normal and E33 MAM-treated ferret neonates. The cell suspensions were taken from ferrets at three stages of development. E33 cells were chosen to match the age of the population of cells disrupted by the MAM treatment. P1 cells were chosen to match the age of the slice cultures, and P3 cells were chosen as controls since neurogenesis is complete in somatosensory cortex at this time.

Fluorescent microspheres are not cytotoxic, show little diffusion, and persist *in vivo* for up to 10 weeks (Katz et al., 1984). These properties make the microspheres suitable for transplantation experiments. After labeling cell suspensions with fluorescent microspheres, individual microspheres were readily visible within the cell body after 18 days in culture (Figure 23). The microspheres did not appear to affect the viability of the transplanted cells, were found in cells

that migrated into the cortical plate (Figure 24), and in cells that achieved mature neuronal phenotype (Figure 25).

Phenotyping transplanted cells with anti-MAP2 antibodies.

To confirm that the transplanted cell suspensions contained neurons, organotypic cultures were immunostained with antibodies directed against MAP2, which recognizes mature neurons in neonatal cortex (Chun and Shatz, 1989). The cultures were examined to determine if MAP2 immunoreactive cells contained microspheres. Sixty-nine percent of the transplanted cells located in the cortical plate of examined slices (5) were positively identified as MAP2 immunoreactive (Figure 25). Phenotypic identity of the remaining microsphere-labeled cells was not confirmed. The MAP2-labeled cells containing microspheres were found up to 1000 μm from injection sites, indicating that transplants were able to migrate significant distances in the slice cultures.

Dextran Injections.

Several organotypic cultures were injected with fluorescent dextrans after 2, 4, or 6 days in culture (DIC) to determine if radial glia maintained their proper orientation and morphology. Previous experiments in this lab demonstrated that injections of fluorescent dextrans into acute slices of neonatal cortex predominantly label radial glial cell fibers (Juliano et al., 1996). An example of a dextran injection into a culture after 6 DIC labeled radial fibers emanating from the ventricular zone reaching toward the pial surface (Figure 26A). Images taken in the same field with a fluorescein filter revealed microsphere labeled cells in close apposition to the dextran-labeled radial fibers (Figure 26B). The radial fibers form a potential substrate for migration of the transplanted cells.

Figure 23. Digitized image demonstrating transplanted cells labeled with fluorescein microspheres in an organotypic culture. The microspheres remained within the cells for the duration of the experiment and were easily identified under fluorescent microscopy. Scale bar=50 μm.

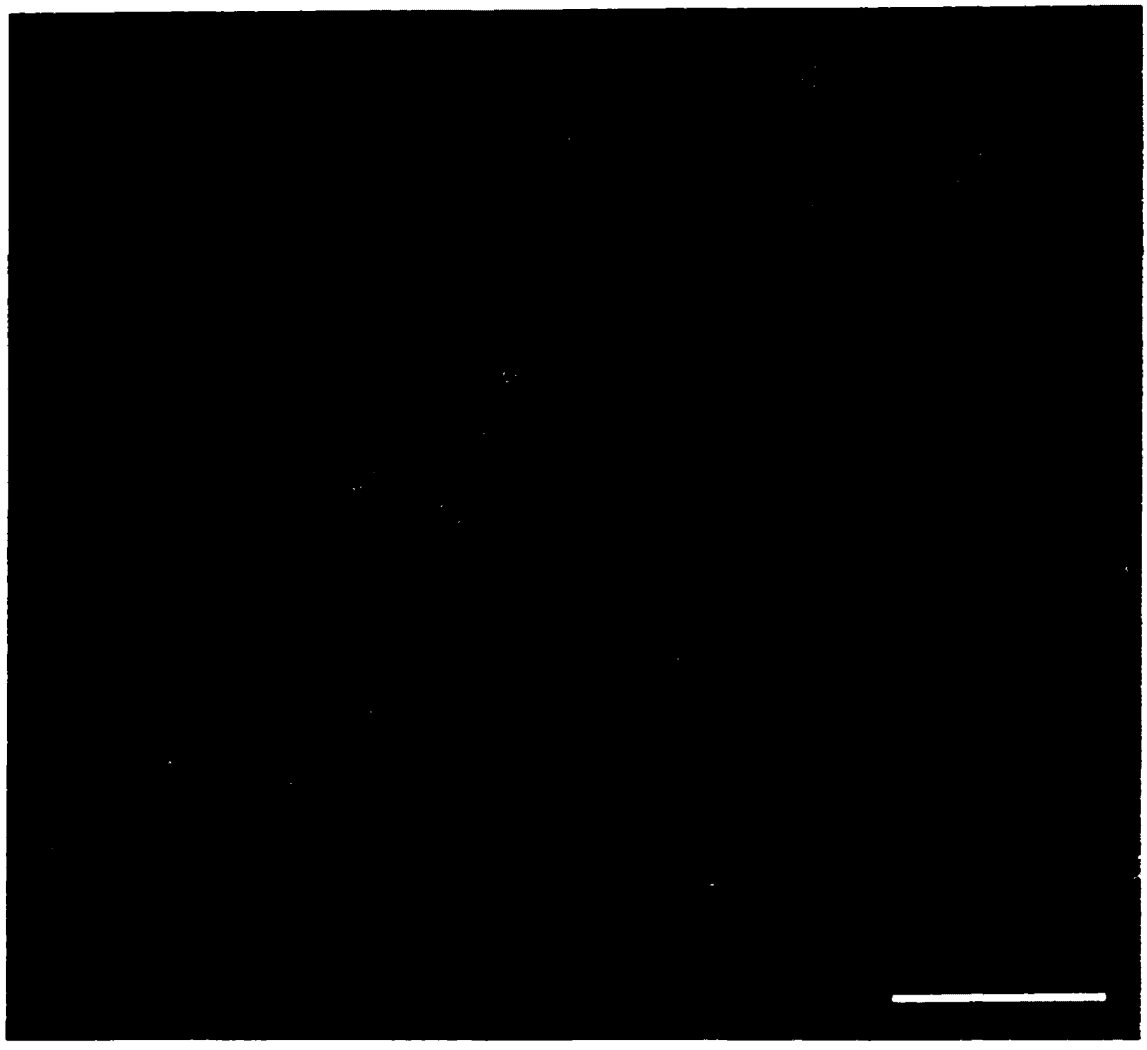


Figure 24. Photomicrograph demonstrating microsphere-labeled cells transplanted into an organotypic slice. The cells were taken from normal ferret embryos at embryonic day 33. Two injections were placed in the ventricular zone of this slice (large arrows). Labeled cells migrated away from the injection sites and can be seen in the cortical plate. VZ, ventricular zone. Scale bar=500 μm.

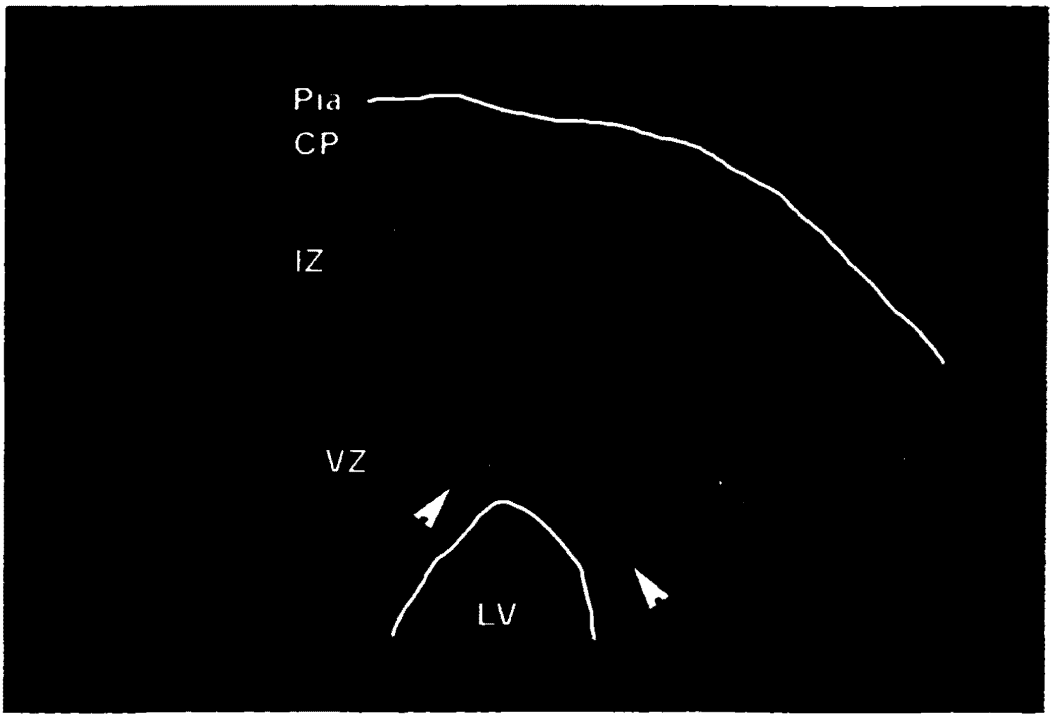

Pia
CP
IZ
VZ
LV

Figure 25. Digitized image of an organotypic culture immunostained with anti-MAP2 antibodies. MAP2-IR was co-localized with microspheres in cells found in the cortical plate more than 1000 µm from the injection site. The inset at the left is a higher power magnification of a MAP2-positive cell containing microspheres (arrows). MZ, marginal zone; CP, cortical plate. Scale bar=100 µm.


mz
cp

Characterizing Cell Suspension Transplants.

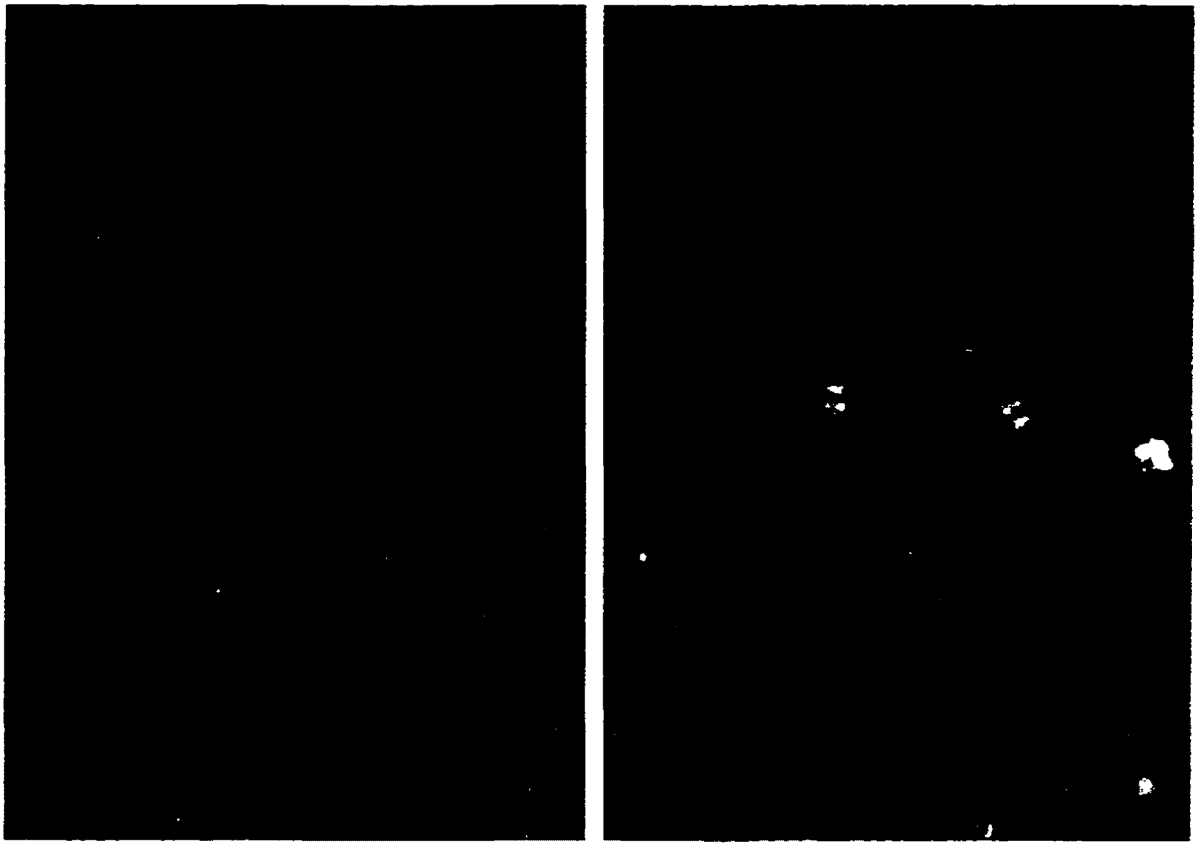
Discrete injections of cell suspensions were placed in the ventricular zone and in deep portions of the intermediate zone within each culture. The total number of injected cells were counted in each culture. The number of labeled cells identified per injection averaged 995 cells; nearly 25% of the transplanted cells migrated away from the injection sites. Of the cells that migrated, an overwhelming majority were directed toward the pial surface.

To test if free microspheres could be transported independently in the slice cultures, control injections of fluorescent microspheres alone were placed into 4 of the slices. None of the control injected microspheres moved away from the injection sites, indicating that residual free microspheres were not transported from the injection sites.

The organotypic cultures were removed from the incubator every other day after the transplantation, up to a maximum of 18 days in culture. No differences were found in the migration or other properties in slices that remained in culture for different numbers of days, so length of time in culture was not a factor in analyzing results.

Since different numbers of VZ cells migrated away from each injection site, analysis compared the percent of VZ cells that migrated into the cortical plate of the cultures and analyzed the relative position of the VZ cells within the cortical plate. Two sets of analysis were conducted. First, the percent of transplanted cells that migrated away from the site of transplantation into the cortical plate

Figure 26. Digitized images taken through the same field of an organotypic culture 3 days *in vitro*, demonstrating (A) presumptive radial glial fibers labeled after injections of Fluororuby (rhodamine filter). (B) in the same field, microspheres inside transplanted cells are seen in the same location as the labeled fibers (fluorescein filter). Radial glial cells may provide the transplanted cells a structure for migrating away from the injection sites in the organotypic cultures. Pial surface is toward the top. Scale bar= 50 μm.



was compared across the type of cell-suspension transplant (i.e. E33, P1, or P3 VZ cells) irrespective of culture type (i.e. slice cultures prepared from normal or E33 MAM-treated animals). This was designed to determine if differences existed in the ability of any of the 3 cell types to migrate. Second, I assessed the ability of each type of cell suspension (i.e. E33, P1, or P3) to migrate in different hosts (normal vs. E33 MAM-treated cultures). In each group the position of the transplanted cells within the cortical plate of the cultured slices was compared. This was designed to test potential differences in normal versus E33 MAM-treated organotypic cultures as hosts.

Comparing Migration of E33, P1, and P3 Ventricular Zone cells across groups

Figure 27 identifies the percent of each cell type that migrated into the cortical plate of the organotypic cultures. Very few of the P3 ventricular zone (VZ) cells migrated into the cortical plate. The few cells found in the cortical plate were located in the deepest portion, none were in the middle or upper portions of the cortical plate. Therefore 100 percent of P3 cells that had migrated into the cortical plate were located in the lower region. Since such a small number of P3 cells actually migrated into the cortical plate, statistical comparisons were not made between P3 and P1, or E33 VZ cells. P1 VZ cells (light grey bars) were found in each of the 3 regions of the cortical plate. However a larger percentage of P1 cells migrated to the upper regions of the cortical plate when compared to E33 VZ cells ($p<0.05$, Mann-Whitney test). The E33 VZ cells (dark grey bars) also to each region of the cortical plate, but a larger percentage of E33 cells were found in the middle portion of the cortical plate when compared to the P1 VZ cells ($p<0.01$, Mann-Whitney test).

Migration of E33 Ventricular Zone Cells in Normal versus E33 MAM-treated Cultures

The transplanted E33 cells migrated comparable distances (a maximum over 1000 μm) from injection sites in organotypic cultures prepared from both normal and E33 MAM-treated ferrets. Within the cortical plate, differences emerged when comparing results from injections into normal and E33 MAM-treated cultures. Figure 28 compares the distribution of E33 cells transplanted cells in the cortical plate of organotypic cultures. A larger percentage of E33 VZ cells migrated into the cortical plate of MAM-treated cultures when compared to E33 cells injected into normal slices, however this difference was not significant ($p=0.12$, Mann-Whitney test). Further subdividing the cortical plate into upper, middle, and lower sections revealed that a larger percentage of E33 VZ cells migrated to the middle portion of the cortical plate in the MAM-treated cultures compared to E33 cells transplanted into normal hosts ($p<0.05$, Mann-Whitney test). There was not a significant difference in the percent of E33 VZ cells residing in the uppermost, or deepest portions of the cortical plate between the normal and MAM-treated cultures.

Migration of P1 Ventricular Zone Cells in Normal versus E33 MAM-treated Cultures

The maximum distance that P1 VZ cells migrated from the injection sites was slightly farther in the E33 MAM-treated cultures compared to the normals, but this difference was not significant (t-test). Figure 29 compares the distributions of transplanted P1 VZ cells in the cortical plate of normal and E33 MAM-treated

organotypic cultures. Similar to the findings after E33 VZ cell transplants, a larger percent of P1 VZ cells were found in the cortical plate of MAM-treated slices, when compared to cultures of normal animals, but this difference was not significant ($p=0.08$, Mann-Whitney test). Further analysis within the cortical plate revealed that a larger percentage of P1 VZ cells had migrated to the most superficial portion of the cortical plate of MAM-treated cultures compared to P1 VZ cells transplanted into normal hosts ($p<0.01$, Mann-Whitney test). Significant differences did not emerge between normal and E33 MAM-treated organotypic cultures in the percent of labeled cells in the middle, or lower portions of cortical plate.

Migration of P3 Ventricular Zone Cells in Normal versus E33 MAM-treated Cultures

Very few P3 VZ cells migrated into the cortical plate. The vast majority of P3 cells that moved away from the injection site stopped in the white matter, and the distribution did not differ between normal and MAM-treated slice cultures.

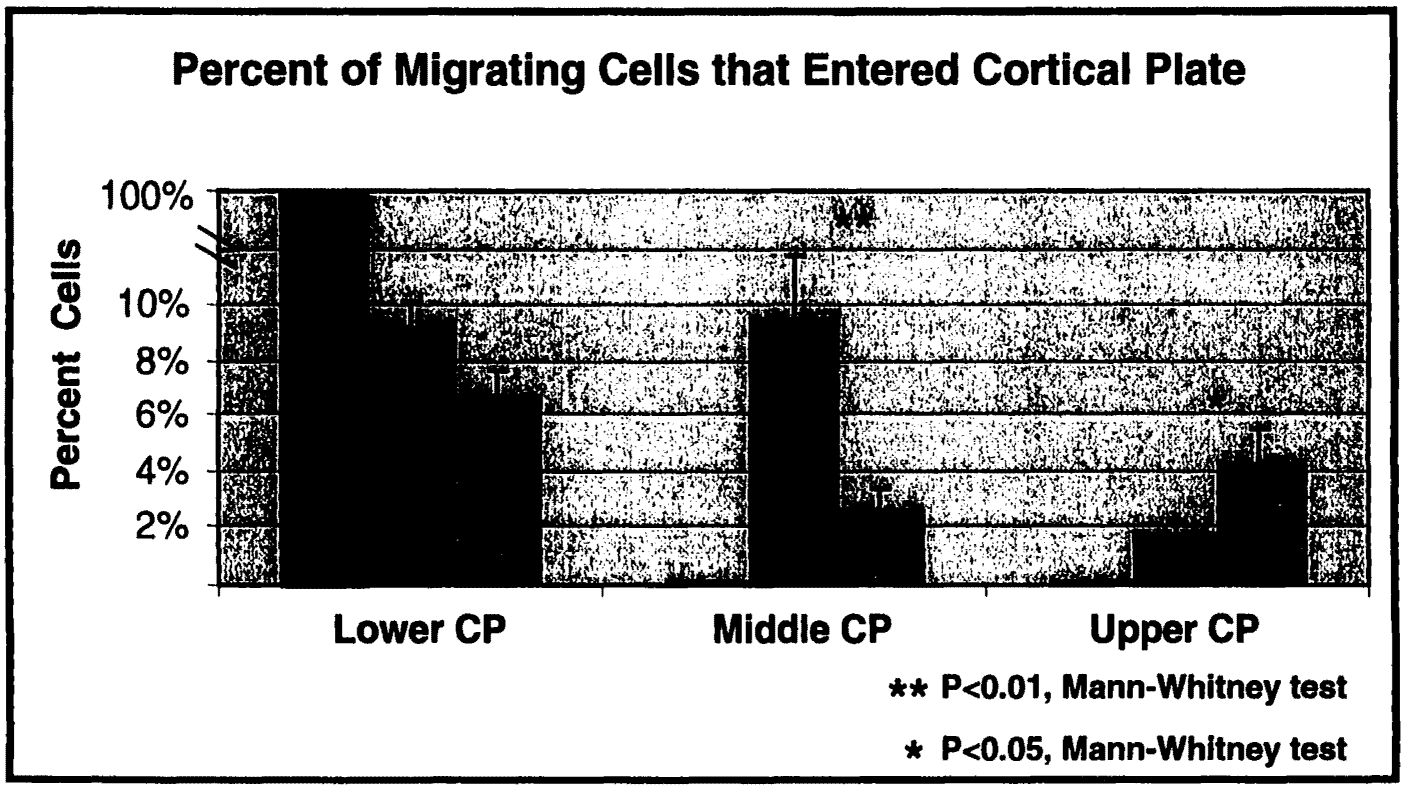
Discussion

In organotypic slice cultures prepared from E33 MAM-treated ferrets, the E33 VZ cells migrated to an appropriate location within the cortical plate both for their age and to the missing layer. The P1 VZ cells also migrated to an appropriate position for their age, but did not change their laminar fate to populate the missing cells within the mid cortical plate.

MAM-treated cultures provided a better substrate for migration of the transplanted cells. A smaller percent of both E33 and P1 VZ cell suspensions

Figure 27. Histogram comparing percent of each type of transplanted cell (E33, P1, or P3) that migrated into the cortical plate of the organotypic cultures, irrespective of slice type (i.e. normal or E33 MAM-treated). Bars represent the percent of each type of transplanted cell that migrated from the site of transplantation into the cortical plate. Bars on the left represent transplanted cells that migrated to the lower portion of the cortical plate, bars in the middle represent cells that migrated to the middle portion of the cortical plate, bars on the right represent the percent of cells that migrated into the most superficial portions of the cortical plate. Dark grey bars represent E33 cells, light grey bars represent P1 cells, black bars represent P3 cells. A larger percent of E33 VZ cells migrated to the middle portion of the cortical plate compared to P1 VZ cells ($p<0.01$, Mann-Whitney test). A larger percent of P1 VZ cells migrated to more superficial locations in the cortical plate of the cultures compared to the E33 VZ cells ($p<0.05$, Mann Whitney test). Very few P3 cells migrated into the cortical plate. VZ, ventricular zone. Error bars represent the standard deviation.

E33 vs. P1 vs. P3 VZ Cells



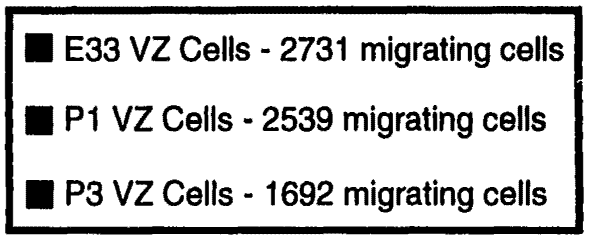
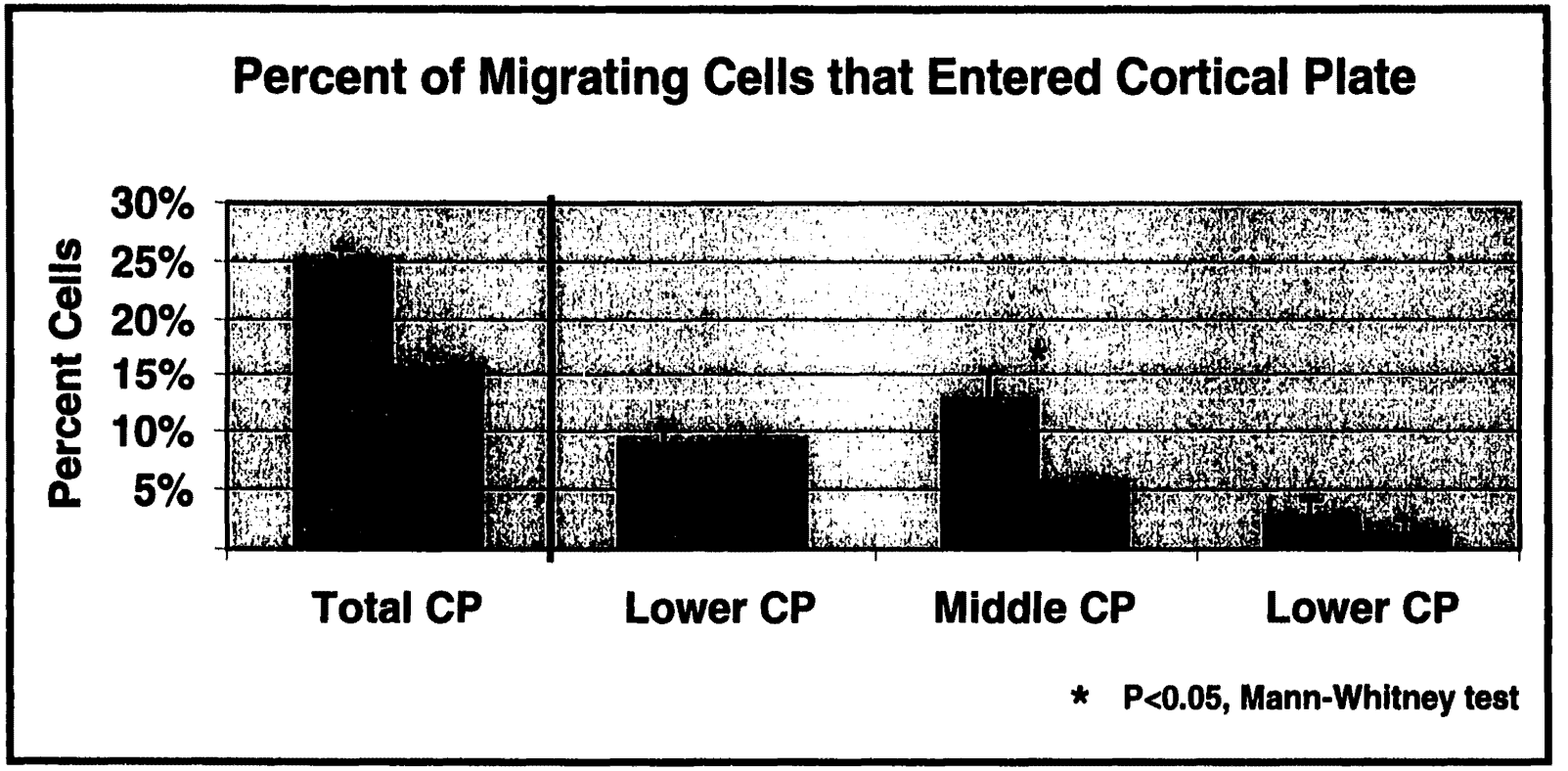


Figure 28. Histogram comparing the percent of E33 VZ cells that migrated into the cortical plate in normal and E33 MAM-treated organotypic cultures. Bars represent the percent of migrating cells that were found in the cortical plate. A larger percent of E33 VZ cells migrated into the cortical plate of organotypic cultures prepared from E33 MAM-treated animals (light grey bars) but this difference was not significant ($p=0.12$, Mann-Whitney test) compared with E33 VZ cells injected into normal cultures (dark grey bars). On the right, further subdividing the cortical plate into lower, middle, and upper sections revealed that a larger percent of E33 VZ cells migrated to the middle portion of the cortical plate in MAM-treated cultures compared to E33 cells transplanted in normal cultures ($p<0.05$, Mann-Whitney test). There was not a difference in the percent of E33 VZ cells that migrated to the upper or lower portions of the cortical plate in normal versus MAM-treated cultures. CP, cortical plate; VZ, ventricular zone. Error bars represent the standard deviation.

E33 VZ cells Transplanted into Normal versus MAM-Treated Cultures

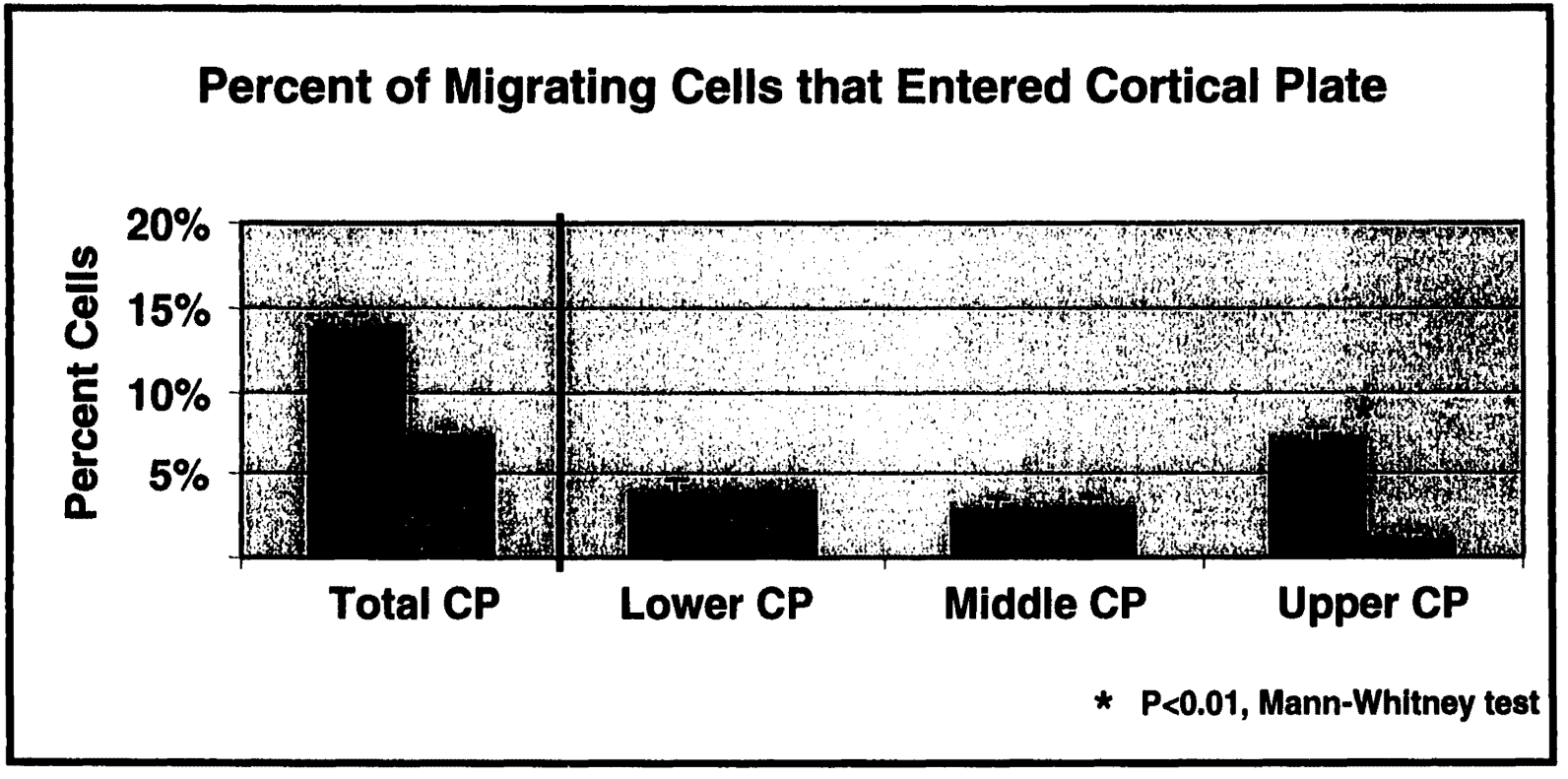


E33 VZ cells transplanted in E33 MAM Cultures

E33 VZ cells transplanted in Normal Cultures

Figure 29. Histogram comparing the percent of transplanted cells prepared from normal P1 ventricular zone that migrated into the cortical plate in normal or E33 MAM-treated organotypic cultures. Bars represent the percent of migrating cells that were found in the cortical plate. A greater percent of P1 cells migrated into the cortical plate of organotypic slices prepared from E33 MAM-treated animals (light grey bars) but this difference was not significant ($p=0.08$, Mann-Whitney test) compared with P1 cells transplanted into normal cultures (dark grey bars). On the right, further subdivision of the cortical plate into upper, middle, and lower sectors revealed that a larger percent of P1 cells migrated to the superficial cortical plate in MAM-treated cultures compared to P1 cells injected in normal cultures ($p<0.01$, Mann-Whitney test). The percent of transplanted cells that migrated to the middle or lower portions of the cortical plate did not differ between normal and MAM-treated cultures. CP, cortical plate; VZ, ventricular zone. Error bars represent the standard deviation.

P1 VZ cells Transplanted into Normal versus MAM-Treated Cultures



migrated into the cortical plate of cultures obtained from normal animals. The E33 MAM-treated cultures provide a better substrate for "replacement" cells, and in these cultures the transplanted VZ cells do not alter their laminar fate, even when presented with a substrate lacking a similar population of cells. McConnell (1988) also found that early generated cortical precursors maintain their laminar fate, and migrate to deep layers of cortex after transplantation into normal neonates. McConnell (1991) later found that cells normally destined for deep layers of cortex alter their laminar fate and migrate to superficial layers of cortex only if the transplanted cells undergo the s-phase of their last cell cycle within the environment of the host ventricular zone. Presumably these early generated cells are multipotential under the right conditions, which include exposure to locally acting environmental factors at a specific time. The findings of McConnell indicate that precursor cells normally destined for deep layers and transplanted quickly after harvesting into a host ventricular zone, may undergo their final cell cycle in their new environment and adopt the fate of their host cohorts, migrating to layers 2/3. In our experiments the precursor cell suspensions were transplanted into the slice cultures approximately 5 hours after harvesting. This closely matches the conditions of the experiments of McConnell and colleagues in which the precursors maintained their original laminar fate when going through the final cell cycle in the presence of their donor cohorts (McConnell and Kaznowski, 1991; Bohner et al., 1997).

McConnell also found that the laminar fate of cortical precursors is progressively restricted during cortical development - early generated cells can

adopt the superficial fate of later generated cells, but not vice versa (Frantz and McConnell, 1996). In our experiments more P1 VZ cells migrated into the cortical plate of the MAM-treated cultures compared to normal cultures, but did not replace the missing layer 4 cells. Instead, they tended to locate in more superficial layers. Whereas MAM-treated cortex may present as a more permissible substrate for migration, the laminar fate of the P1 VZ cells may have been determined already.

It is not entirely clear why the normal organotypic slice cultures did not provide as supportive a substrate for migration. Several studies report that normal, healthy CNS does not provide as strong an environment for transplants of all types. For example, McLoon and McLoon (1984) found that transplanted cells formed more extensive fiber outgrowth into host tissue when they where placed in a lesioned animal compared to cells transplanted in a normal environment. Production of neurotrophic factors increases after certain CNS lesions, which may help ensure the survival of transplanted cells in a lesioned environment (Gage and Bjorklund, 1987). Furthermore, cells transplanted into lesioned CNS may have an advantage over similar cells transplanted in normal structures in that they do not have to compete with host cells for migrational substrates and/or trophic factors. The studies of McConnell and colleagues that were described were all performed *in vivo*, while our *in vitro* conditions, although allowing ease of manipulation, may not have provided as supportive an environment for the transplanted cells.

CHAPTER 4. DISRUPTING FORMATION OF EARLY GENERATED LAYERS OF FERRET SOMATOSENSORY CORTEX WITH METHYLAZOXYMETHANOL

Introduction

The layers generated during early cortical neogenesis may play a key role in further neocortical development. Of the early born layers, the subplate participates in a number of processes that help the cortical architecture to mature. Its neurons extend pioneering axons centrifugally to the thalamus to guide thalamic afferents into the cortex (De Carlos and O'Leary, 1992; Erzurumlu and Jhavari, 1992; McConnell et al., 1989); subplate neurons participate in early functional circuits that link thalamic afferents with the subplate and the subplate with layer 4 (see Allendoerfer and Shatz, 1994 for review). In visual cortex, the subplate also appears to be important in directing thalamic afferents into ocular dominance columns, since deletion of subplate neurons in neonatal cats prevents segregation of geniculocortical afferents into distinct clusters (Ghosh et al., 1990; Ghosh and Shatz, 1992a,b; 1994).

It is not clear whether the early generated layers, including the subplate and deep neocortical layers, are important to subsequent laminar formation and migration of neurons into the cortex. A genetic model of distorted cortical layer formation, the *reeler* mouse, suggests that layers form without certain positional cues, although in a misplaced manner. In *reeler* mice, the preplate, which normally splits and allows the subsequent layers to form between its deepest element (the subplate) and its most superficial element (layer 1), does not divide and the normally occurring inside out pattern of laminar genesis does not take

place (Caviness and Rakic, 1978). Instead, cortical layers build up beneath each other in an upside down fashion and the subplate remains superficial, towards the pia. Even in *reeler* mice, however, the subplate appears to play an important role in cortical development, since axons from the thalamus grow directly into the superficially located subplate and then downward into layer 4, the normal recipient layer from the thalamus (Frost and Caviness, 1980; Caviness and Frost, 1983; Molnar and Blakemore, 1995).

One way to test the impact of early generated cortical layers on later-born layers is to investigate the maturation of cerebral cortex in animals that continue to develop after interfering with the early produced layers. Other studies have evaluated the impact of subplate deletion on subsequent formation of cortical architecture by removing the subplate postnatally, after a substantial portion of cortex has already formed (Ghosh et al., 1990; Ghosh and Shatz, 1992a; 1994). Although this method is highly useful and important in evaluating structures that mature later in cortical development, such as thalamocortical distribution, it does not allow assessment of the impact of the subplate on earlier events. To test the role of early generated layers on subsequent events the development of the subplate and/or deeper cortical layers was disrupted by *in utero* injection of methylazoxymethanol acetate (MAM) into pregnant ferrets on appropriate gestational days. As reported in Chapters 2 and 3, MAM is a toxin that prevents cells from dividing for a short period of time, thereby effectively preventing the birth of a given population of cells that would normally divide at the time of the injection (Matsumoto and Higa, 1966; Matsumoto et al., 1972; Johnston et al.,

1979; Zedeck et al., 1970; Cattabeni and Di Luca, 1997). Using this method can therefore interfere with the formation of a specific layer of neocortex (Johnston et al., 1981; Jones et al., 1982; Virgili et al., 1988; Fasolo et al., 1992). The results presented here indicate that MAM injections early during cortical development result in severely disrupted cortex together with dramatic alteration of radial glial cell morphology, and Cajal-Retzius cell position, in contrast to later injections of MAM, timed to disrupt layer 4 development, which cause more subtle cortical changes and do not produce dramatic alterations in cortical structure or radial glia.

Materials and Methods

Methylazoxymethanol was injected into pregnant ferrets on embryonic day 24 (E24) or 28 (E28), which disrupted the development of the subplate or deep portions of layer 6 (for the E28 injection) of ferret somatosensory cortex. Injections were also made on E33, which interfered with layer 4 development. These dates were decided based on results of birthdating studies using bromodeoxyuridine (BrdU), an analog of thymidine (see Chapter 1, Noctor et al., 1997). Several animals received injections of BrdU 0 to 12 days after MAM injections to determine the viability and migration of cells after MAM treatment.

Live slices were prepared from three groups of animals ranging in age from postnatal day 0-7 (P0-7, day of birth is considered to be P0): (I) normal kits (n=12), (ii) kits whose mothers received MAM treatment on E24 or 28 (early group n=11), and (iii) kits whose mothers received MAM treatment on E33 (late group, n=19). The structure of the somatosensory cortex was studied in slices

using patterns of intracortical connections, analysis of cytoarchitecture, and assessment of the relations between the ventricular zone (VZ) and the neocortex. Additional animals in each group were reacted for immunoreactivity against BrdU, vimentin, glial fibrillary acidic protein (GFAP), or the CR-50 antibody which labels Cajal-Retzius neurons in the marginal zone. Western blots were made using tissue obtained from P0 animals treated with MAM on E24 and from normal P0 kits to test for the presence of GFAP. In one pregnant animal, MAM was injected at E24 and the embryos evaluated at E27 to determine the effect of MAM a short time after the *in utero* injection.

BrdU and MAM Injections.

BrdU injections were made into pregnant ferrets on different embryonic days after MAM injections on E24 or E33. Timed pregnant ferrets were obtained from Marshal Farms (New Rose, NY). The injections were made IP under halothane anesthesia (2%) and delivered 60 mg/kg of BrdU (Sigma) dissolved in saline with 0.007 N NaOH. After the kits were born, BrdU-labeled cells were revealed using standard immunohistochemical techniques (see below). In this set of experiments, kits were examined on P0 or P1, except for several E33 MAM-treated animals that received BrdU injections, which were also assessed at P28 and P42, when the laminar pattern of the somatosensory cortex is histologically mature (Juliano et al., 1996). These animals were assessed to determine the final position of BrdU-positive cells. On the appropriate date, each kit received an IP injection of Pentobarbital Na (50 mg/kg) and when insensitive to pain, was perfused through the heart with saline followed by 4% paraformaldehyde in

0.01 M phosphate buffer. The brains were removed and sunk in 10% and then 20% sucrose in phosphate buffer at 4°C and then frozen in isopentane at –35°C and kept in a freezer at –70°C, until processed as below. In most instances, when the brains were cut, alternate sections were saved for immunohistochemistry, or Nissl staining (see below).

MAM injections were made into pregnant ferrets on appropriate days of gestation. Ferrets were anesthetized with halothane (2%) and the MAM injected IP; each ferret received 12 mg/kg of MAM (Sigma) dissolved in saline. Kits were also examined on P0 or P1 as described above.

Immunohistochemistry.

The brains were cut in a cryostat at –16°C at 30 µm thickness. Monoclonal antibodies against vimentin (Boehringer Mannheim, anti-vimentin, clone V9), MAP2 (Boehringer Mannheim, anti-MAP2), GFAP (Boehringer Mannheim, anti-GFAP), or CR-50 (gift from Dr. Nakajima) were applied to the sections after blocking with 10% normal horse serum, 0.2% gelatin plus 0.1% triton X (Sigma). After 12 hr incubation with the primary antibody (vimentin and GFAP, diluted 1:4; MAP2 and CR-50, diluted 1:100; with 2% normal horse serum and 0.1% triton X), the sections were rinsed with 0.1 M PBS pH 7.4, and incubated in the secondary antibody (Vector, antimouse IgG conjugated to fluorescein or Texas Red at 1:100 in 2% normal horse serum and 0.1% triton X or Vector Vectastain Elite ABC kit) for 2 hr. At this point, the resulting label was visualized with fluorescent microscopy or using a Vector VIP peroxidase substrate kit. Control sections received no primary antibody.

For BrdU immunohistochemistry, sections were treated with 95% ETOH and 5% acetic acid for 30 minutes, 1 mg/ml pepsin (Sigma) in 2N HCl for 1 hour at 370 C, then rinsed in 0.1 M PBS pH 8.5. Sections were incubated in the primary antibody (anti-BrdU Boerhinger-Mannheim; 1:20 with 2% normal horse serum and 0.1% triton X-100) at 40 C overnight. The following day, sections were rinsed in 0.1 M PBS pH7.4 and then incubated in the secondary antibody (Vector Vectastain Elite ABC kit) for one hour, rinsed in 0.1 M PBS pH7.4, and finally incubated in ABC for one hour. The immunoreaction product was visualized with DAB.

Acute slices.

Slices were prepared at P1-7 by injecting each kit with 50 mg/kg pentobarbital, IP; the brains were then removed under chilled conditions. The somatosensory cortex was cut into 400 µm thick coronal slices using a tissue chopper and placed directly in an oxygenated chamber perfused with artificial CSF. At this point, injections of fluorescent dextrans (dextran fluorescein or fluoruby, Molecular Probes, Inc.) were made iontophoretically (3 µ amp, alternating positive current for 4 minutes) through pipettes with a tip thickness of 10-15 µm. The slices remained in the chamber for 5-8 hours to allow for transport. In most instances, the slices were fixed overnight in 4% buffered paraformaldehyde with 10% sucrose and further sectioned into 40 µm thick sections. The label resulting from the dextran injections was visualized using either conventional fluorescent microscopy or confocal microscopy (BioRad, MRC 600). Many injections were drawn from the digitized confocal images (x20)

to visualize further details across several sections using the Neurolucida reconstruction program (MicroBrightField, Inc.).

Western blots.

Tissue samples were obtained from either normal or E24 MAM-treated P0 kits; regions of the telencephalon were dissected that included the cortex, the intermediate zone and the ventricular zone. The brain pieces were homogenized in 10mM tris buffer at pH 7.6; total soluble protein was determined (protein detection kit, Bio-Rad). The samples and sample buffer were boiled and loaded (100 µg total protein) onto 12% acrylamide gels alongside low molecular weight non-stain markers. A Bradford assay was performed to ensure that the total protein concentration in each sample was the same. After running the gels, they were transferred to a Hybond-ECL membrane (Bio-Rad). To detect GFAP, an anti-GFAP antibody was used at 1:1000 and visualized using an ECL western blotting analysis system (Amersham) and exposed to Hyperfilm-ECL.

RESULTS

In normal animals, injections of fluorescently labeled dextrans into acute live slices resulted in characteristic and precise patterns of label that were strongly radial in nature. This distribution of label occurred after injections into the VZ or the intermediate zone. The labeled elements consisted largely of radial glia, which extended long distances from the ventricular zone to the pial surface (Figure 30A; Figure 31A); neurons migrating to the neocortex were studded along the glial processes (Juliano et al., 1996). The cytoarchitecture of the somatosensory cortex at postnatal day 0 to 7 (P0-7) in normal animals consists

of a dense cortical plate (i.e. developing and differentiating upper and middle layers of cerebral cortex) and a poorly formed layer 5 and 6; the subplate is also observable. At this age, layer 4 cannot be distinguished from the other layers that make up the dense cortical plate (Figure 32A).

In slices obtained from animals treated with MAM on embryonic day 33 (E33; the late group, layer 4 disruption) the architecture of the cortex looked similar to that in normal animals, i.e., a poorly formed layer 5 and 6 were present, as well as a dense cortical plate. The subplate is also substantial and present (Figure 32B). The cortex is slightly thinner than normal, however, with a mean value of 380 μm [n=5] in thickness from pia to bottom of the cortical plate (i.e., layer 6), compared with mean value of 411 μm in thickness for the normal [n=4]). (These values were not significantly different.) Dextran injections into E33 MAM-treated slices resulted in label similar to that in normal animals. Presumptive radial glia were distinctly spoke-like, extending from the VZ toward the cortical surface (Figures 30B, 31A). Although the later MAM injection (E33) had little obvious early effect on newborn kits, these injections result in a poorly formed layer 4 when kits are observed at cortical maturity (see Chapter 2).

In the group of animals that received early MAM injections, two dates were used: E24 or E28. Injections on either of these days resulted in severely disorganized cortical architecture. The cortical plate was very thin, with a mean value of 103 μm in thickness compared with a mean value of 411 μm in thickness in normal animals at P1. Neither a clear laminar pattern, nor a distinct subplate were found (Figure 32C). Occasional clusters of cells were seen in

Figure 30. Label resulting from injections of fluorescent-tagged dextrans in live slices of ferret somatosensory cortex taken at P1. Injections were made in the ventricular zone (VZ) of the slices; in the normal animal (A), the label extends in a column-like array, extending from the ventricular edge toward the cortical surface. The label is comprised of radial glial cells and migrating neurons. The injection illustrated in (B) is taken from a kit that received a dose of MAM on E33; the resulting label looks nearly identical to the normal pattern. The drawing in (C) is obtained from an animal that received a MAM injection on E24; rather than a precise point-to-point distribution, the label fans out from the injection site and does not head directly toward the pia. The blue color indicates the pattern of processes or fibers, the pink color indicates a cell body. Identifiable layers of cortex are indicated with numbers. VZ, ventricular zone; IZ, intermediate zone; CP cortical plate. Scale =100 μm.

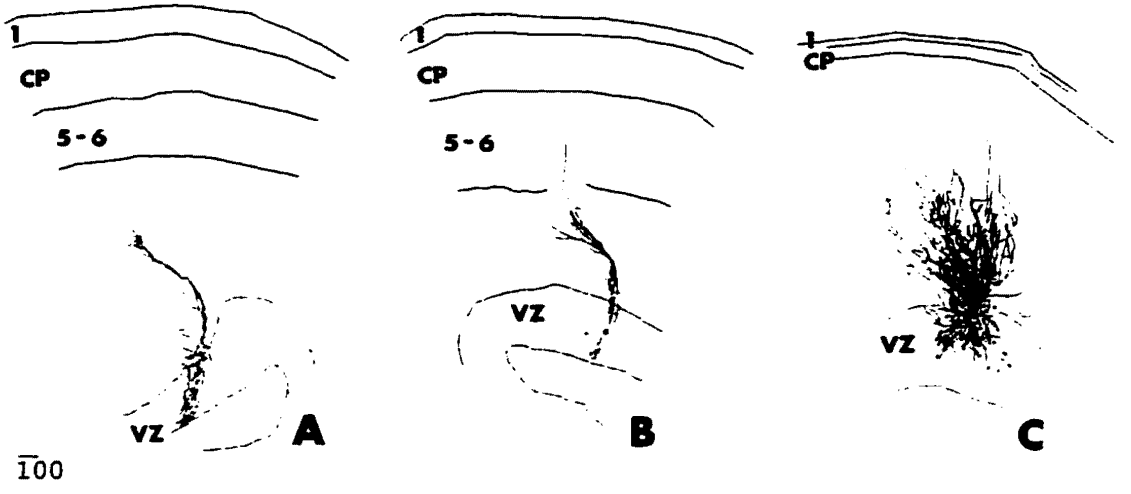

1
CP
5-6
VZ
A
100
1
CP
5-6
VZ
B
1
CP
VZ
C

Figure 31. (A) is a digitized confocal image of a dextran injection into a live slice taken from a P1 normal ferret kit; the label extends in a radial manner toward the cortex. (B) is a similar injection in a slice obtained from a ferret kit treated with MAM on E24; the resulting label fans out and does not extend directly toward the pia. VZ, ventricular zone; IZ, intermediate zone. Pial surface is toward the top. Scale = 100 μm.

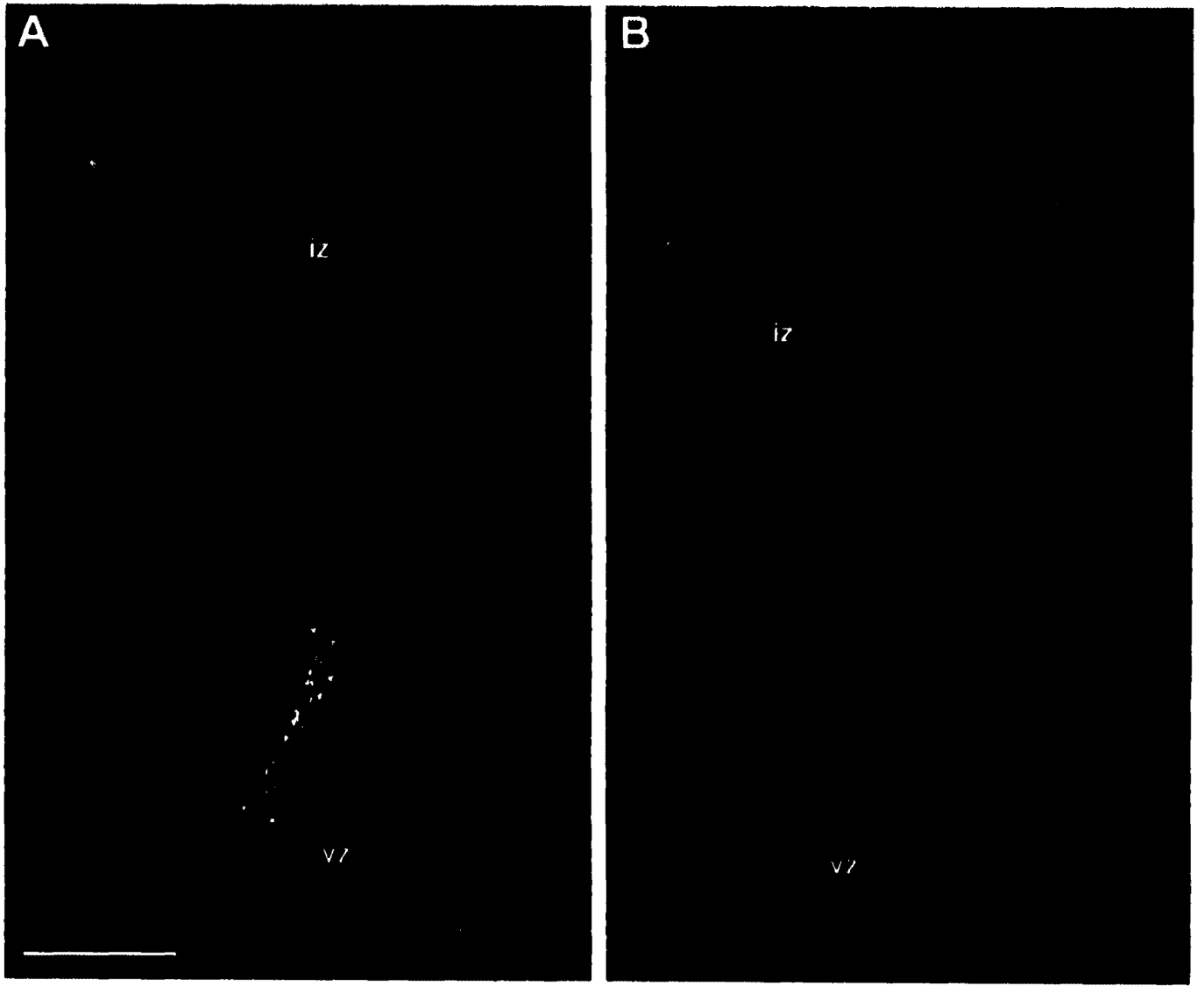
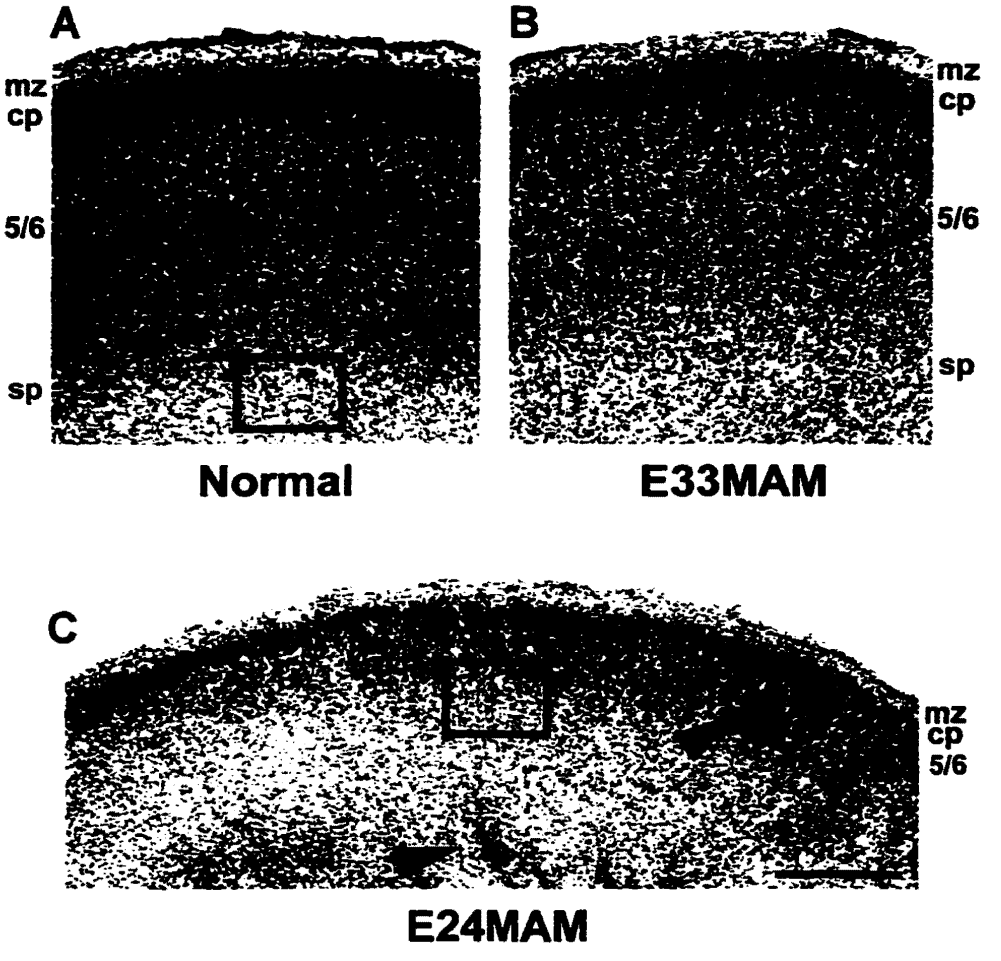

A
iz
vz
B
iz
vz

Figure 32. These are Nissl-stained sections of ferret somatosensory cortex cut in the coronal plane at P1. A) is taken from a normal kit, B) is taken from a kit that received a dose of MAM at E33, C) is from a kit that received a dose of MAM at E24. In the newborn ferret somatosensory cortex, layers are poorly formed; visible in A and B are the dense cortical plate (CP), the poorly formed layers 5-6, and the subplate (SP). In C) taken from a kit treated with MAM at E24, cortical layers fail to form and a thin sheet of cells is present in place of the normally-layered cortex. Arrows point to ectopic clusters of cells that form after E24 MAM treatment. The rectangles in A and C indicate the regions chosen for analysis of MAP2 immunoreactive neurons in Figures 7 and 8. MZ, marginal zone; CP, cortical plate; SP, subplate. Scale = 250 μm.


A
mz
cp
5/6
sp
Normal
B
mz
cp
5/6
sp
E33MAM
C
mz
cp
5/6
E24MAM

abnormal arrangements, suggesting that cells were not able to migrate to their proper location. Dextran injections into slices obtained from these animals resulted in a severely disorganized pattern of label, in comparison with the distribution found in normal or E33 MAM-injected animals (Figure 30B, 30C; Figure 31B). Rather than a radial flow, the labeled elements formed a fan-like configuration spread over a wide angle, not the point-to-point like distribution seen in the normal or E33 MAM-treated animals.

Injections of BrdU were made into pregnant ferrets both before and after MAM treatment on E33. In both situations (BrdU delivered E30 or E38) cells were generated and migrated into distinct laminar patterns appropriate for the gestational age of injection. Figure 19 (Chapter 2) shows an example of BrdU positive cells observed after an E33 MAM injection followed by BrdU administration on E36. The labeled cells are located in a distinct laminar pattern appropriate for the time of BrdU injection.

BrdU administration after E24 MAM treatment, indicated that neurons continued to be born and migrate into the cortex after early MAM injections as well. BrdU injections were delivered on several dates (E24, E28, E30, and E38) after the early MAM injections. Data derived after BrdU injections are shown in Figures 33 and 34. Figure 33 contains photomicrographs of neocortical regions containing densely labeled BrdU-positive cells. These were taken after BrdU injections into normal or MAM-treated jills on either E28 (shown on the left, corresponding to the normal birthdate of layer 6) or E38 (shown on the right, corresponding to the normal birthdate of layer 2; Chapter 2). The inserts in

Figure 33 show the overall position of the BrdU-labeled cells. In the two left panels, the BrdU positive cells reside largely in a distinct band corresponding to layer 6 in the normal animal, whereas in the E24 MAM-treated animal, the labeled cells distribute throughout the much thinner cortical plate. Labeled cells are also found beneath the cortical plate in the intermediate zone. After a BrdU injection on E38, the distribution of resulting label in the normal animal occurs throughout the thickness of the cortex and in the intermediate zone, as many of the cells generated on this day have not yet reached their final resting place in layer 2 (see Chapter 2, Noctor et al., 1997). After E24 MAM treatment and E38 BrdU injection, the BrdU positive cells are also scattered throughout the intermediate zone, but fewer of them reach the cortical plate.

These data are quantified in Figure 34. To compare the distribution of labeled cells across similar distances through the cortex in normal and MAM-treated animals, the number of BrdU positive cells were counted in bins 500 μm wide and 50 μm deep starting from the top of the dense cortical plate and presented in histograms as the percent of the total number of cells counted (Figure 34). In the MAM-treated animals, the bins extended into the intermediate zone, as the cortical plate is much thinner in these animals.

To quantify the effect of early MAM treatment on the number of neurons populating the subplate underlying somatosensory cortex on P0, the number of MAP2 positive cells in the region of the subplate in normal and E24 MAM-treated animals were examined. MAP2 has been identified as a marker for subplate neurons in ferret (Ghosh and Shatz 1992a; 1993). Substantially more MAP2

positive cells were found in normal subplate compared to a similar region in animals treated with MAM at E24 (Figures 35 and 36). Figure 35 shows MAP2 immunoreactivity in normal (Figure 35B) and E24 MAM-treated (Figure 35C) P0 somatosensory cortex. In both groups, MAP2 labeled cells were observed in the subplate with the characteristic inverted pyramidal shape (Antonini and Shatz, 1990; Valverde and Facal-Valverde, 1988). The regions chosen for analysis are indicated with rectangles in Figure 32A and C. Figure 35A illustrates BrdU positive cells found in the subplate of P0 normal ferrets after BrdU injection on E24. Figure 36A demonstrates that in normal neonates there are similar numbers of BrdU positive cells (after BrdU treatment on E24) and MAP2 positive cells in the analyzed subplate region. Neurons that reside in the subplate region of ferret somatosensory cortex are born on several days, including E24 (see Chapter 2). The histogram in Figure 36B demonstrates that E24 MAM-treated ferrets contained approximately 1/3 the number of MAP2 positive neurons beneath the cortical plate when compared to normal neonates.

Since the spoke-like labeling pattern revealed after dextran injection is largely composed of radial glia, it was predicted that the processes of these cells would be specifically disrupted in animals treated with MAM on E24 or E28. The normal morphology of radial glia consists of cell bodies in the VZ, with processes that extend toward the pia (Ramon y Cajal, 1911; Schmechel and Rakic, 1979; Voigt 1989; Juliano et al., 1996). During development, some of these cells migrate toward the cortex. As the cortex matures, radial glia transform into astrocytes, which populate the adult cerebral cortex (Schmechel, Rakic, 1979; Levitt, Rakic,

1980; Voigt, 1989; Culican et al., 1990; Raff, 1989). To study the effect of MAM treatment on these cells, sections of ferret brains were incubated with antibodies directed against vimentin, which is an intermediate filament protein established as a marker for radial glia in developing ferret brain (Voigt 1989). The normal pattern of vimentin immunoreactivity (IR) can be seen in Figure 37A; in neonatal ferrets the distribution consists of elongated fibers that extend from the ventricular zone to the pia. The pattern of vimentin-IR assessed in animals treated with MAM on E33 appears relatively normal, also comprised of elongated fibers that extend from the VZ to pia (Figure 37B). After MAM treatment on E24 or E28, however, the distribution of vimentin-IR was distorted; the precise radial pattern of staining is gone revealing stained fibers that are not aligned in radial arrays (Figure 37C).

Since it is well accepted that as the cortex matures, radial glia differentiate into astrocytes, the study examined if interruption of the early generated cortical layers also results in the presence of astrocytes early in the development of ferret somatosensory cortex. To do this, sections were studied for immunoreactivity against GFAP (an established marker for astrocytes). In normal P0 kits, there is a small amount of positive staining near the pia (Figure 38A). The staining pattern is similar in P0 animals treated with MAM on E33, that is a few immunoreactive fibers occur near the pia, but very little additional positive staining exists in the cortex or intermediate zone. In kits treated with MAM on E24, however, there are many GFAP positive cells (presumptive astrocytes) throughout the thickness of the cortex and intermediate zone, which appear

Figure 33. Photomicrographs of BrdU-labeled cells in the positions indicated by the boxes in the inset drawings. The distributions of BrdU-positive cells on P0 in normal and E24 MAM-treated animals after BrdU injection at E28 (left panels) or E38 (right panels) are shown. In a normal animal after E28 BrdU administration the labeled cells reside in a distinct band corresponding to layer 5-6, whereas in E24 MAM-treated animals the BrdU cells are scattered throughout the cortical thickness. After E38 BrdU injections, the cells generated on that day have not yet reached their final position on P0 (birth occurs after 41-42 days of gestation in the ferret) but distribute throughout the cortical thickness in normal animals. On P0 in the E24 MAM-treated animal, cells labeled with BrdU on E38 are also scattered throughout the thin cortical plate and in the ventricular zone. CP, cortical plate. Scale bar = 100 μm for photomicrographs; 250 μm for drawings.

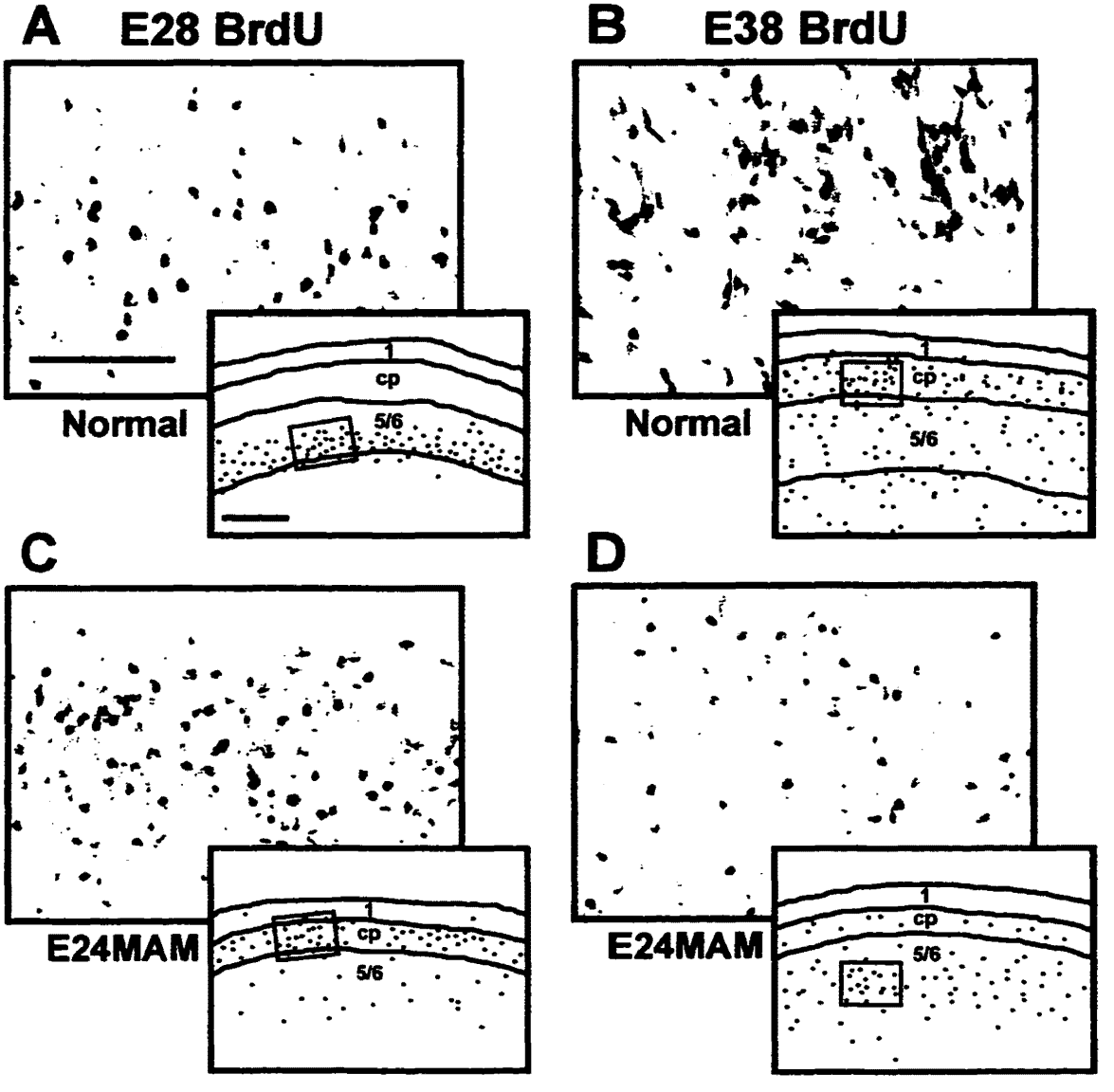
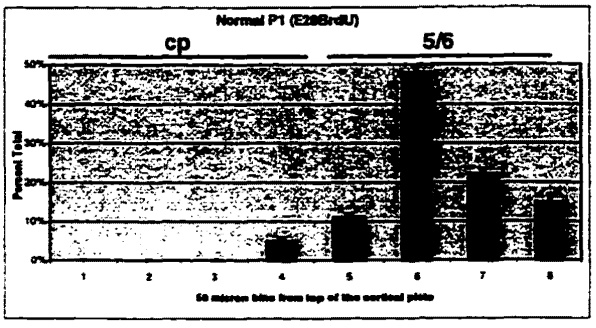
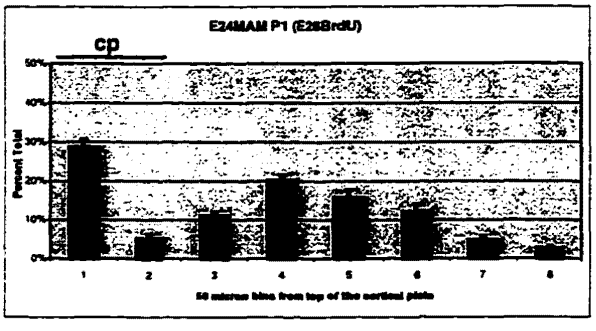

A
E28 BrdU
Normal
1
cp
5/6
B
E38 BrdU
Normal
1
cp
5/6
C
E24MAM
1
cp
5/6
D
E24MAM
1
cp
5/6

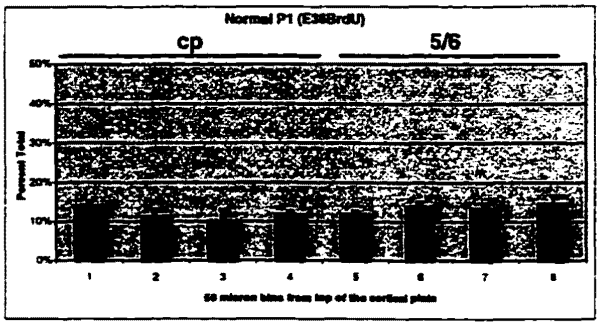
Figure 34. These histograms display the relative percentages of BrdU-positive cells found in the somatosensory cortex on P0 after BrdU injections on E28 or E38 in normal and E24 MAM-treated animals. Each bar represents the relative percentage of labeled cells counted in a bin 500 μm wide and 50 μm deep. The cells were counted in bins extending from the pia to a depth of 400 μm. The top two panels show that in normal animals the greatest percentage of cells generated on E28 are in layer 5 on P0, whereas after E38 injections the cells incorporating BrdU have not yet reached their final layer by P0. In the E24 MAM-treated animals, the greatest percentage of counted cells reach the cortical plate after an E28 injection, whereas after an E38 injection many fewer cells reach the cortical plate, but are scattered through the intermediate zone in the E24 MAM-treated animal.

E28 BrdU





E38 BrdU



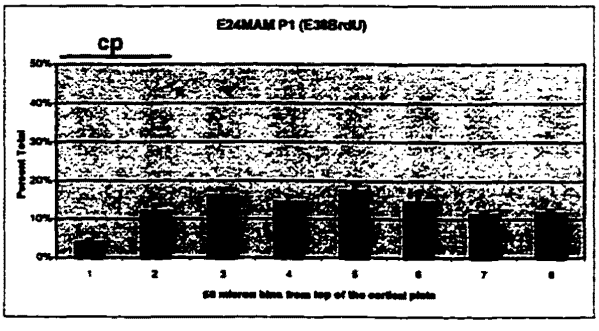


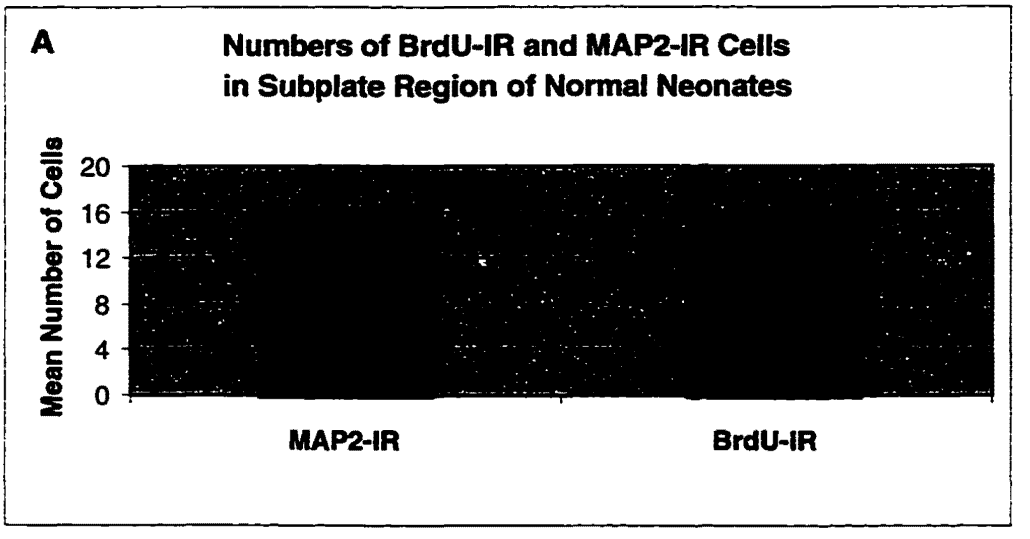
Figure 35. (A) is a digitized image of BrdU labeled cells found in the subplate, taken from the region indicated by the rectangle in Figure 32A, of a normal P0 animal injected with BrdU on E24; subplate cells that populate ferret somatosensory cortex are born over several days, including E24. (B) is an image of MAP2 immunoreactivity taken from the same region in a P0 normal ferret. (C) is an image of MAP2 immunoreactivity taken from a P0 ferret treated with MAM on E24 in the region indicated by the rectangle in Figure 32C. Pial surface toward the top. Scale = 50 μm.

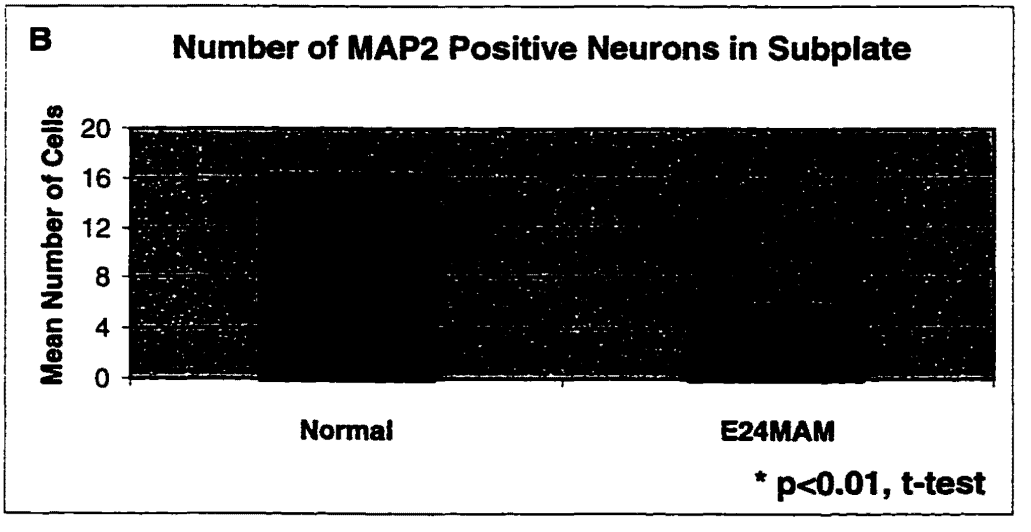
Normal P1
E24 BrdU

Normal P1
MAP2

E24MAM P1
MAP2

Figure 36. Histograms representing the number of MAP2 positive or BrdU positive cells in the subplate region of normal and E24 MAM-treated P0 ferrets. Cells were sampled in a 200 μm x 150 μm sector of 5 sections through the subplate region of the somatosensory cortex in each animal (see rectangles in Figure 32A & 32C). Three animals in each group were used for counts; the groups include [i] normal-BrdU-IR, [ii] normal-MAP2-IR, [iii] E24 MAM-treated-MAP2-IR. Panel (A) demonstrates that the mean number of BrdU positive cells (after labeling on E24) is similar to the mean number of MAP2 positive cells in the region of the subplate sampled for analysis (t-test). A mean of 15.1 (S.E. 1.140) BrdU-IR cells and 16.13 (S.E. 1.396) MAP2-IR cells were found. Panel (B) demonstrates that the mean number of MAP2-IR neurons in the subplate was significantly reduced in the E24 MAM-treated cortex (light grey bars) compared to normal animals (dark grey bars). This difference was significant at the $p<0.01$ level, (t-test). IR, immunoreactive.


A
Numbers of BrdU-IR and MAP2-IR Cells
in Subplate Region of Normal Neonates
Mean Number of Cells
20
16
12
8
4
0
MAP2-IR
BrdU-IR


B
Number of MAP2 Positive Neurons in Subplate
Mean Number of Cells
20
16
12
8
4
0
Normal
E24MAM
* p<0.01, t-test

comparable in overall distribution to the vimentin immunoreactivity in similar animals (Figure 38B).

To further verify the presence of GFAP in E24 MAM-treated kits, western blots were prepared on tissue obtained from normal and E24 MAM-treated brains on P0. The samples were taken from the frontoparietal cortex and underlying white matter in the ventricular zone. These data are presented in Figure 39, and demonstrate that although small amounts of GFAP are present in normal P0 ferret brains, the MAM-treated brains contain substantially greater amounts of this protein. Two bands immunoreactive for GFAP were observed, one at 55Kd and one at 49Kd. This was not unusual compared to previous studies, and is likely to be a result of degradation of the molecule (Bigbee et al., 1983; Sheng et al., 1994).

To help determine the time course of events in the abnormal vimentin and GFAP immunoreactivity, MAM was injected on E24 and the embryos removed by cesarean section on E27, for one set of kits. This allowed a relatively short time between the administration of MAM and the assessment of radial glial morphology, as revealed by staining with antibodies directed against vimentin. In this case, the pattern of immunoreactivity appeared similar to that seen in both the normal and E33 MAM-treated animals but somewhat sparser (Figure 40). Although the pattern of staining was sparser than that seen in normal P0 animals and fetuses at later embryonic dates (e.g. E33), the stained processes were distinctly radial and extended from the ventricular zone to the pial surface. Nissl stains of this material demonstrated that the elements of the cortical plate

Figure 37. Sections stained with an antibody directed against vimentin and obtained from 3 different ferret kits at P0. (A) is taken from a normal ferret kit and displays the normal radial pattern of staining for the intermediate filament protein. (B) is taken from an animal that received a MAM injection on E33, and appears similar to the normal pattern of vimentin immunoreactivity. (C) is a photomicrograph of vimentin immunoreactivity obtained from a ferret kit that received a dose of MAM on E24. The pattern of staining in this animal is distorted and does not display the normal radial characteristics. Pial surface toward the top. Scale = 100 μm.

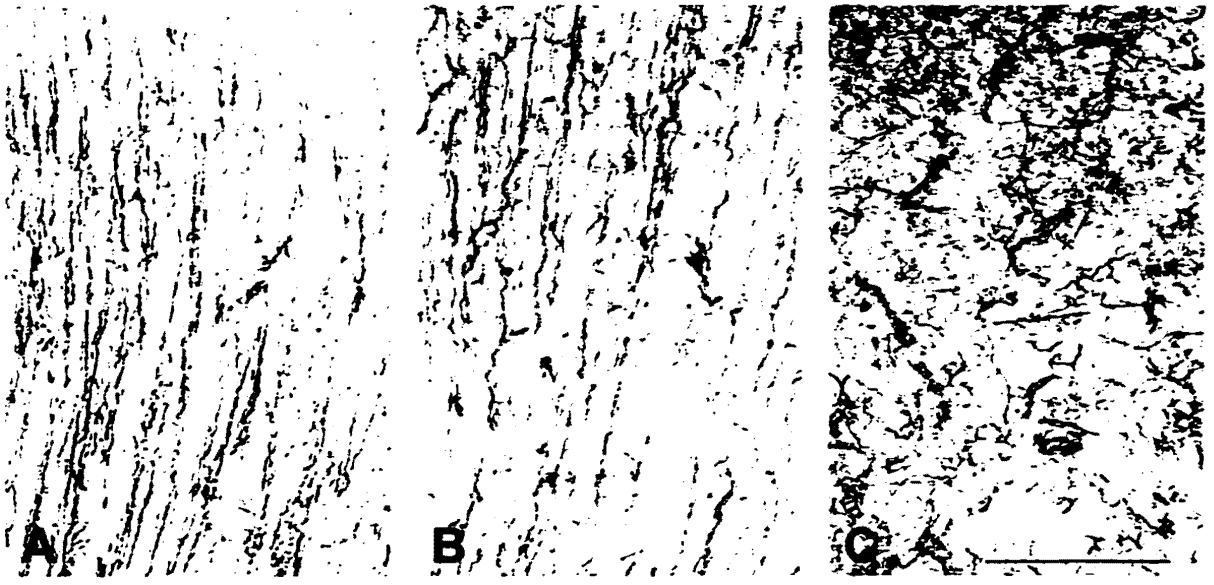
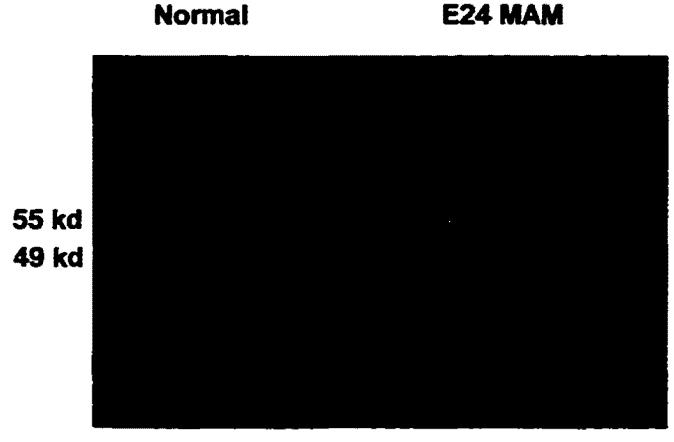

A
B
C
Normal
E33MAM
E24MAM

Figure 38. GFAP immunoreactivity on P0 in sections taken from normal (A) and E24 MAM-treated ferrets (B). In the normal somatosensory cortex, small amounts of anti-GFAP staining can be observed in the outer part of layer 1, near the pial surface. In the E24 MAM-treated brain, many immunoreactive cells that appear morphologically similar to astrocytes are observed scattered through the cortical thickness and intermediate zone. MZ, marginal zone; CP, cortical plate. Scale = 100 μm.


mz
cp
5/6
A
B
mz
cp
Normal
E24MAM

Figure 39. Western blot of normal and E24 MAM-treated brains at P0 demonstrating the presence of GFAP (MW 55 kDa and 49 kDa); two immunoreactive bands are often found in western blots identifying GFAP. Substantially more GFAP is present in the E24 MAM-treated brain; two different dilutions are shown with the strongest concentrations for both the normal and E24 MAM-treated animals in the center. The weaker concentrations (by 10x) are on the extreme right and the extreme left. Numbers on the left indicate the molecular weight of the standards run with the gel.

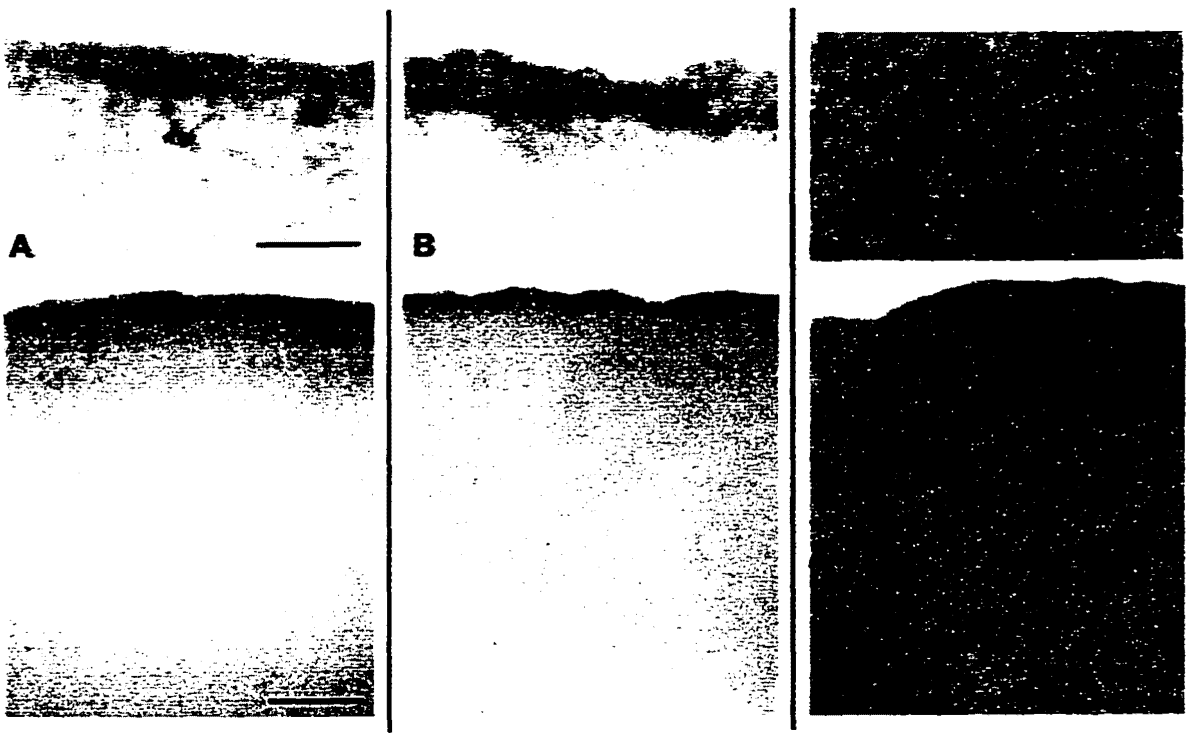

Normal
E24 MAM
55 kd
49 kd

formed by E27 appeared relatively normal. At this time point, although very thin, there was no obvious disruption of the cortical plate; layer 1 was present and contained cells, including presumptive neurons.

Since the superficial and deep components of the preplate are thought to be generated at nearly the same time Bayer and Altman, 1990), it was interesting to find that layer one appeared to be intact both soon after the MAM treatment and even in some cases 2 weeks after the MAM treatment at birth. To further investigate the effects of an early MAM treatment on layer 1, sections of tissue from E24 MAM-treated ferrets were incubated with the CR-50 antibody, which recognizes Cajal-Retzius neurons in the marginal zone of developing embryos. The normal pattern of CR-50 immunoreactivity (Figure 41A) labels fusiform cell bodies in the marginal zone of cerebral cortex, with cell processes extending horizontally within the marginal zone. The pattern of CR-50-IR observed in the E33 MAM-treated ferrets appears normal containing fusiform shaped cell bodies located in the marginal zone (Figure 41B). After MAM treatment on E24, however, the distribution of CR-50 positive neurons was altered. While the labeled cells demonstrate a normal fusiform shape, the orientation and distribution of the cells were greatly altered. CR-50 positive neurons were found throughout the thickness of the cortical plate and in the intermediate zone in addition to their normal location in the marginal zone (Figure 41C).

Figure 40. Immunoreactivity against vimentin in a section taken from an E27 embryo that was previously treated with MAM on E24. At this age after MAM treatment, the radial glia are present and organized similar to their distribution in normal P0 kits, but the distribution is sparse. Pial surface toward the top. Scale bar = 100 µm.

Figure 41. Photomicrographs demonstrating the distribution of presumptive Cajal-Retzius neurons that stained positively for CR-50 antibody. Panel (A) displays the morphology and position of CR-50 positive cells in normal ferret neonatal cortex; fusiform shaped cells, which are found only in the marginal zone of neocortex, can be seen at high-power on the top, and at lower power on the bottom. Panel (B) demonstrates that CR-50 positive neurons have the same phenotype and location in ferret neocortex treated with MAM on E33. (C), is taken from a ferret treated with MAM on E24. CR-50 positive cells have a fusiform morphology after E24 MAM treatment, but the cells are distributed throughout the thickness of the neocortex. Scale bar = 100 μm for top images; 250 μm for bottom images.


A
B

Discussion

Effects of MAM treatment on laminar pattern.

MAM has been used recently by a number of researchers to interfere with the development of a specific neocortical layer or layers. Those studies, and the present study indicate that MAM treatment is relatively discrete, and that MAM acts for a restricted window of time (Kind et al.,1992; Woo et al., 1996; Woo and Finlay, 1996; Cattabeni and Di Luca, 1997). In the current study, the differences between injections of MAM at early and mid-gestational points during cortical neogenesis are dramatic. Later injections (E33) result in a relatively distinct disruption of layer-specific development (see Chapter 2). Subsequent injections of BrdU indicate that cells continue to be generated and migrate into the cortex in a layer specific manner after MAM treatment.

Early injections, however, result in severe disturbances in the formation of cortical layers and in alterations of radial glial and Cajal-Retzius cells. Injections of MAM early during cortical genesis almost certainly interfere with the division of precursor cells as well as cells undergoing their final division at the time of injection, which could contribute to a poorly formed laminar pattern. It cannot be ruled out that the thin and disarrayed cortical plate after early MAM treatment is due to the elimination of a portion of precursor cells that act as a source of subsequently born cells. Cells continue to be born and migrate into the cortical plate, however, after early MAM treatment. BrdU is incorporated into dividing cells at several time points after the MAM injection (e.g. Figures 33 and 34) suggesting that additional mechanisms contribute to the jumbled cortical pattern.

The MAM treatment may also interfere with the development of other neural centers that influence the neocortex, most notably the thalamus. Although it is possible that an alteration in thalamocortical projection influenced the development of cortical layering, ongoing parallel studies have found a strong projection from the thalamus to the neocortex in similar E24 MAM-treated animals (Palmer et al., 1996, 1997). In addition, Algan and Rakic (1997) using a different model of cell cycle disruption, ionizing radiation, find that delivering doses of X-rays during the development of the lateral geniculate nucleus does not substantially reduce the thickness of the visual cortex or the overall laminar pattern, suggesting that these particular features are not dramatically altered by reduced input from the thalamus. This treatment does, however, reduce the total surface devoted to area 17.

Shortly after early MAM injection (on E27 after E24 injection) both the radial glia and cortical plate look relatively normal. This suggests that the process of disorganization does not begin immediately, but takes place over a period of some days. In addition, injections of BrdU at different dates after early MAM treatment indicate that neurons continue to be born and make their way to the cortical plate. In fact, four days after (E28) the early MAM injection, BrdU injections reveal that cells continue to be born and migrate into cortex in numbers at least equal to the normal animal. They form a quasi laminar pattern, with the greatest percentage of cells born on that day reaching the cortex (Figures 33 and 34). BrdU injections at a later time (E38) after early MAM injection, show that cells continue to be born, but fewer are found in the cortical

plate at P0. Even in the normal animal at this age the migrating cells have not attained their discrete laminar arrangement and maintain a substantial presence in the intermediate zone. They would normally reside in layer 2 if further development and migration were permitted. Since the disrupted pattern of radial glia takes place gradually after E24 MAM treatment, earlier born neurons may travel into cortex more easily than later generated neurons.

Effects of MAM treatment on radial glia.

One of the more dramatic features of early MAM injections are the disorganized radial glia. Why would early MAM injections result in a distorted distribution of radial glia, while the late MAM injections have no effect on these cells? It is generally assumed that radial glia are generated early in cortical development and are already present when neurons that populate the cerebral cortex are born (Schmechel and Rakic, 1979; Altman and Bayer, 1991). They then provide, at least partially, a scaffold for neurons to reach the neocortex (Rakic, 1971; 1972; 1977; 1990; 1995). This seems to be validated by this study, since several days after early MAM injection at E24, the radial glia are present and relatively intact. The radial glia are apparently in place at the time of MAM injection at E24 and the process of radial glial distortion takes place over a period of several days, since 4 days after injection the cortical structure appears relatively normal, but by birth is highly abnormal in appearance. MAM-treatment alone does not seem to cause specific distortion or death of radial glia, since injections at later times do not result in radial glial distortion (e.g. injections on E33). Therefore a specific aspect of interfering with early, but not later,

developing cortex results in this process. The observation that early, but not late, injections of MAM result in the radial glial distortion also argues against the idea that the distortion of radial glia is due to reactive gliosis. If the glial distortion was due to reactive gliosis, as a result of MAM injection alone, one would expect this finding after later injections of MAM as well.

It seems highly likely that the radial glia are differentiating into astrocytes earlier than normal. It is widely accepted that many radial glia become astrocytes after they are no longer necessary as guides for neurons traveling to the neocortex (Schmechel and Rakic, 1979; Voigt, 1989, Culican et al., 1990; Raff, 1989). The demonstration here of a change in morphology, plus increased expression of the characteristic marker for astrocytes, GFAP, strongly suggests that the radial glia of early MAM-treated animals differentiate into astrocytes. If this is the case, there are several mechanisms that might cause this conversion to occur. Early interruption of the generation of cortical cells will block (at least partially) the formation of the precociously generated cortical layers, including the subplate. It is not clear which comes first: the failure of layers to form or the disruption of radial glia. It is likely that the lack of some feature normally supplied by the early generated neocortex causes the radial glia to differentiate into astrocytes. The subplate is a substantial component of the early generated neocortex and contains many cell adhesion and extracellular matrix molecules that are not present in other parts of the developing cortical plate (see Allendoerfer and Shatz, 1994, for review). These molecules may be important for maintenance of radial glial attachment, as well as for axon guidance or other

growth processes. Interestingly, Hunter and Hatton (1995) demonstrated that a diffusable factor present in mouse embryonic neocortex can induce astrocytes to alter their cell phenotype into radial glia. They suggest an inhibitory factor may be present during cortical neogenesis and layer formation, which maintains radial glia in their proper morphology. With cortical maturity, this factor is withdrawn and radial glia are free to differentiate into astrocytes. It is possible that in this study, the early MAM treatment induces withdrawal of the normally present inhibition resulting in early formation of astrocytes. The subplate, with its high complement of trophic substances may be a source for the factor causing radial glia differentiation. In normal ferrets, transformation of radial glia into astrocytes is coincident with the developmental period in which subplate neurons disappear from the cortex (Voigt, 1989; Allendoerfer and Shatz, 1994; Juliano et al., 1996). Although the study of E27 embryos after E24 MAM treatment suggests that the cortical layers begin to form normally after MAM treatment, perhaps after transformation of the radial glial guides, newly generated cortical neurons cannot find their way to the cortex, leading to the severely disrupted cortical pattern observed. A recent study by Hunter-Schaedle (1997) reports the interesting finding that radial glia in the *reeler* mouse are also disrupted and display early differentiation into astrocytes. She suggests that poorly developed radial glia may participate in the disturbed formation of layers in the *reeler* neocortex.

Abnormal radial glia may also be induced due to an incomplete formation of the preplate at the time of early MAM injection. During early cortical genesis, the first generated component of the neocortex is the preplate, which consists of the

marginal zone (future layer 1) and the subplate (which largely dies in the adult). As cortical neurons are generated, the preplate splits into a superficial marginal zone and a deeper subplate to allow neurons of the cortical layers to insinuate themselves in between the two preplate layers. If early MAM treatment interferes with normal marginal zone formation, a usual substrate for radial glia would not be intact and might cause early astrocytic differentiation. The results presented here, however, indicate that layer 1 is present, both in embryos shortly after MAM administration (i.e., on E27) and in P0 early MAM-treated animals, suggesting a sufficient presence for the structural relations of radial glia. It is possible, that although present, the preplate is immature and susceptible to permutation, contributing to the radial glial transformation. The findings of misplaced CR-50 positive cells after E24 MAM treatment indicate that this may be the case. Many researchers have suggested that layer 1, and particularly the Cajal-Retzius neurons, may play an important role in attracting and guiding subsequent neurons to their proper positions (e.g., Caviness and Rakic, 1978; Marin-Padilla, 1984; Ogawa et al., 1995). Deficits in layer 1, or misplaced layer 1 neurons may also contribute to a poorly formed cortex. Furthermore, a recent study by Soriano et al. (1997) reports that Cajal-Retzius cells strongly influence the phenotype of radial glia in adult and developing cerebellum.

The results obtained from the *reeler* mouse mutation also suggest that a specific feature of the preplate is important for subsequent formation of neocortex. Although in *reeler*, the preplate fails to split and cortical layers pile up underneath the superficial structure (Caviness and Sidman, 1973), the presence

of the preplate itself is consequential to several features of subsequent cortical development. In this model, thalamocortical afferents first touch the subplate before turning and terminating in layer 4, suggesting that the presence of the preplate is an important feature for cortical development (Frost and Caviness, 1983). If the formation of this region is disrupted after early MAM treatment, it may also result in disruption of both cortical layers and radial glia.

These results suggest that early generated cortex is necessary for proper laminar formation in the neocortex. The early generated layers appear to provide cues to the radial glia about where to maintain their processes and when to differentiate into astrocytes. Without the substrate normally provided by the first born layers of neocortex, the radial glia lose their spoke-like orientation and begin astrocytic differentiation.

CHAPTER 5. RELATIVE CONTRIBUTIONS OF EARLY GENERATED VERSUS LATER GENERATED LAYERS TO THE DEVELOPMENT OF LAMINAR PATTERNS OF NEOCORTEX.

E24 MAM treatment disrupts formation of the cortical laminae

Evidence presented here suggests that early-generated layers of neocortex provide essential cues for laminar development, whereas later-generated layers may be important for establishing proper extrinsic and intrinsic connectional patterns. E24 MAM treatment causes a dramatic disorganization of neocortex accompanied by poorly formed laminae. Several additional features occur with the disorganized cortex: ①Neurons generated within a few days of the E24 MAM treatment were likely to migrate into the poorly formed cortical plate. ②Neurons generated in mid and late stages of corticogenesis do not appear to reach the cortical plate. ③The radial glia are severely disrupted and show evidence of early differentiation into astrocytes, but this process occurs over a period of several days. ④Cajal-Retzius (CR) cells are also highly disordered and distributed throughout the cortical plate and intermediate zone.

These observations suggest that interruption of the early generated neocortex prevents proper formation of subsequently born neocortical layers. The inability of proper laminar formation to occur appears to be due to a sequence of events that begins with the failure of early layers to be born. Subsequently, radial glia differentiate into astrocytes and CR cells move to abnormal positions. The distortion of radial glia may be due to withdrawal of a radialization factor associated with the early generated layers. The combination of abnormal radial glia and misplaced CR cells creates an environment that disrupts neuronal migration.

It is not clear what causes the Cajal-Retzius cells to become displaced. One possibility is that the CR cells are born after the MAM treatment and as a result of the radial glial disruption cannot migrate to their proper location in the marginal zone. This seems unlikely since several studies describe the Cajal-Retzius cells as among the first neocortical cells to be born. It is also possible that the cells are displaced during the process of the radial glial transformation. This might occur if the CR cells maintain a close association with the radial glia, and are pulled into cortex as the radial glia retract their processes. Recent evidence indicates that Cajal-Retzius cells function to stop migrating neurons, their altered positions in the MAM-treated animals would further disrupt neocortical migration (see Frotscher, 1997).

Effects of E33 MAM treatment on cortical formation

In contrast to the drastic alterations in cortical structure observed after the early MAM treatment, E33 MAM treatment did not prevent subsequent laminar formation. The overall laminar properties of adult cortex were maintained despite the absence of much of layer 4. Since the cortical layers continue to develop in a relatively normal fashion after E33 MAM treatment, what effects do interruption of layer 4 have? Ongoing parallel studies in our lab are investigating the development of connections between thalamus and cortex in normals and MAM-treated ferrets. In E33 MAM-treated ferrets, after a 70 percent reduction in layer 4, thalamic afferents are still driven to make connections with the developing neocortex (Palmer et al., 1996, 1997). This supports the findings of Woo and Finlay (1996), and Jones et al., (1982), who found that thalamic

afferents colonize neocortex after MAM treatment that disrupts formation of layer 4 in rats and hamsters. While these results indicate that layer 4 neurons are not responsible for attracting thalamic afferents into neocortex, these cells may be required for proper connections to form between sensory cortical neurons and thalamus. In E33 MAM-treated animals, we find that thalamic afferents reach the cortical plate by birth as they do in normals. By 1 week of age, however, rather than terminating specifically in layer 4, thalamic afferents in E33 MAM-treated ferrets are more likely to be distributed throughout the full cortex (Palmer et al., 1996, 1997). The altered distribution of the thalamic afferents may result from the absence of a proper stop signal for the ingrowing thalamic axons in layer 4, a mechanism thought to guide the formation of connections between thalamic axons and layer 4 neurons (Molnar and Blakemore, 1995). Studies in this lab also indicate that E33 MAM-treated adult ferret contains a normal somatotopic organization (Mclaughlin and Juliano, 1998), matching the findings of Yurkewicz et al. (1984). In the Yurkewicz study, rat somatosensory cortex topographic order was maintained, despite layers 2-4 disruption after MAM treatment.

Despite normal somatotopic organization, microelectrode recordings reporting current source density (CSD) profiles find that initial responses to stimulation in E33 MAM-treated ferret cortex are distributed through the cortical layers, rather than focusing in the middle layers as in the normal adult (McLaughlin and Juliano, 1998). These functional responses correspond with the anatomical results providing evidence that afferent terminations are distributed throughout the cortical depth after E33 MAM treatment.

We also have evidence that the distribution of $GABA_A$ receptors is altered after E33 MAM treatment. In normal animals $GABA_A$ receptors are present in all layers, but display a peak in layer 4. After E33 MAM treatment, the pattern of $GABA_A$ receptor binding was not focused in any specific layer, but display a slight increase in layer 3 (Palmer et al., 1998). Evidence indicates that $GABA_A$ receptor expression is regulated by thalamic afferents in neocortex (Paysan et al., 1997), and the altered distribution of thalamic afferents may also result in a redistribution of $GABA_A$ receptors. Appropriate numbers of layer 4 neurons may be necessary for the afferents to form proper connections with the cortex. The decreased numbers of layer 4 neurons may evoke compensatory connections to form between somatosensory cortex and thalamus that cause atypical responses to stimuli. Preliminary evidence gathered in our lab also indicates that development of intrinsic connections is altered in the E33 MAM-treated ferrets. We hypothesize that intrinsic connectivity will be altered by the disruption of layer 4 neurons.

These results indicate that the presence of layer 4 neurons may not be necessary for thalamic terminations to enter cortex, or for subsequent laminar formation. Their absence, however, results in disordered thalamic afferents and altered laminar response profiles.

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APPENDIX A: Statistical analysis on Nissl-stained sections obtained from normal versus E33 MAM-treated adult ferret somatosensory cortex.

Thickness of Cortex Analysis

Using the Image Pro Plus® program, the thickness of cortex from the pial surface to the bottom of layer 6 was measured in 5 coronal sections each in somatosensory cortex of 3 normal, and 3 E33 MAM-treated, 12 week old ferrets. The data was entered into Microsoft Excel® and mean values obtained from the 5 measurements for each animal. T-tests were performed to test for significant differences between the mean values of the normal versus E33 MAM-treated animals.

Total Cortex

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	1519.866667	1135.933333
Variance	1544.573333	27562.45333
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	3.897781814	
P(T<=t) one-tail	0.029980963	
t Critical one-tail	2.91998731	
P(T<=t) two-tail	0.059961927	
t Critical two-tail	4.302655725	

Layer Thickness Analysis

Using the Image Pro Plus® program, the thickness of each layer was measured in 5 coronal sections each in somatosensory cortex of 3 normal, and 3 E33 MAM-treated, 12 week old ferrets. The data was entered into Microsoft Excel®, mean values obtained from the 5 measurements for each animal, and t-tests performed to test for significant differences between the mean values of the normal versus E33 MAM-treated animals.

Layer 1
t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	152.7933333	148.86
Variance	19.52413333	23.1916
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	1.042383304	
P(T<=t) one-tail	0.178044043	
t Critical one-tail	2.131846486	
P(T<=t) two-tail	0.356088087	
t Critical two-tail	2.776450856	

Layer 2
t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	180.8733333	206.82667
Variance	1224.748933	256.22333
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-1.16810077	
P(T<=t) one-tail	0.163576865	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.32715373	
t Critical two-tail	3.182449291	

Layer Thickness Analysis

Layer 3

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	175.84	295.18667
Variance	2338.6188	140.89613
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-4.15133286	
P(T<=t) one-tail	0.026709625	
t Critical one-tail	2.91998731	
P(T<=t) two-tail	0.053419251	
t Critical two-tail	4.302655725	

Layer 4

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	267.6	83.393333
Variance	55.3852	98.653333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	25.70699326	
P(T<=t) one-tail	6.80061E-06	
t Critical one-tail	2.131846486	
P(T<=t) two-tail	1.36012E-05	
t Critical two-tail	2.776450856	

Layer Thickness Analysis

Layer 5

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	163.2666667	162.23333
Variance	121.3737333	607.57813
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0.066290553	
P(T<=t) one-tail	0.475658498	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.951316995	
t Critical two-tail	3.182449291	

Layer 6

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	376.9533333	398.92667
Variance	67.22573333	2346.4981
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-0.77466294	
P(T<=t) one-tail	0.259791963	
t Critical one-tail	2.91998731	
P(T<=t) two-tail	0.519583925	
t Critical two-tail	4.302655725	

Cell Packing Density Analysis

To examine whether the E33 MAM treatment affected the density of cells within each layer of somatosensory cortex, the number of cells was counted within a grid of boxes 500 µm wide by 100 µm deep extending from the top of layer 2 through each layer using the Image Pro Plus® program. Counts were obtained in 5 coronal sections each of 3 normal and 3 E33 MAM-treated, 12 week old ferrets. Only cells in which the full profile was stained with the Nissl substance and a single nucleoli was visible within the nucleus were counted. The data was entered into Microsoft Excel®, mean values obtained from the 5 measurements for each animal, and t-tests performed to test for significant differences between the mean values of the normal versus E33 MAM-treated animals.

Layer 2
t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	47.63333333	45.06666667
Variance	6.303333333	12.41333333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	1.027580478	
P(T<=t) one-tail	0.181111324	
t Critical one-tail	2.131846486	
P(T<=t) two-tail	0.362222649	
t Critical two-tail	2.776450856	

Cell Packing Density Analysis

Layer 3

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	33.57777778	33.16666667
Variance	2.783703704	13.04333333
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0.1789864	
P(T<=t) one-tail	0.434676974	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.869353949	
t Critical two-tail	3.182449291	

Layer 4

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	35.96666667	28.4
Variance	8.823333333	39.24
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	1.890419926	
P(T<=t) one-tail	0.077545098	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.155090197	
t Critical two-tail	3.182449291	

Cell Packing Density Analysis

Layer 5

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	28.22222222	26.6
Variance	2.161481481	16.43111111
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0.65162989	
P(T<=t) one-tail	0.280554069	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.561108137	
t Critical two-tail	3.182449291	

Layer 6

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	30.90521886	31.77763158
Variance	3.887051406	9.199001039
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-0.41771329	
P(T<=t) one-tail	0.3521262	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.7042524	
t Critical two-tail	3.182449291	

Cell Size Analysis

To examine whether the E33 MAM treatment affected the size of cells within somatosensory cortex, the size of cell profiles were measured within a grid of 500 µm wide, 100 µm deep boxes extending from the top of layer 2 through cortex with the Image Pro Plus® program. Data were obtained from 5 coronal sections each of 3 normal and 3 E33 MAM-treated, 12 week old ferrets. Only cells in which the full profile was stained with the Nissl substance and a single nucleoli was visible within the nucleus were included for analysis. The data was entered into Microsoft Excel®, mean values obtained from the 5 measurements for each animal, and t-tests performed to test for significant differences between the mean values of the normal versus E33 MAM-treated animals.

Layer 2

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	117.2158667	103.6347333
Variance	40.12216422	117.5894168
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	1.87311675	
P(T<=t) one-tail	0.078882926	
t Critical one-tail	2.353363016	
P(T<=t) two-tail	0.157765851	
t Critical two-tail	3.182449291	

Cell Size Analysis

Layer 3

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	143.0843407	134.6125333
Variance	78.5178369	1.091068213
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	1.644583301	
P(T<=t) one-tail	0.120892806	
t Critical one-tail	2.91998731	
P(T<=t) two-tail	0.241785612	
t Critical two-tail	4.302655725	

Layer 4

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	128.5016667	125.9238667
Variance	75.4906781	6.036559773
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	0.494491103	
P(T<=t) one-tail	0.334968591	
t Critical one-tail	2.91998731	
P(T<=t) two-tail	0.669937183	
t Critical two-tail	4.302655725	

Cell Size Analysis

Layer 5

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	160.0394889	154.5010667
Variance	160.2532693	134.2332206
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	0.559002823	
P(T<=t) one-tail	0.302987838	
t Critical one-tail	2.131846486	
P(T<=t) two-tail	0.605975676	
t Critical two-tail	2.776450856	

Layer 6

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	129.0813374	126.327145
Variance	70.08829579	3.661949695
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	0.555486053	
P(T<=t) one-tail	0.317201708	
t Critical one-tail	2.91998731	
P(T<=t) two-tail	0.634403417	
t Critical two-tail	4.302655725	

APPENDIX B: Since different numbers of cells migrated away from each injection site, non-parametric statistical analyses compared the percent of VZ cells from each injection that migrated into the cortical plate of the cultures. In addition, the relative position of VZ cells within the cortical plate (upper, middle, or lower cortical plate) was compared between groups. The upper cortical plate included bins within 200 μm from the pial surface. The middle cortical plate included bins within 200 to 400 μm from the pial surface, and the lower cortical plate included those bins within 400 to 600 μm from the pial surface (see Figure 22). The number of cells that migrated away from each injection sites was set to 100 percent. For the first set of statistical analyses - *Comparison of E33 vs. P1 Cells in Lower, Middle, and Upper Cortical Plate* - the percent of E33, P1, and P3 VZ cells that migrated into the cortical plate of the organotypic cultures (normal and MAM-treated cultures combined) was compared. For the second set of statistical analyses - *Comparison of the percent of E33 (or P1) Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals* - the percent of each type of VZ cell (E33 or P1) that reached the cortical plate was compared in normal versus MAM-treated organotypic cultures. Very few P3 cells migrated into the cortical plate, and therefore these cells were not included in statistical analyses. Statistical tests were performed with the SigmaStat® program and results from these tests are included below.

Comparison of E33 vs. P1 Precursor Cells in Lower Cortical Plate

Mann-Whitney Rank Sum Test - Data source: E33 vs P1 Data in Notebook

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.810)

Group	N	Median	25%	75%
E33 Lower	8	0.102	0.0727	0.113
Nor Lower	6	0.0759	0.0522	0.135

T = 42.000 n(small)= 6 n(big)= 8 P(est.)= 0.747 P(exact)= 0.755

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.755).

Comparison of E33 vs. P1 Precursor Cells in Mid Cortical Plate

Mann-Whitney Rank Sum Test - Data source: E33 vs P1 Data in Notebook

Normality Test: Failed (P = 0.001)

Group	N	Median	25%	75%
P1 Middle	12	0.0196	0.00555	0.0379
E33 Middle	14	0.0700	0.0448	0.108

T = 102.000 n(small)= 12 n(big)= 14 (P = 0.002)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Comparison of E33 vs. P1 Precursor Cells in Upper Cortical Plate

Mann-Whitney Rank Sum Test - Data source: E33 vs P1 Data in Notebook

Normality Test: Passed (P > 0.200)

Equal Variance Test: Failed (P = <0.001)

Group	N	Median	25%	75%
P1 Upper	12	0.0496	0.0144	0.0836
E33 Upper	14	0.0160	0.00435	0.0279

T = 201.500 n(small)= 12 n(big)= 14 (P = 0.045)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.045).

Comparison of the percent of E33 Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals.

Non-parametric tests were performed to compare the percentage of E33 cells that migrated into the cortical plate of cultures prepared from E33 MAM-treated neonates versus cultures prepared from normal neonates.

Total cortical plate

Mann-Whitney Rank Sum Test - Data source: E33 cells arranged in Notebook

Normality Test:		Failed	(P = 0.008)	
Group	N	Median	25%	75%
E33 total	24	0.0761	0.0250	0.108
Nor total	18	0.0483	0.0217	0.0690

T = 326.000 n(small)= 18 n(big)= 24 (P = 0.124)

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.124).

Comparison of the percent of E33 Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals.

Lower Cortical Plate

Mann-Whitney Rank Sum Test - Data source: E33 cells arranged in Notebook

Group	N	Median	25%	75%
E33 Lower	8	0.102	0.0727	0.113
Nor Lower	6	0.0759	0.0522	0.135

T = 42.000 n(small)= 6 n(big)= 8 P(est.)= 0.747 P(exact)= 0.755

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.755).

Middle Cortical Plate

Mann-Whitney Rank Sum Test - Data source: E33 cells arranged in Notebook

Normality Test: Passed (P = 0.022)

Equal Variance Test: Passed (P = 0.101)

Group	N	Median	25%	75%
E33 Mid	8	0.0991	0.0700	0.190
Nor Mid	6	0.0512	0.0391	0.0690

T = 29.000 n(small)= 6 n(big)= 8 P(est.)= 0.045 P(exact)= 0.043

The difference in the median values between the 2 groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.043).

Comparison of the percent of E33 Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals.

Upper Cortical Plate

Mann-Whitney Rank Sum Test - Data source: E33 cells arranged in Notebook

Group	N	Median	25%	75%
E33 Upper	8	0.0190	0.00375	0.0519
Nor Upper	6	0.0108	0.00430	0.0217

T = 40.000 n(small)= 6 n(big)= 8 P(est.)= 0.561 P(exact)= 0.573

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.573).

Comparison of the percent of P1 Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals.

Non-parametric tests were performed to compare the percentage of P1 cells that migrated into the cortical plate of cultures prepared from E33 MAM-treated neonates versus cultures prepared from normal neonates.

Total Cortical Plate

Mann-Whitney Rank Sum Test - Data source: P1 Cells Arranged in Notebook

Normality Test: Failed (P = 0.004)

Group	N	Median	25%	75%
Nor Total	18	0.0118	0.000	0.0429
E33 Total	18	0.0339	0.0111	0.0784

T = 278.000 n(small)= 18 n(big)= 18 (P = 0.084)

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.084).

Comparison of the percent of P1 Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals.

Lower Cortical Plate

Mann-Whitney Rank Sum Test - Data source: Data 2 in Notebook

Group	N	Median	25%	75%
E33 Lower	6	0.0170	0.000	0.0784
Nor Lower	6	0.0471	0.000	0.0539

T = 37.000 n(small)= 6 n(big)= 6 P(est.)= 0.810 P(exact)= 0.818

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.818).

Middle Cortical Plate

Mann-Whitney Rank Sum Test - Data source: Data 2 in Notebook

Group	N	Median	25%	75%
E33 Mid	6	0.0196	0.0111	0.0339
Nor Mid	6	0.0213	0.000	0.0419

T = 39.000 n(small)= 6 n(big)= 6 P(est.)= 0.936 P(exact)= 1.000

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Comparison of the percent of P1 Precursor Cells that migrated into the cortical plate of cultures prepared from normal versus E33-MAM treated animals.

Upper Cortical Plate

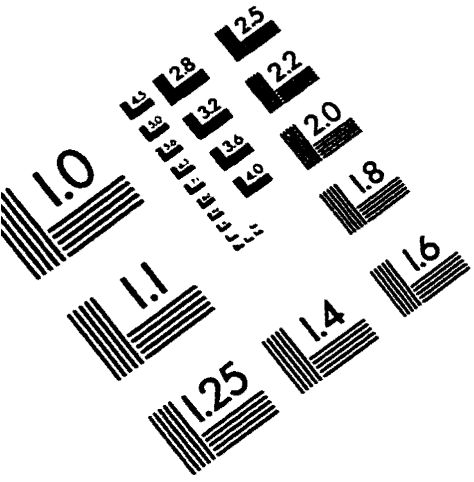
Mann-Whitney Rank Sum Test - Data source: Data 2 in Notebook

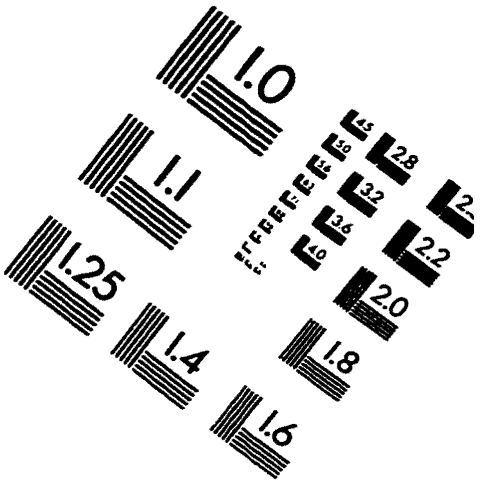
Group	N	Median	25%	75%
E33 Upper	6	0.0733	0.0593	0.115
Nor Upper	6	0.000	0.000	0.00480

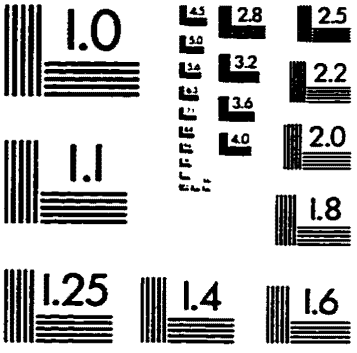
T = 57.000 n(small)= 6 n(big)= 6 P(est.)= 0.005 P(exact)= 0.002

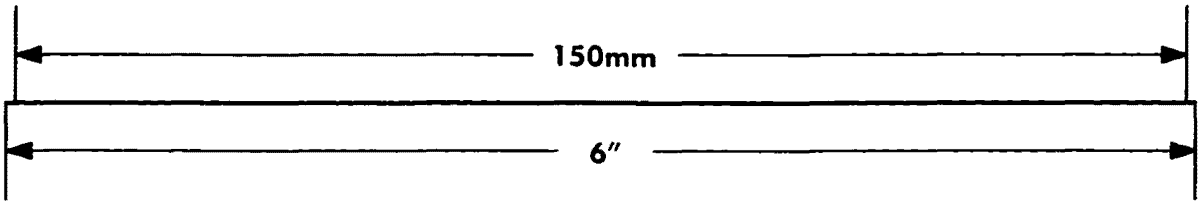
The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

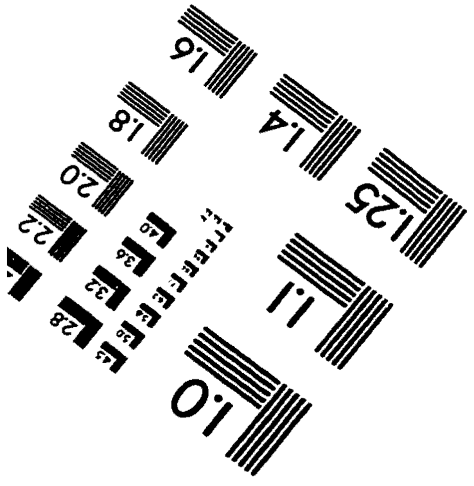
IMAGE EVALUATION
TEST TARGET (QA-3)

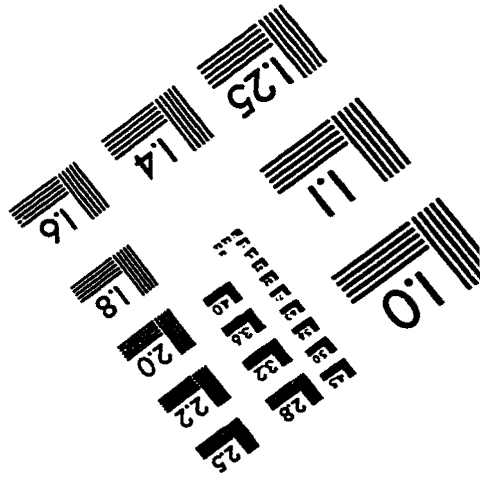












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