Cortical Target Depletion and the Developing Lateral Geniculate Nucleus: Implications for Trophic Dependence

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The dependence of the developing dorsal lateral geniculate nucleus (LGd) on visual cortex for survival has been well documented. Complete removal of visual cortex during early postnatal development results in degeneration of the LGd. To further explore the nature of this trophic relationship, we depleted variable proportions of the principal targets of geniculocortical axons, layer IV neurons, and also variable proportions of the supragranular neurons by intraperitoneal injections of different dosages of a mitotic inhibitor MAM (methylazoxymethanol acetate) into pregnant hamsters at the time when these neurons were being generated in the ventricular zone. We demonstrate that after more than 75% loss of layer IV there is no reduction in cell number in the LGd. HRP (horseradish peroxidase) injections into the LGd in adult animals reveal an essentially normal pattern of termination without evidence of rerouting of geniculocortical axons to other cortical areas, nor compensatory increase in arborization in layer VI and VIb (subplate). Geniculocortical axons terminate principally in the middle stratum of the depleted cortex above layer V, with obvious reduction in both the extent and density of arborization. After higher dosages of MAM treatment resulting in more severe cell loss in layers II–IV with the apparent loss of layer IV, the extent and density of geniculocortical arborization are further reduced. Reduction in size as well as total number of geniculate neurons become detectable. Above depletions of 75% of layer IV neurons, the number of surviving LGd neurons is linearly related to the total number of remaining layer II–IV neurons in the cortex. These findings are discussed in light of the possible trophic mechanisms that match cell populations in number during development.

Naturally occurring cell death is ubiquitous in the developing nervous system, and in many locations serves as one of the factors that regulates relative numbers of cells in interconnected regions (Cowan et al., 1984; Finlay and Pallas, 1989; Oppenheim, 1991; Finlay, 1992). In the neocortex, while cell death has been generally ruled out as the mechanism pruning the widespread axonal connections of the developing cortex (O'Leary et al., 1981; O'Leary and Stanfield, 1986), in the early development of the subplate and the cortical plate, particularly granular and supragranular layers, cell death is prominent, locally variable and contributes to determine adult neuron numbers in both normal and experimental conditions (Kostovic and Rakic, 1980; Finlay and Slattery, 1983; Luskin and Shatz, 1985; Chun and Shatz, 1989; Windrem and Finlay, 1991; Woo et al., 1991; for review, see Finlay, 1992).

In most developing neural systems, neurons must derive trophic support from their targets or afferents to survive (Purves, 1988; Jacobson, 1991; Linden, 1994). Lateral geniculate neurons in the developing visual system depend critically on visual cortex for survival. Ablation of visual cortex during development at the time when geniculocortical axons are establishing permanent connections with their presumptive targets, layer IV neurons, results in nearly complete degeneration of the LGd (Schneider, 1970; Cunningham et al., 1979; Raabe et al., 1986). Further, LGd neuron survival can be modulated by a diffusible trophic factor derived from the cortex (Cunningham et al., 1987, 1988). The cellular source, time course, and mode of action of the presumptive diffusible trophic factor is not understood, nor whether it is the only trophic interaction of thalamus and cortex.

During development of the cerebral cortex, the earliest arriving axons are from the dorsal thalamus (Catalano et al., 1991; De Carlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1990, 1992; Miller et al., 1993). At the time when these axons arrive at the subplate compartment, their future targets, layer IV neurons, have yet to migrate into the developing cortical plate (Lund and Mustari, 1977; Rakic, 1974, 1982; Luskin and Shatz, 1985; Shatz and Luskin, 1986; Bayer and Altman, 1991; Erzurumlu and Jhaveri, 1992; Miller et al., 1993). Thalamic axons then establish transient synaptic connections with at least a subpopulation of subplate neurons (Friauf et al., 1990; Hermann et al., 1991) before finally invading the cortical plate after the arrival of layer IV neurons and establishing permanent connections with these neurons. A variety of possible functions of this transient synaptic circuit have been suggested (Friauf et al., 1990; Ghosh et al., 1990; Kostovic and Rakic, 1980; Ghosh and Shatz, 1992, 1993, 1994; for review, see Allendoerfer and Shatz, 1994). Trophic interactions between subplate neurons and the "waiting" thalamic axons might be instrumental in sustaining the survival of the two populations of neurons during the waiting period.

In the present study, we attempted to further explore the mechanisms involved in the control of cell number in the lateral geniculate in light of the possible trophic interactions between geniculate neurons and their major targets, layer IV neurons, during development by removing variable proportions of layer IV and supragranular neurons in the hamster cortex by means of application a potent mitotic inhibitor MAM (methylazoxymethanol acetate) at the time when these neurons were being generated (Johnston and Coyle, 1979, 1980, 1982; Jones et al., 1982; Ashwell, 1987; Ciaroni et al., 1989, 1992). We have quantified the amount of LGd cell survival in relation to the number of neurons in layers IV and II/III over a wide range of depletions, and any secondary effects granular and supragranular cell depletions might have on the cortical subplate. We have also examined the altered location and density of the mature geniculocortical projection, with particular attention to the possibility of a stabilized thalamocortical projection to the subplate region.

Preliminary results of this series of studies have been published in abstract form (Woo and Finlay, 1991; Woo et al., 1992, 1993).

Materials and Methods

A total of 49 golden hamsters of different postnatal ages were used in this study. Differential depletion of upper cortical layers (principally layer IV, with variable cell loss in layers II/III, depending on the dosage as described below) was achieved by intraperitoneal (i.p.) administration of different dosages of MAM (Sigma) to timed-pregnant dams on embryonic (E) day 14 when the major population of layer IV neurons and part of layer II/III neurons are normally being generated, but the generation of geniculate neurons has been com-

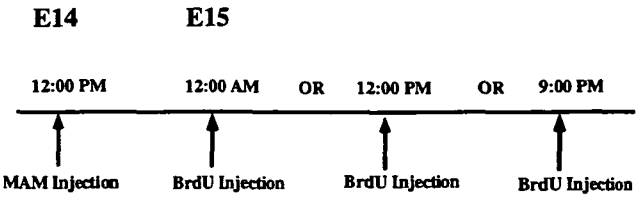


Figure 1. Schematic drawing showing BrdU injection paradigm. Animals were injected with MAM on E14. They were then divided into three groups receiving BrdU injections 12, 24, or 33 hr later, respectively. The same BrdU injection paradigm applies to the control animals except that they did not receive any MAM injections on E14.

pleted (generation of the geniculate begins at E9.5 and ends at E12; Crossland and Uchwat, 1982; unpublished tritiated thymidine data).

Administration of MAM

Timed-pregnant hamsters from our breeding colony were injected intraperitoneally with different dosages (20, 25, 30 mg/kg, or double doses of 20 mg/kg each with the second dose injected 9–12 hr after the first dose given on E14) of MAM on E14. After MAM injections, all of the animals delivered following the normal gestational time course except that about 30% of animals that had received the highest dosages of MAM (i.e., 30 mg/kg or double doses) either did not deliver on time or died before delivery. Only pups that were delivered following the normal gestational schedule were included in this study.

BrdU Immunohistochemistry

To assess the effectiveness and the time course of action of MAM on cortical depletion, we injected the DNA analogue 5-bromo-2′-deoxyuridine (BrdU) at different time points (Fig. 1) after injections of 20 mg/kg MAM (the smallest dosage that we used) so that we could quantitatively estimate the extent and relative amount of loss of layer IV. Pregnant animals received MAM injections of 20 mg/kg on E14 as described above. These experimental animals were then divided into three groups, which received BrdU intraperitoneal injections on 12, 24, or 33 hr, respectively, after MAM injections. BrdU (75 μg/g body weight) was dissolved in 0.007 M NaOH in normal saline to make the injection solution with the final concentration of 5 mg/ml. Normal controls were also divided into three groups receiving identically timed BrdU injections except that they did not receive any MAM treatment.

After pups were delivered, they were allowed to survive until P20, when they received a lethal dose of intraperitoneal injection of pentobarbital sodium. They were then perfused through the heart first with normal saline for 10 min and then with 70% alcohol for 20 min. Brains were then removed from the skull and were postfixed in 70% alcohol for a week before they were dehydrated through serial changes of alcohol and embedded in paraffin. Brains were then cut at 10 μm thickness on a sliding microtome. Sections were mounted, dried, dehydrated, deparaffinized, and rehydrated before being processed for immunohistochemistry.

Immunohistochemistry was performed based on the protocol by Miller and Nowakowski (1988; Takahashi et al., 1992). Rehydrated mounted sections were incubated with 0.07 M NaOH for 2 min or 2 M HCl for 30 min to denature ds-DNA and expose BrdU binding sites. They were then washed in 0.1 M phosphate buffer (pH 8.5 if used NaOH and pH 6.0 if used HCl) for 30 sec and incubated with anti-BrdU antibody (Becton Dickinson, diluted 1:20 in pH 7.4 phosphate buffer) for 30 min. After washing in phosphate buffer (pH 7.4) for 10 min, sections were then incubated with a biotinylated anti-IgG antibody (Vectastain ABC kit, Vector) for 30–45 min. After a second wash with phosphate buffer (pH 7.4) for 10 min, the ABC solution (Vectastain ABC kit, Vector) was added to the sections for 45–60 min. After a final wash with phosphate buffer (pH 7.4), sections were incubated first in 3,3′-diaminobenzidine (DAB) solution (0.15 gm/300 ml pH 7.4 phosphate buffer plus 7.5 ml of 1% cobalt chloride and 6 ml of 1% nickel ammonium sulfate) for 10 min before hydrogen peroxide (0.64 ml 3% H_2O_2/50 ml DAB) was added. After incubation for 20 min, sections were rinsed with phosphate buffer (pH 7.4), dried, dehydrated, and coverslipped with Permount (Fisher).

Tissue Processing for Cell Counting

Normal and MAM-treated pups of 20-d-old (P20) were injected with a lethal dose of pentobarbital sodium and were then perfused through the heart first with normal saline for 15 min followed by Perfix (Fisher) for 20 min. Brains were removed from the skull and were postfixed for at least 1 week. They were then dehydrated, embedded in paraffin, and cut at 10 μm on a sliding microtome. Sections were mounted, dried, dehydrated, deparaffinized, rehydrated, stained with cresyl violet, and coverslipped with Permount (Fisher). Sections were examined under a Leitz Diaplan or Olympus BH-2 microscope and cells were counted with the aid of a computerized camera lucida system attached to a BH-2 Olympus microscope.

Quantification of Cell Number in the Neocortex and Lateral Geniculate Nucleus

Neocortex

Since layer IV is still identifiable (see Results for details) after the lowest dosage (20 mg/kg) of MAM treatment, we were able to estimate the absolute number of neurons in this layer in this condition alone. Layer IV was distinguished from layer III and V based on morphological and cytological criteria. Layer IV is principally composed of granule neurons with relatively small soma size compared with the large pyramidal neurons of layer V below and the small to medium sized pyramidal neurons of layers II and III above. Furthermore, neuronal density of this layer also tends to be higher than adjacent layers. In normal and small dose MAM brains, these criteria allowed us to identify layer IV and thus estimate cell number within this particular layer. However, after 25 mg/kg or higher dosages of MAM, layer IV is no longer clearly identifiable based on cytoarchitectonic criteria. We thus estimated the total number of all the cells above layer V and below layer I, for instance, layers II–IV, for our analysis and this population of cells are referred to as layer II–IV cells in the rest of the article. In both cases, we first identified the rostrocaudal and mediolateral extent of visual cortex based on cytoarchitectonic criteria with reference to the rat atlas by Paxinos and Watson (1986) and our own atlas for hamsters (Windrem and Finlay, unpublished observations). In animals in which layer IV is no longer identifiable, we based our identification of tangential borders of visual cortex on extrapolating data from normal and 20 mg/kg animals and compared them with the corresponding anatomical landmarks. We then measured the cross-sectional areas of five equally spaced sections throughout the rostrocaudal extent of visual cortex and estimate the volume of the layer in visual cortex by applying the Cavalieri's estimator:

$$V_{total} = d[\Sigma(y_i)] - (t)y_{max},$$

where d is the distance between analyzed sections, y_i is the cross-sectional area of the ith section, t is section thickness, and y_{max} is the maximum value of y (see Rosen and Harry, 1990, for details of different methods of volumetric analysis).

We then counted the number of neurons in the layer(s) of interest using the same five sections for volumetric analysis. Mean cell density was then computed by dividing absolute cell number by the corresponding volume. Counting error and split cell error were corrected for by applying the Floderus modification of the Abercrombie (1946) correction. Multiplication of cell density and volume gave the estimated total cell number of layer IV or layer II–IV for each brain.

The subplate was assessed both early in development and after the main period of cell loss in the hamster cortex. We traced the developmental history of subplate neurons in the whole cortex by measuring subplate cell number on P4 (before the normal cell death period), P6 and P7 (during the normal cell death period), and finally on P20 in animals that received 20 mg/kg MAM on E14, resulting in substantial loss of layer IV (see Results). These data were then compared to the subplate cell death curve in normal animals (Woo et al., 1991). Subplate cell number was estimated as above. We also estimated subplate cell number in P20 visual cortex in both normal animals and animals receiving all the different dosages of MAM.

Lateral Geniculate Nucleus

The same brains used for neocortical quantification were used for the estimation of cell number in the lateral geniculate nucleus. We first performed a three-dimensional reconstruction of the lateral geniculate nucleus. We then identified five equally spaced sections and

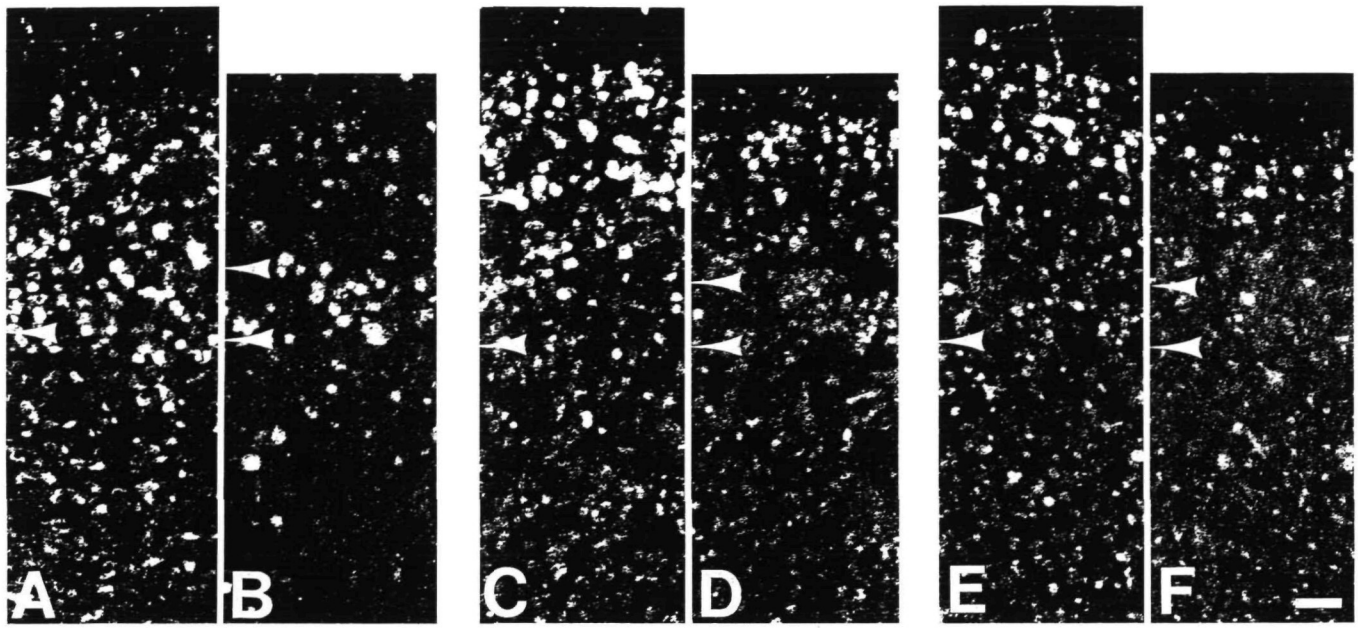


Figure 2. Dark-field photomicrographs of BrdU label in visual cortex at various time points after 20 mg/kg MAM injections. Twelve hours after MAM injections, in the normal cortex (*A*), BrdU label is seen mainly in layer IV, although some label is also present in layers V and III. In the MAM cortex (*B*), the amount of BrdU label is severely reduced and layer IV is most severely affected. Twenty-four hours after MAM injections, in the normal cortex (*C*), BrdU label is concentrated in layer IV and layer II/III. Again, after MAM treatment (*D*), amount of label is much reduced. Thirty-three hours after MAM injections, the majority of BrdU label is seen in layer II/III in the normal cortex (*E*). In the MAM cortex (*F*), there is still reduction in the amount of label even though the direct toxic effect of MAM should have subsided by this time. This observation is likely to be due to the reduction in the number of progenitor cells in the ventricular zone resulting from previous toxic effects. *Arrowheads* mark the upper and lower boundary of layer IV determined by comparing with adjacent sections stained with cresyl violet. Scale bar, 50 μm.

measured their areas, computing cell number and density as described above for the neocortical layers.

Adult Pattern of Geniculocortical Arborization

Horseradish peroxidase (HRP) was used to label geniculocortical projections in adult hamsters. A total of five animals including three normal and two MAM (20 mg/kg) animals were used. Animals were anesthetized by intraperitoneal injections of pentobarbital sodium and were then stabilized in stereotaxic equipment. A small hole was drilled through the skull at the coordinates determined by our own stereotaxic atlas for hamsters (unpublished observations). A small volume (0.2–0.5 μl) of 30% HRP solution (dissolved in distilled water) was injected focally into the LGd using a Hamilton syringe. After surgery, animals were allowed to recover from anesthesia and were returned to the colony. Forty-eight to 64 hr after the surgery, animals were deeply anesthetized with an overdose of pentobarbital and perfused through the heart first with cold normal saline for 10 min followed by a mixture of 2% glutaraldehyde and 1.5% paraformaldehyde at 4°C for 20 min. Brains were then removed from the skull and postfixed for 6 hr in the same fixative before being put into 30% sucrose solution overnight for cryoprotection. After brains sank in the sucrose solution, they were embedded in albumin/gelatin and were cut coronally at 120–150 μm on a freezing microtome. Sections were collected into phosphate buffer (0.1 M, pH 7.4) and free-floating sections were then incubated in DAB solution (100 mg DAB/200 ml pH 7.4 phosphate buffer plus 5 ml 1% cobalt chloride and 4 ml 1% nickel ammonium sulfate) for 20 min. Hydrogen peroxide (0.64 ml 3% H_2O_2 solution/50 ml DAB solution) was then added to the DAB solution and the reaction was allowed to proceed for 15 min. Sections were then rinsed in phosphate buffer (pH 7.4) for several minutes. They were then mounted, dried, dehydrated, and finally coverslipped with Permount (Fisher). Sections were then examined under a Leitz Diaplan microscope.

Results

Specificity and Time Course of Cortical Depletion

MAM has its effect through the selective methylation of the guanine in position C_7 thus inhibiting DNA replication during S phase of the cell cycle; therefore, only cells that are dividing are subject to its direct toxic effects (Johnston and Coyle, 1982). In previous studies, the mitotic inhibitory effect of MAM begins about 2 hr postinjection and lasts for about 24 hr, as shown by experiments of quantification of DNA contents in the ventricular zone at different times following systemic administration of the drug (Matsumoto et al., 1972). Since both neurons undergoing their "birthday" at the time of MAM activity and dividing precursor cells in the ventricular zone will die as a result, both the neurons generated on the targeted birthday and the total number of cells in the remaining precursor pool that will give rise to later-generated cells will be depleted. Neurons and other cell populations with earlier birthdays should not be directly affected.

For the present study, on E14 in the hamster, layer IV and also part of layer II/III of the posterior (presumptive visual) cortex are actively being produced while the neurons comprising layers V and VI and the subplate have already been generated (Shimada and Langman, 1970; Crossland and Uchwat, 1982; Woo et al., 1991); thus, application of MAM at this time should have selective effects on the upper layers, most severely layer IV, but have no direct effect on the infragranular layers. The LGd is generated on embryonic day 9.5 through 12 in the hamster, and similarly should not be affected (Crossland and Uchwat, 1982; Woo et al., 1991).

We verified the time course of the direct MAM effect on the cortex by BrdU immunohistochemistry for the 33 hr following MAM administration. BrdU injections at 12 and 24 hr after MAM injections confirm that layer IV was the major site of cell loss after 20 mg/kg MAM (Fig. 2). Twelve hours after MAM injection, layer IV was the major population of cells being generated as shown in the control cortex (Fig. 2*A*), although a small number of layer III and V cells were also being generated at the same time. Major reduction in the number of BrdU-labeled neurons is shown in Figure 2*B*, in the MAM-

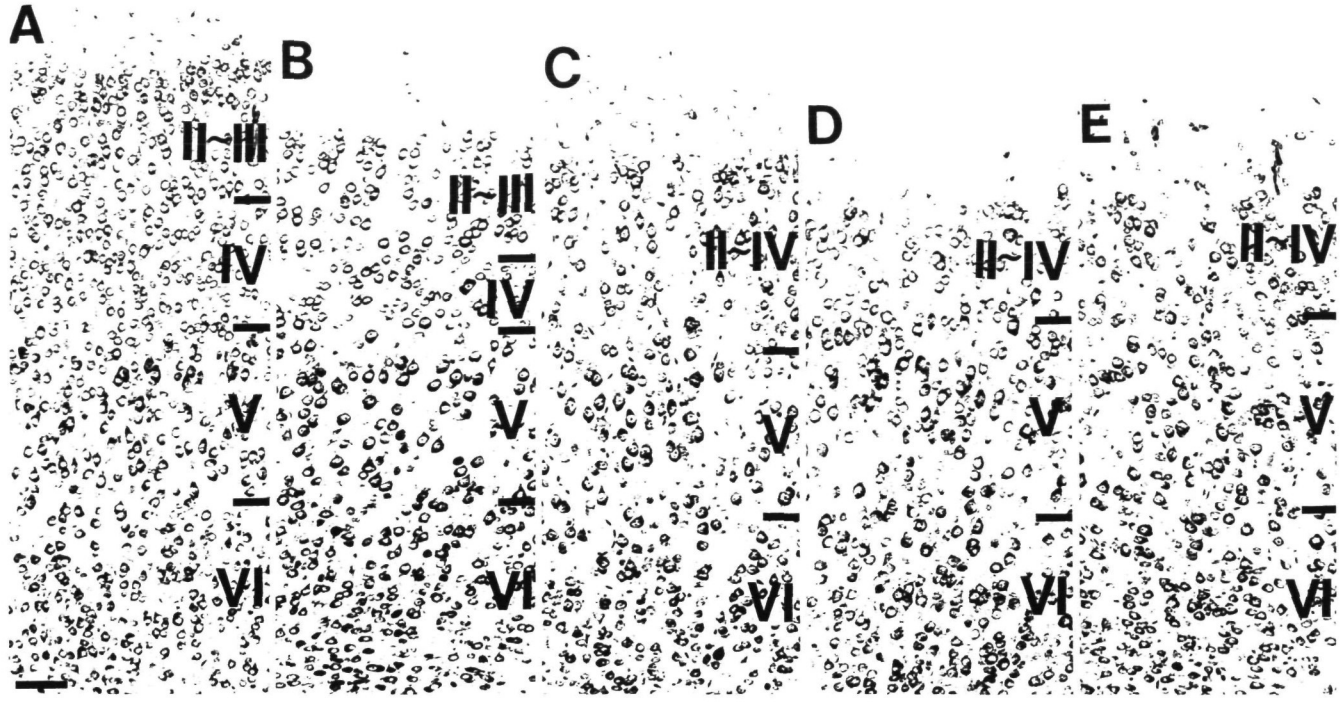


Figure 3. Photomicrographs of Nissl-stained coronal sections of visual cortex of P20 normal and MAM animals receiving different dosages of MAM treatment. *A*, Normal visual cortex. *B*, MAM cortex, 20 mg/kg. Thickness of upper cortical layers, especially layer IV, is reduced. *C*, MAM cortex, 25 mg/kg. Layer IV is no longer distinguishable in most of the brains after this dosage of treatment. *D*, MAM cortex, 30 mg/kg, and *E*, MAM cortex, double doses of 20 mg/kg. Layer IV is apparently absent and the whole upper cortex is much thinner. In all of the above cases, layers V and VI are relatively unaffected. All photomicrographs are cropped at the bottom of layer VI. Scale bar, 50 μm.

treated cortex. On E15 in normal development, neurogenesis is centered in layer III with some layer IV and II cells also produced (Fig. 2*C*). Figure 2*D* shows the reduction in the number of BrdU-labeled cells on E15, 24 hr after MAM treatment on E14. In the normal cortex on day 15.5 (Fig. 2*E*), layer II and III neurons are being generated, in reduced amount as corticogenesis ends. In the MAM cortex on E15.5, 33 hr after injection (Fig. 2*F*), the amount of BrdU label is still reduced compared to that in the normal brain. Since previous work shows that the direct neurotoxic effect of MAM should have subsided by this time, the reduction in neurogenesis indicated by the reduction of BrdU label is likely due to reduction in the number of progenitor cells in the ventricular zone on E14–15. A compensatory upregulation of the precursor pool after MAM depletion is a possible outcome of the treatment, and we find no evidence for this possibility.

Effect of MAM on Cortical Cell Number

Different dosages of MAM treatment result in large differences in the amount of depletion of layer IV and the supragranular layers. The effects of these different dosages will be described in turn.

20 mg/kg MAM

This dosage, the smallest used, results in significant cell loss in all upper cortical layers, including layers II, III, and IV. The layer affected most visibly is layer IV (Fig. 3*B*), as determined by morphological and cytoarchitectural change of the cortex and by reduction in absolute cell number. Morphologically, layers V and VI are relatively unaffected, and there is no significant change in thickness of these layers (mean total thickness ± SD of layers V and VI: normal = 387.94 ± 17.87 μm, MAM = 382.31 ± 13.42 μm, $t = 0.57, n = 6, p = 0.59$). Layer IV, which is normally composed of small neurons with relatively high density, is still easily identifiable, but much thinner. There is no obvious cytological derangement of residual layer IV. Layers II and III have been minimally affected. There is no sign of disruption of cytoarchitecture or neuronal arrangement except that the two layers are slightly thinner than normal (total thickness of layers II and III: normal = 188.40 ± 5.46 μm, MAM = 173.42 ± 5.56 μm, $t = -5.76, n = 8, p = 0.0007$). We estimated the absolute number of neurons of layer IV alone and the total neuronal number of layers II–IV as a whole. There is 76% cell loss in layer IV alone (Fig. 4*A*; normal = 88,738 ± 11,494; MAM = 21,583 ± 2413; $n = 3, t = 10.53, p = 0.008$) and cell loss is 39% when neurons in layers II and III are also included in the cell count [Fig. 5; ANOVA, $F(4) = 70.06, p < 0.00001$]. Thus, application of this particular dosage of MAM on E14 results in major, but not total cell loss in layer IV, with minimal loss in layers II–III. Layers V and VI appear relatively unaffected.

Higher Dosages of MAM

With higher dosages of MAM, it was difficult to identify layer IV on cytoarchitectural criteria, particularly for brains receiving 30 mg/kg or double doses of MAM. Therefore, in all the analyses, instead of attempting to identify layer IV, as we did in 20 mg/kg brains, we simply counted all the neurons above layer V as a single layer to estimate cell loss (collectively referred to as layer II–IV). Because E-14 MAM injections appear to have a minimal effect on layer V cells at all dosages, the border between layer V, which is composed of large pyramidal neurons with relatively low density, and layer II–IV, of variable composition, above could be identified consistently in all the brains we had examined (Fig. 3*C–E*).

25 mg/kg MAM

After 25 mg/kg MAM injection, the average cell loss in layer II–IV is 59% [Fig. 5; ANOVA, $F(4) = 70.06, p < 0.00001$]. Morphologically, although thickness of layer II–IV is much re-

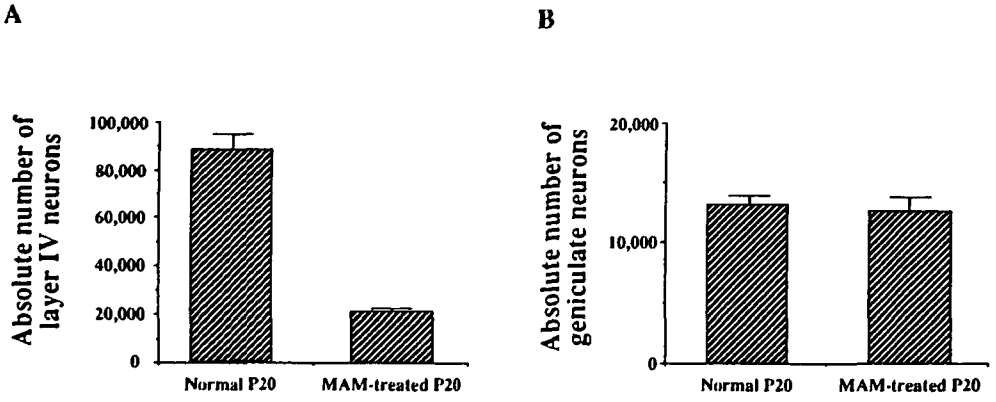


Figure 4. *A*, Absolute number of neurons in layer IV after 20 mg/kg MAM treatment on E14 compared with that in the normal cortex. Cell loss in layer IV is 76% (normal = 88,738 ± 11,494, $n = 3$; MAM = 21,583 ± 2413, $n = 4$; $t = 10.53$, $p = 0.008$). *B*, Absolute number of neurons in the lateral geniculate after 76% of layer IV cell loss compared with that in the normal brain. There is no significant cell loss in the geniculate (normal = 13,112 ± 785, $n = 4$; MAM = 12,599 ± 1,222.9, $n = 4$; $t = 0.548$, $p = 0.63$).

duced, there is no significant disruption of neuronal arrangement. In some of the brains, layer IV is still identifiable as a distinct layer.

30 mg/kg MAM

In animals that received higher dosages of MAM treatment, the thickness of the whole upper cortical layer above layer V is much reduced (Fig. 3*D*). In the visual cortex of a normal brain, layers II to IV comprise about 50% of the thickness of the whole cortex. In the 30 mg/kg MAM-treated brains, this ratio is reduced to about 20%. Absolute cell loss in layer II-IV in these brains is 71% [Fig. 5; ANOVA, $F(4)$ eq 70.06, $p < 0.00001$], with layer IV unidentifiable. Again, layers V and VI are relatively unaffected.

Double Doses of 20 mg/kg MAM

Effect of cortical depletion after double doses of MAM is very similar to that of 30 mg/kg animals. Briefly, thickness of the upper cortical layer is much reduced (Fig. 3*E*) and cell loss in layer II-IV is 74% [Fig. 5; ANOVA, $F(4) = 70.06$, $p < 0.00001$]. No significant morphological change in layers V and VI can be observed.

Effect of Cortical Depletion on Geniculate Cell Number

After 76% of cell loss in layer IV (39% of cell loss in layers II-IV; 20 mg/kg MAM), the major target layer of thalamic afferents, we did not observe any change in cell number (Fig. 4*B*; normal = 13,112 ± 785, MAM_{20} = 12,599 ± 1,222.9; $t = 0.548$, $n = 4$, $p = 0.63$) or density (Fig. 6) in the lateral geniculate nucleus, nor is there any significant reduction in geniculate neuronal diameter (Fig. 7; normal = 11.86 ± 0.20 μm, MAM = 11.46 ± 0.54 μm; $t = 1.816$, $n = 4$, $p = 0.167$). However, after 59% of cell loss in layers II-IV, with the apparent loss of the whole layer IV in most cases (25 mg/kg MAM), we begin to detect significant cell loss (25%) in the LGd (Fig. 8; normal = 13,112 ± 785, MAM_{25} = 9954 ± 539; $t = 7.253$, $n = 4$, $p = 0.005$). There is also a significant reduction in neuronal diameter (Fig. 7; normal = 11.86 ± 0.20 μm, MAM_{25} = 10.31 ± 0.49 μm; $t = 8.785$, $n = 4$, $p = 0.0031$). After more extensive amount of cortical depletion (i.e., 71-74% of cell loss after 30 mg/kg or double doses of 20 mg/kg MAM), cell loss in the LGd becomes 31-42% (Fig. 8; normal = 13,112 ± 785, MAM_{30} = 9043 ± 394; $t = 5.459$, $n = 4$, $p = 0.01$; $MAM_{20\text{-}20}$ = 7591 ± 1345, $t = 6.350$, $n = 3$, $p = 0.02$). There is no further reduction in neuronal diameter (Fig. 7; MAM_{25} = 10.31 ± 0.49 μm, MAM_{30} = 10.33 ± 0.19 μm, $MAM_{20\text{-}20}$ = 10.19 ± 0.20 μm; $F = 0.17$, $p = 0.85$). When cell loss in the LGd becomes detectable (i.e., after 25 mg/kg or higher dosages of MAM), the number of surviving geniculate neurons becomes linearly dependent on the amount of remaining upper cortex (Fig. 9; $R = 0.896$, $F = 53.15$, $p < 0.00001$).

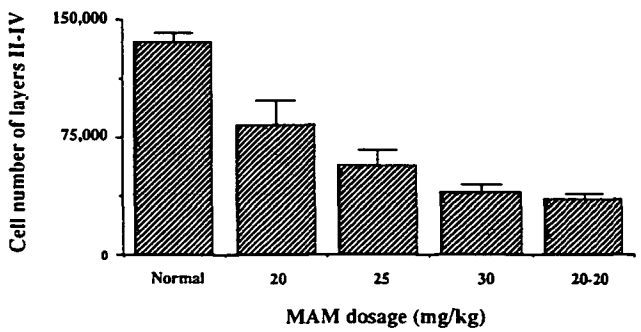


Figure 5. Upper cortical depletion effect of different dosages of MAM. All quantification was done on P20 animals. After 20, 25, 30, and double doses of treatment with MAM, cell loss in layers II–IV is 39%, 59%, 71%, and 74%, respectively (normal = 136,930 ± 5890, $n = 4$; MAM_{20} = 83,050 ± 16,460, $n = 4$; MAM_{25} = 56,360 ± 10,550, $n = 4$; MAM_{30} = 35,615 ± 3500, $n = 4$; $MAM_{20\text{-}20}$ = 39,900 ± 4900, $n = 4$; ANOVA, df = 4, $F = 70.06$, $p < 0.00001$).

Effect of Cortical Depletion on Subplate Cell Number

After 20 mg/kg MAM treatment resulting in up to 76% of layer IV depletion, there is no change in cell death in the subplate in the whole cortex at different postnatal ages compared to normal brains (Fig. 10; Woo et al., 1991). In the visual cortex alone, estimations of total number of neurons in the subplate layer show that no significant increase in subplate neurons' survival in P20 animals can be detected after different dosages of MAM treatment (Fig. 11; mean subplate neuronal number: normal = 26,555 ± 1977; MAM_{20} = 25,141 ± 1272; MAM_{25} = 26,130 ± 1378; MAM_{30} = 26,811 ± 1084; $MAM_{20\text{-}20}$ = 28,204 ± 3002; ANOVA: $F = 1.360$, $p = 0.297$). Therefore, subplate neurons die following the normal developmental schedule and there is no increase in their survival after severe depletion of layers IV and II/III.

Adult Pattern of Geniculocortical Arborization after More Than 75% of Cell Loss in Layer IV

It was surprising to find that substantial depletion of layer IV (after 20 mg/kg MAM administration) results in no excess cell death in the lateral geniculate. We therefore injected HRP into the geniculate and investigated the pattern of arborization of the geniculocortical pathway. Figure 12 shows the arborization pattern in both normal and MAM-treated visual cortex. After MAM treatment, geniculocortical axons terminate in layer VI and the middle cortical stratum including the remaining layer IV and the bottom part of layer III, showing a clear bilaminar pattern of termination, as in the normal cortex. The major difference between the normal and the MAM pattern is that in the MAM cortex both the extent of axonal arbori-

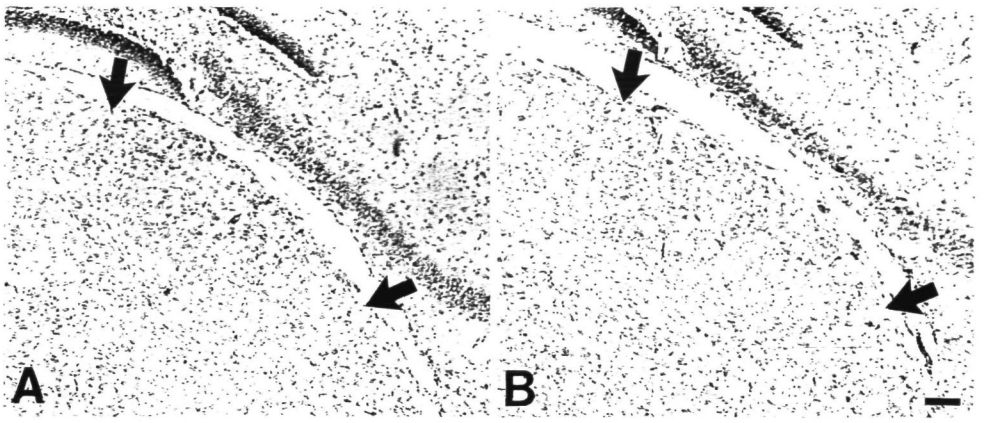


Figure 6. Photomicrographs of Nissl-stained coronal sections of the lateral geniculate nucleus in a normal (*A*) and a MAM (*B*, 20 mg/kg) P20 animal. There is no change in volume or neuronal density after severe cortical target depletion. *Arrows* outline the medial and lateral boundary of LGd. Scale bar, 100 μm.

zation and the arbor density in the upper lamina seem to have been reduced. However, there is no evidence of any compensatory increase in arbor density in layers VI and VIb (remaining subplate), nor is there any evidence of presence of geniculocortical axons in other cortical areas. In the animals receiving the highest dosage of MAM treatment (30 mg/kg), DiI was used to trace the development and the final establishment and arborization pattern of the geniculocortical axons and similar results were found (see following companion article, Woo and Finlay, 1996).

Discussion

Summary of Principal Conclusions

Our results have shown that even after more than 75% of cell loss in the target layer of geniculocortical axons, layer IV, there is no detectable cell loss in the dorsal lateral geniculate nucleus, showing that the possible trophic relationship between the numbers of cells is not a linear one. Reduction in density, and not repositioning of lateral geniculate axonal arbors in the middle cortical stratum, suggests that alteration in axon arbor size is the first mechanism employed to match connections in numerically disparate populations, as we have observed in population matching in the retinotectal connection (Xiong et al., 1994). When cell loss from 59% to 74% is induced in the whole layers II–IV, with layer IV no longer identifiable, geniculate cell number linearly decreases with decrease in the population of layer II–IV neurons. The observation that there is neither change in subplate neuron number under any of these conditions nor stabilization of thalamic axons in the subplate zone rules out the hypothesis that subplate neurons and layer IV neurons compete for trophic support from thalamic afferents and that it is the loss of subplate neurons to layer IV neurons in this competition process that results in subplate neurons' death.

Methodological Considerations

Time Course and Specificity of Neuronal Depletion

Due to the specific mode of action of MAM (Johnston and Coyle, 1982), only cells that are dividing during the time of administration of the drug should be directly affected. Reduction of the number of cells produced after the period of MAM administration is also likely in that MAM will kill both dividing precursor cells and cells undergoing terminal divisions; secondary effects on existing neurons through alteration in trophic support or potentially, supporting non-neural tissues are the subject of this study. The timing of the generation of cortical layers and the thalamus is favorable for the use of MAM for the questions posed here. Neurogenesis in the lateral geniculate body concludes before neurogenesis in layer IV of

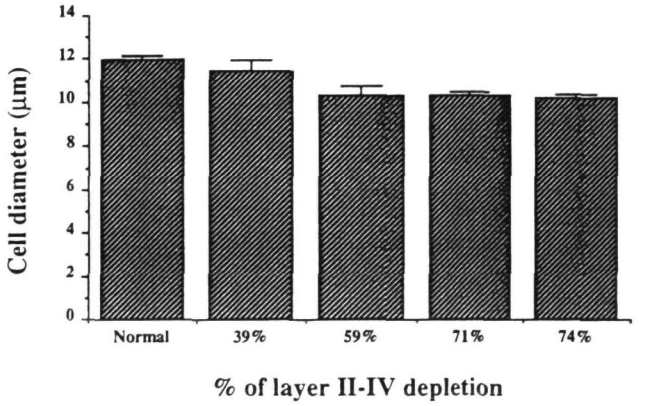


Figure 7. Effect of different degrees of cortical depletion on geniculate neuronal diameter. After 39% of cell loss in layers II–IV (20 mg/kg MAM), there is no reduction in neuronal diameter in the geniculate (normal = 11.86 ± 0.20 μm, MAM_{20} = 11.46 ± 0.54 μm, n = 4, t = 1.816, p = 0.167). After 59% of layer II–IV loss (25 mg/kg MAM), there is a significant reduction in neuronal diameter (MAM_{25} = 10.31 ± 0.49 μm, n = 4, t = 8.785, p = 0.0031). However, after more severe cortical depletion (30 mg/kg or double doses of MAM), there is no further reduction in neuronal diameter (MAM_{25} = 10.31 ± 0.49 μm, n = 4; MAM_{30} = 10.33 ± 0.19 μm, n = 4; $MAM_{20\text{-}20}$ = 10.19 ± 0.20 μm, n = 4; df = 3, F = 0.17, p = 0.85). All quantification was done on P20 animals.

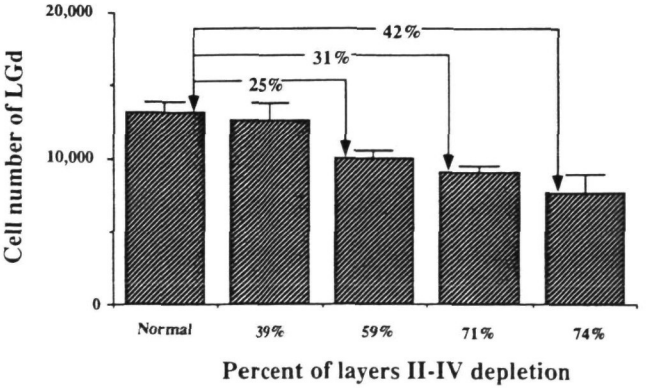


Figure 8. Effect of different amounts of cortical depletion on geniculate cell loss. After 39% of loss of layer II–IV neurons (20 mg/kg MAM), there is no cell loss in the geniculate. When cell loss in the cortex reaches 59% (25 mg/kg MAM), significant cell loss (25%) in the geniculate becomes detectable (normal = 13,112 ± 785, MAM_{25} = 9954 ± 539, n = 4, t = 7.253, p = 0.005). After 71–74% of cortical depletion (30 mg/kg and double doses of MAM respectively), cell loss in the geniculate becomes 31–42% (normal = 13,112 ± 785, MAM_{30} = 9043 ± 394, n = 4, t = 5.459, p = 0.01; $MAM_{20\text{-}20}$ = 7591 ± 1345, n = 4, t = 6.350, p = 0.02). All quantification was done on P20 animals.

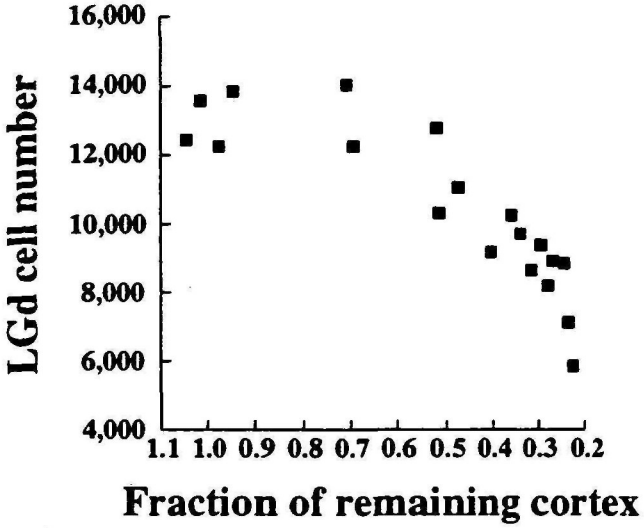


Figure 9. Cell loss in the lateral geniculate nucleus as a function of the amount of remaining upper cortex (layers II–IV). Geniculate cell number is initially relatively insensitive to cortical loss. However, as cortical loss reaches 59%, with layer IV no longer distinguishable, geniculate cell number becomes linearly dependent on the amount of remaining upper cortex.

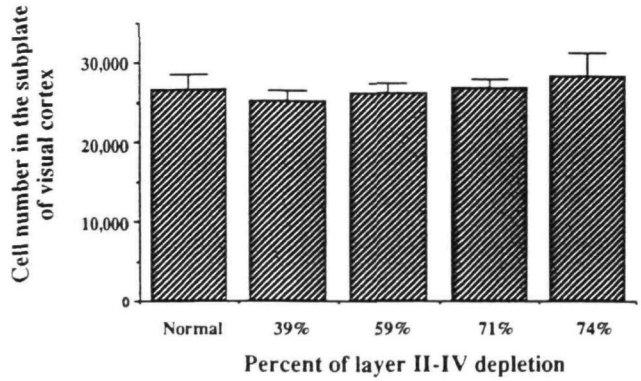


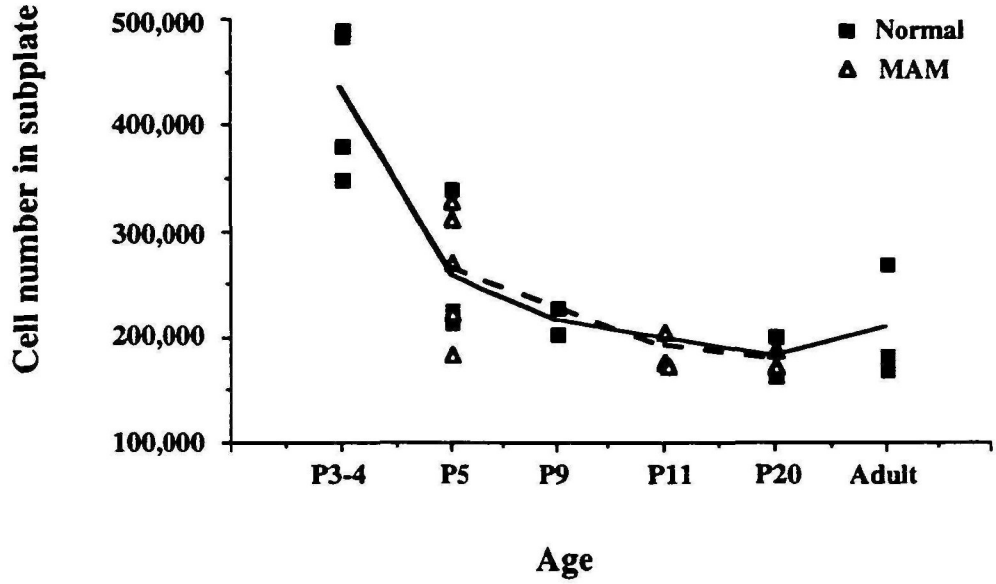
Figure 11. Number of subplate neurons in visual cortex in P20 normal and MAM animals. After different dosages (20, 25, 30 mg/kg, and double doses of 20 mg/kg) of MAM resulting in various amounts of layer II–IV loss (39%, 59%, and 71%, and 74%, respectively), there is no change in subplate neurons' survival (normal = 26,555 ± 1977, n = 4; MAM_{20} = 25,141 ± 1272; n = 4; MAM_{25} = 26,130 ± 1378, n = 4; MAM_{30} = 26,811 ± 1084, n = 4; $MAM_{20\text{-}20}$ = 28,204 ± 3002, n = 4; ANOVA: df = 3, F = 1.36, p = 0.297).

the neocortex starts (Shimada and Langman, 1970; Crossland and Uchwat, 1982). The observation that 20 mg/kg MAM causes no cell loss in the LGd also rules out direct toxic effects of MAM on the geniculate. As is well known, in the radial domain, neurogenesis in the cortex proceeds in an inside-out pattern (Angevine and Sidman, 1961; Shimada and Langman, 1970; Rakic, 1974; Caviness, 1982; Bayer and Altman, 1991). Layer IV can be depleted without altering directly the generation of cells in subplate, layer VI, and layer V; however, the generation of layers II and III will necessarily be affected by the depletion of their precursor pool. Our conclusions will thus always include the considerations that we cannot assume that layer IV is completely removed, nor that layers II–III are unaffected (the "identity" of cells produced after resumption of neurogenesis after MAM is addressed in the following companion article, Woo and Finlay, 1996). All of our observations proved MAM's effect to be in accord with our experimental intent; we observed no direct toxic effect of MAM on the lateral geniculate or infragranular cortical layers.

Cortical Cell Number Quantification

The accuracy and validity of the interpretation of our experimental results depend on consistency of placement of cortical laminar borders and visual cortex boundaries. In the 20 mg/kg brains, layer IV is distinct, and identification of cytoarchitectonic borders were done without complication. However, in the 25 mg/kg brains, layer IV in a large part of the visual cortex is very thin and in the most severely depleted brains (30 mg/kg or double doses), layer IV is completely absent, with severe reduction in the thickness of the whole layer II–IV. However, a number of regional markers are still available. Because of the rostrocaudal gradient of neurogenesis, layer IV in the rostral-most part of visual cortex, close to the border between OcI and OcII, is still identifiable in even the most severely depleted cortices, while much thinner. The caudal boundary of OcI extends to the very end of the posterior cortex and thus could be identified without difficulty. In all of the cases, identification of the lateral and medial borders

Figure 10. Reduction in subplate cell number in the whole cortex during development in normal and MAM animals. After 20 mg/kg MAM treatment resulting in 76% loss of layer IV cells, there is no compensatory increase in subplate neurons' survival.



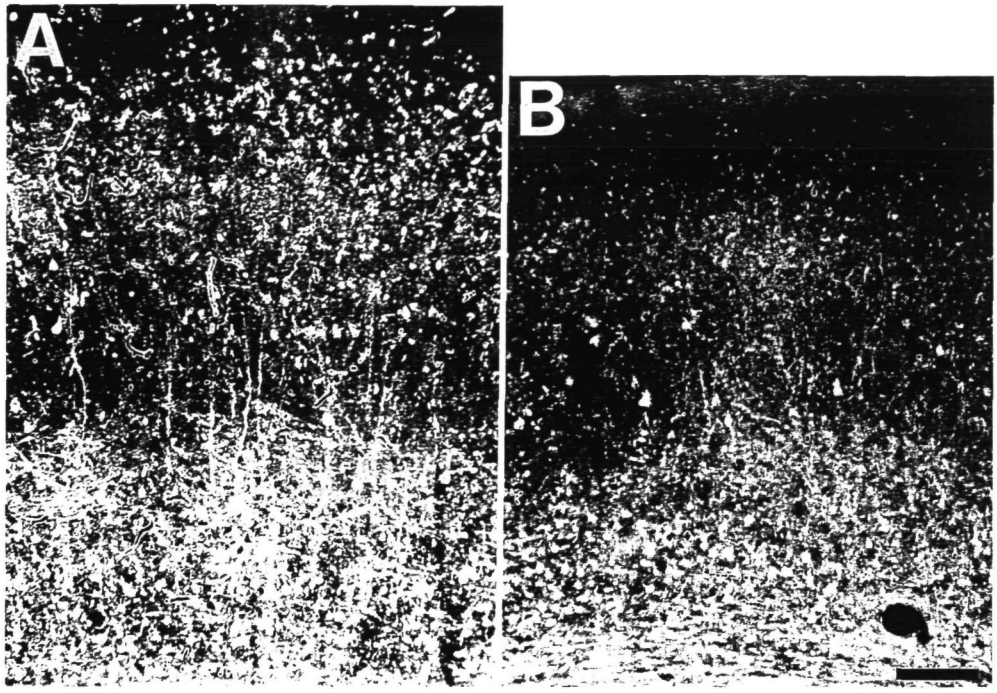


Figure 12. Dark-field photomicrographs of HRP-labeled geniculocortical arborizations in a P20 normal (*A*) and a P20 MAM (*B*, 20 mg/kg) animal. The bilaminar pattern of arborization is apparent in both the normal and MAM cortex. However, both the extent and density of arborization in the upper lamina in the MAM cortex are reduced. Arbor density in the lower lamina in the MAM cortex is not different from that in the normal cortex, showing no evidence of any compensatory increase in arborization in layer VI and subplate (layer VIb) after massive cortical depletion. Also note that cortical thickness is much reduced after MAM treatment. Scale bar, 50 μm.

between OcI and OcII, particularly the dorsomedial border, turned out to be the most difficult. To identify the lateral and medial borders, we took advantage of our knowledge that in the normal cortex layer V tends to be more prominent in the area OcII and cell density in this layer also tends to be higher, compared to OcI. These differences in cytoarchitecture between the two areas are quite well preserved in the MAM brains. Based on these criteria, and extrapolating the normal relative anatomical positions of the borders with other landmark structures such as various parts of the hippocampus, we feel confident that both the medial and lateral borders were identified with reasonable consistency and without systematic deviation in the most severely cell depleted brains.

To calculate neuron number in thalamus and cortex, we used one of the simplest methods, the split cell with "caps"" correction first described by Abercrombie and modified by Floderus. While a detailed account of the validity of different counting methods is beyond the scope of this article (the reader is referred to the review by Coggeshall, 1992), this method yields satisfactory results in light microscopy with the section thicknesses employed in this study. Even if the actual number of cortical and geniculate neurons have been misestimated in some unsuspected manner, our interest in this study is in the relative, and not the absolute covariation of geniculate cell number with different degrees of cortical loss.

Why Massive Cell Loss in Layer IV Results in No Cell Death in the Lateral Geniculate Nucleus

That loss of more than 75% of the neurons in layer IV resulted in no detectable cell loss in the LGd is surprising, given previous literature that emphasizes the specificity and codependence of the thalamocortical circuit. Lateral geniculate neurons project almost exclusively to the visual cortex (Caviness and Frost, 1980; Jones, 1985; Lund et al., 1985; Peters, 1985; Rosenquist, 1985). During development, thalamocortical axons arborize selectively in layer IV, showing little exuberance into layers V and II–III (Erzumulu and Jhaveri, 1990; Catalano et al. 1991; Miller et al., 1993); in coculture studies, thalamic axons stop at and arborize in layer IV, regardless of the direction of approach (Yamamoto et al., 1992; Molnar and Blakemore, 1991; Boltz and Gotz, 1992; Bolz et al., 1992). After removal of the whole visual cortex, the lateral geniculate nucleus degenerates (Schneider, 1970; Cunningham et al., 1979; Raabe et al., 1986). Infusion of conditioned culture medium of posterior cortical explants after visual cortical removal rescues geniculate neurons suggesting this trophic dependence might be mediated through a diffusible factor (Cunningham et al., 1987; Eagleson et al., 1990, 1992). Similarly, lateral geniculate nucleus explants sustain nonpyramidal neurons in culture (Repka and Cunningham, 1987). Many reconciliations of these observations are possible. Tropism of thalamic axons for a particular zone might be entirely dissociable from their source of trophic support; the location of layer IV glia, the dendritic processes of other cortical neurons and the extracellular matrix as well as layer IV neurons. The loss of lateral geniculate nuclei after complete visual cortical lesions might be the result of direct damage to thalamocortical axons and not loss of trophic support. Many other compensatory mechanisms well documented in other neural systems after major target loss can produce nonlinear relationships between number of neurons in connecting populations; we will consider two kinds of axonal remodeling.

One possible compensatory mechanism is through stabilization or establishment of anomalous or novel projections, but this does not seem to occur in the geniculocortical projection. For the hamster particularly, there is ample evidence of plasticity of termination site of retinofugal projections; early ablation of some of the principal targets of ganglion cell axons results in the establishment of novel projections from the retina to other visual or nonvisual thalamic nuclei either through reactive sprouting of new connections or stabilization of transitory projections (Schneider, 1970, 1973; Crain and Hall, 1980; Frost, 1981, 1982, 1986). In the present study, after injections of HRP into the lateral geniculate, we visualize an essentially normal laminar projection pattern of geniculocortical axons; for instance, they terminate in the middle cortical layers (the depleted layer IV and the bottom part of layer III) with collaterals in layer VI without compensatory increase in arborizations in any layer; neither is there is evi-

dence for anomalous projections to other cortical areas. We have seen a similar lack of plasticity in the tangential domain of the remaining thalamocortical pathway after early thalamic lesions (Miller et al., 1991).

Axon Arborization Variability

Axon arbors both increase and regress in normal cortical development, showing a wide range of final sizes. Recently, it has been underlined that both axonal growth/elaboration and axonal elimination (Antonini and Stryker, 1993a,b) are operating simultaneously during the normal process of geniculocortical development in the cat and that the progressive growth in complexity of individual arbors persists even in the absence of neural activity. During the normal development of geniculocortical arbors in the hamster (Naegele et al., 1988), there is a progressive increase in both the size and complexity of individual axonal arbors. Studies in the retinocollicular system have provided insights into the constraints and dynamics of axonal growth. After ablation of the superior colliculus in the hamster, resulting in up to 75% of loss of tissue, cell death in the retina is also minimal (Wikler et al., 1986), as in the present study. This manipulation has no significant effects on either neuronal or synaptic density in the remaining colliculus (Pallas and Finlay, 1991; Xiong and Finlay, 1993). The only change is substantial reduction in the arbor size of individual retinal ganglion cell axons (Xiong et al., 1994) consistent with the preservation of receptive-field properties and sizes of collicular neurons (Pallas and Finlay, 1989). These findings suggest that alteration of axonal arbor, and not alteration of dendritic arbor, synaptic density, or cell survival, may be the first mechanism employed by the developing nervous system to map together numerically disparate populations. In the present study, we observed a reduction in total density of axonal arborization (Fig. 12). It will be interesting to label single geniculocortical axons in MAM animals to see definitively whether these findings can be extended to the geniculocortical system; the point of interest here is the extremely wide range of axonal arborization over which cells remain viable.

Population Matching between the Geniculate and the Cortex

After severe depletion of layers II–IV, cell loss in the lateral geniculate begins to be detectable, ranging from 25% loss after 59% cortical depletion to 42% loss after 74% depletion. We suggest there are at least two stages involved in population matching between the geniculate and its cortical target. Within a certain limit of cortical loss, the geniculate adjusts for target depletion by reducing its axonal arborization. When arborization can be reduced no further, some geniculate cells fail to establish cortical arbors and die.

The linear response of geniculate neurons to cortical neuron number after large depletions resembles the linear relationship of motoneurons to muscle, which is not confined to large depletions (although there are a number of major departures from linearity in the motor system as well; Lamb, 1980). After surgical removal of variable proportion of the soleus muscle in the mouse, Habgood et al. (1984) reports that there is a close correlation between the amount of remaining muscle and the number of surviving motoneurons. In their studies in the chick, quail, and the chick-quail chimera, Tanaka and Landmesser (1986) again establish a linear correlation between the number of myotubes before the onset of naturally occurring cell death of spinal motoneurons and the final number of motoneurons surviving the cell death period, extending the original finding in the chick by McLennan (1982). In the central nervous system, a similar correlation has also been observed during development between the granule cell population and the Purkinje cell population in the cerebellum in both wild-type and chimeric mutant mice, where Purkinje cells are the exclusive targets of granule cells in the cerebellum (Wetts and Herrup, 1983; Herrup and Sunter, 1987). In many parts of the nervous system trophic factors or synaptic sites may be in excess with respect to the minimum requirements of any one axon, as in the retinotectal system or geniculocortical system, while others may rest at their minimum.

In the lateral geniculate nucleus in the hamster there are two peaks of naturally occurring cell death, although the amount of cell death is relatively small (Sengelaub et al., 1985). The first peak is at P4, which coincides with the cell death period in both the retina and the superior colliculus, another major target of retinal ganglion cells, suggesting that cell death in the LGd may be the result of death of retinal ganglion cells at the time when afferent and efferent connectivity is being established. The second peak is at P7, coincident with the major period of cell death in the cortex (Finlay and Slattery, 1983). We hypothesized that the second peak of cell death in the LGd might reflect the interaction of cortical cell death and the establishment of synaptic connections between geniculocortical axons and cortical neurons. However, the insensitivity of geniculate neurons to cortical cell number makes this argument unlikely, leaving the function of LGd cell death mysterious.

Why Do Subplate Neurons Die?

Our observation that there is no increase in subplate neurons' survival or stabilization of thalamic afferents in the subplate when layer IV is gone refutes the hypothesis that layer IV and the subplate compete for thalamic afferents. In the following companion article (Woo and Finlay, 1996), we show that the transposition of thalamic axons from the subplate to the cortical plate and the migration and arrival of layer IV neurons are independent processes. The death of subplate neurons might still be caused by loss of thalamic afferents, although what triggers the translocation of these afferents is unresolved. In the hamster (Woo et al., 1991) and other rodents (Valverde et al., 1989; Bayer and Altman, 1990, 1991; Wood et al., 1992) a significant population of subplate neurons survive into adulthood and form a layer beneath layer VI. The heterogeneity of the subplate population in terms of neurotransmitter or neurotrophic factor expressions and species differences in the relative timing of corticogenesis and thalamocortical ingrowth may help explain the causes of the death of these neurons.

Notes

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