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MATURATION OF VISUAL CALLOSAL CONNECTIONS IN VISUALLY DEPRIVED KITTENS: A CHALLENGING CRITICAL PERIOD[1]

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Abstract

The number of callosally projecting neurons (callosal neurons) which can be labeled in cortical areas 17 and 18 by horseradish peroxidase (HRP), injected in the contralateral visual cortex, is reduced to about 50% of normal in cats reared with their eyelids bilaterally sutured. In the same animals the density of HRP anterogradely transported to areas 17 and 18 is also decreased. The apparent loss of callosal neurons is limited to layers III and IV (subzone a), whereas layer VI (subzone c) is unaffected. The effect is obtained after 3 months or more but not after 1 month of deprivation. Two months of visual experience following deprivation do not restitute a normal number of callosal neurons. However, 10 days of normal visual experience preceding the deprivation are sufficient to prevent the effects of the latter. Animals deprived of vision after a short period of normal visual experience and animals allowed normal vision after 1 month of visual deprivation have a more widespread distribution of callosal neurons than do normal animals; in this way they are similar to previously described cats reared with convergent or divergent strabismus, monocular enucleation, or monocular eyelid suture.

The results suggest that: (*i*) vision is actively responsible for both the maintenance and the elimination of fractions of the juvenile callosal connections; (*ii*) the elimination which normally takes place during the second postnatal month requires normal binocular vision; and (*iii*) activity-dependent competition between callosal and other axons can explain the role of vision.

Visual experience appears to affect the development of visual callosal connections. Different paradigms of visual deprivation have different effects: in kittens raised with their eyes sutured shut, fewer callosally projecting neurons (callosal neurons) are found in areas 17 and 18 than in normal adult cats (Innocenti and Frost, 1979, 1980). Similarly, dark-reared kittens seem to have fewer terminating callosal axons in areas 17 and 18 (Lund and Mitchell, 1979). In contrast, in kittens raised with convergent or divergent strabismus, monocular enucleation, or monocular eyelid suture, callosal neurons acquire a more widespread distribution than in normal kittens (Innocenti and Frost, 1978, 1979); in kittens raised with strabismus, the callosal terminals also become widespread (Lund et al., 1978). The explanation of these experience-dependent modifications of callosal connections may be similar to that proposed for the modifications of eye dominance column width induced by monocular deprivation in area 17 (Rakic, 1976; Hubel et al., 1977): vision could modify the elimination of transitory callosal axons (Innocenti, 1981) which in normal development leads to the characteristically restricted tangential distribution of callosal neurons (Innocenti et al., 1977; Innocenti and Caminiti, 1980).

The effects of bilateral eyelid suture are especially interesting. In newborn kittens and monkeys eyelid suture deprives the retina of finely patterned stimuli but not of coarse changes in illumination (Loop and Sherman, 1977; Spear et al., 1978), reproducing a condition similar to that of early bilateral cataract or loss of corneal transparency. When these animals reach maturity, neurons in their striate cortex show severely abnormal response properties (for references see Spear et al., 1978; Mower et al., 1981). The anatomical basis for these defects is unknown. Counts of synapses in the visual cortex of cats raised with bilaterally sutured eyelids show retardation of development and possibly a slight permanent deficiency (Winfield, 1981). The effects of dark rearing on cortical morphology have been more widely studied in different species and generally appear to be subtle and/or reversible (see "Discussion"), although an arrested segregation of the ocular dominance columns has been observed (Swindale, 1981). However, dark rearing affects the response properties of striate neurons less severely than eyelid suture (Mower et al., 1981) and is less comparable to conditions of human pathology such as cataract or loss of corneal transparency.

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The elimination of transitory projections seems to be a general trait in the development of corticocortical and corticofugal connections (Chow et al., 1981; Ivy and Killackey, 1981; Stanfield et al., 1982; Clarke and Innocenti, 1983), and the effects of visual experience on this process may explain deprivation-dependent modifications in the response properties of cortical neurons. It is possible that other corticocortical connections may be affected by eyelid suture in the same way the callosal ones are. For example, neurons in the parietal cortex become visually unresponsive in monkeys raised with their eyelids bilaterally sutured, suggesting defective maturation of the visual cortical input to this area (Hyvärinen et al., 1978, 1981).

In re-examining the effects of bilateral eyelid suture on the development of callosal connections it became clear to us that a number of important questions were still unanswered: in particular, whether the effects are: (*i*) independent of the sensitivity of the substrate used for HRP visualization, (*ii*) restricted to developing animals, (*iii*) reversed by reopening the eyes, (*iv*) affected by periods of normal vision preceding the deprivation, and (*v*) involve aspecifically all visual callosal neurons.

In this paper the questions above are answered, respectively, yes, yes, no, yes, and no.

Materials and Methods

Experimental design. As shown in Figure 1, this study is based on 17 experimental cats divided into four groups. Cats BD (binocularly deprived) 26, 20, 18, and 16 (continuous deprivation group; CD) were visually deprived for their entire lives (respectively, 235, 173, 92, and 42 days), the eyelids having been bilaterally sutured prior to natural opening. Cats BD 19, 22, and 21 (long deprivation, then vision group; LDV) remained bilaterally sutured until day 90; their eyes were then reopened and they were allowed normal visual experience until sacrifice (respectively, on days 166, 163, and 160; day 0 is the day of birth). Cats BD 12, 11, and 27 (short deprivation, then vision group; SDV) remained bilaterally sutured until days 38, 37, and 37, respectively; the eyes were then reopened and the animals were allowed normal visual experience until sacrifice (respectively, on days 152, 147, and 146). Cats BD 28, 24, 25, 26A, 8, and 13 (vision, then long deprivation group; VLD) opened their eyes naturally (on days 7 to 10) and had variable periods of normal vision (respectively, 10, 10, 12, 16, 15, and 21 days) followed by bilateral eyelid suture maintained until sacrifice (respectively, on days 118, 109, 103, 123, 190, and 234); in addition, the eyes of one adult animal (BDA) were closed for 90 days. Two days before the end of the rearing period, each cat received unilateral injections of horseradish peroxidase (HRP) into its visual cortex. Finally, five normal adult cats and one kitten aged 29 days at sacrifice were used to collect control data on callosal connections using techniques similar to those used for the deprived animals.

Surgery, animal care, and histological techniques. Eyelid suture was performed under Ketalar anesthesia (30 to 40 mg/kg, i.m.) supplemented, when necessary, with inhalation of Penthrane. The operation consisted of cutting the distal 1 to 2 mm of the full lengths of the upper and lower eyelids and joining them with three to four stitches of 4-0 silk or prolene thread, taking care to leave an opening of about 1 mm at the medial canthus for drainage of the eye. The size of this opening is very critical since too small an opening can provoke infection and accumulation of fluid under the eyelids, while an excessively large one probably allows some peripheral vision, especially since its size increases with the growth of the kitten. After surgery the kittens were returned to their mothers and were checked twice a day for swelling of the eyelids and opening of the sutures. Swelling can be alleviated by drainage of the accumulated fluid followed by one or two injections of antibiotics. Small holes in the sutured eyelids were repaired within a few hours of their discovery.

Reopening of the eyes involved a simple incision along the original palpebral rim (same anesthetic as before). Before and after eye opening, kittens lived in an artificial light cycle of 8 hr of dark and 16 hr of light at approximately 100 lux.

In the terminal experiment (same anesthetic as above), the lateral and postlateral gyri were infiltrated with 5 μl of a 50% HRP solution, distributed in 10 regularly spaced injections of 0.5 μl each. Some of the animals (PC 3, BD 8, 11, 12, 18, 19, 22, and BDA) also received two to three injections of [^{3}H]proline and [^{3}H]leucine (total radioactivity ~700

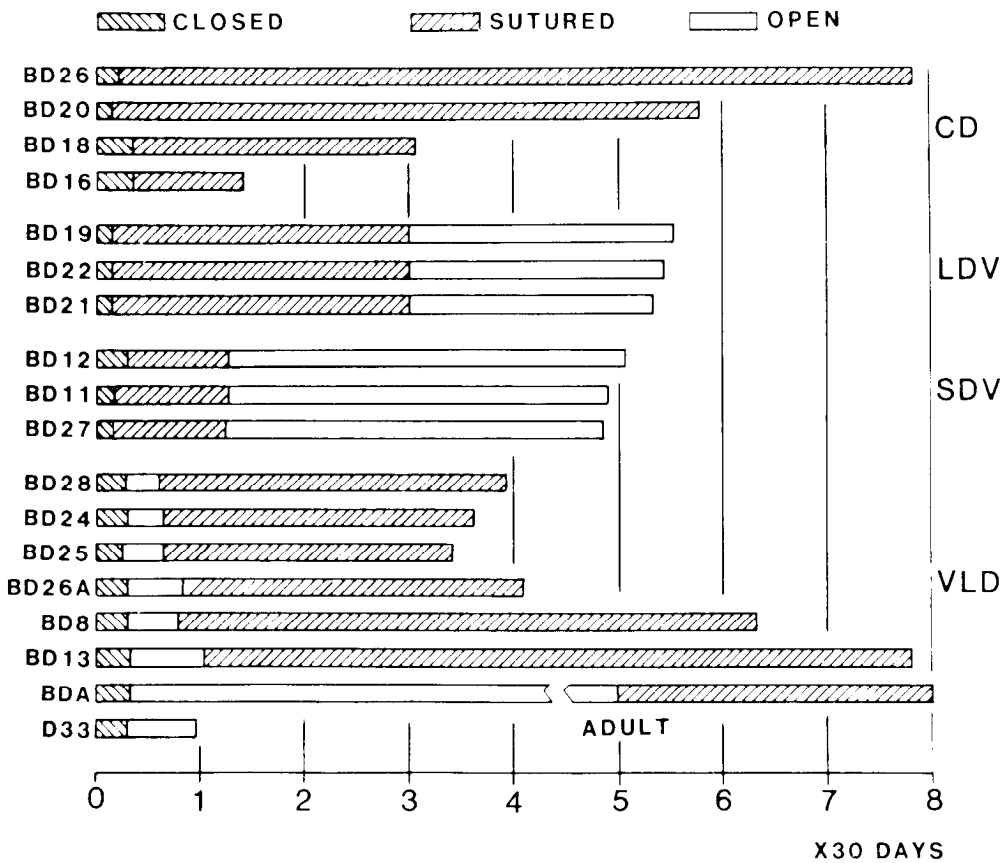

Figure 1. Rearing paradigms of the 17 experimentally manipulated cats and one normal kitten used in this study. The graph does not include five additional adult cats of unknown age, which presumably had experienced normal vision since natural eye opening. The *horizontal axis* indicates the ages of the cats. (The word *adult* under the *bar* corresponding to case *BDA* indicates that the scale does not apply to this adult cat, whose exact age was not known.) *Letters* at *right* indicate experimental groups defined under "Materials and Methods": *CD*, continuous deprivation; *LDV*, long deprivation, then vision; *SDV*, short deprivation, then vision; *VLD*, vision, then long deprivation. *Hatching* slanting downward from *left* to *right* indicates intervals during which the eyelids were naturally closed; *hatching* slanting downward from *right* to *left* indicates periods during which the eyelids were sutured shut; *open spaces* indicate intervals when both eyes were open.

μCi), the results of which will not be described here (for details of injection procedure, see Innocenti, 1980).

Forty-eight hours after the injections, all animals were perfused with phosphate-buffered 3% paraformaldehyde (pH 7.6), followed by cold buffered 20% sucrose; the brains were removed and stored at 4°C in buffered 20% sucrose. After 24 to 48 hr the brains were cut frozen at 80μm. Two adjacent series of sections were reacted with tetramethylbenzidine (TMB; Mesulam, 1978) and with diaminobenzidine tetrahydrochloride preceded by incubation in $CoCl_2$ (DAB-Co; Adams, 1977).

As judged from DAB-Co-reacted sections, all of the sites of HRP injection are very similar in size, location, and intensity of labeling and resemble those obtained previously (and described in detail) with the same injection and reaction procedure (Innocenti and Frost, 1980). The HRP precipitate fills most of the gray and white matter of the lateral and postlateral gyri; occasionally, a lighter precipitate extends into restricted portions of the suprasylvian gyrus. There is retrograde and anterograde transport of HRP to most of the dorsal nucleus of the lateral geniculate body (dLGN), excluding its caudalmost tip.

Data analysis. In each brain, labeled neurons were viewed at × 200 to 300 and their distribution was charted using a computer-microscope (Glaser and Van der Loos, 1965) in 14 to 20 sections reacted with TMB, evenly spaced (480 to 880 μm apart), and encompassing the caudal 10 to 15 mm of the brain (about 500 sections and more than 80,000 cells were analyzed in total). Criteria for the identification of labeled neurons were similar to those previously described (Innocenti and Frost, 1980). Histograms of the number of labeled neurons per section as a function of rostrocaudal level were constructed by a computer which also provided flattened reconstructions of the distribution of labeled neurons (Innocenti, 1980). Briefly, in each coronal section, the labeled neurons were projected onto a line running 400 μm below the pial surface. The line was divided into 100-μm segments and the number of neurons projected onto each segment was indicated by vertical lines whose lengths were proportional to the number of neurons. The lines representing each section were aligned using the convexity of the lateral gyrus as a larndmark. Additional sections between those used for the reconstructions were inspected. The cytoarchitectonic border between areas 17 and 18 was determined on selected counterstained sections.

The significance of differences in the number of neurons per section in differently reared animals was determined using the Mann-Whitney *U* test (see Table I).

Results

Distribution of callosal neurons in areas 17 and 18 of normal adult cats

The distribution and morphology of labeled callosal neurons were similar to those previously found with less sensitive substrates for HRP visualization (Innocenti, 1980; Innocenti and Frost, 1980; Segraves and Rosenquist, 1982). Therefore, only those aspects relevant to the present quantitative analysis will be described here.

In normal adult cats, callosal neurons are distributed within a band running rostrocaudally along the border between areas 17 and 18 and extending mediolaterally 1 to 2 mm over each area (Figs. 2 and 8). The region containing callosal neurons (callosal efferent zone) is flanked by unlabeled (acallosal) regions corresponding to most of area 17 and the lateral part of area 18. Because of this distribution, callosal neurons in most of areas 17 and 18 can be counted separately from those in area 19 or in the splenial sulcus where other callosal zones exist (Innocenti, 1980; Segraves and Rosenquist, 1982). However, at different rostrocaudal levels in different animals, one or two bridges of callosal neurons stretch across the full mediolateral extent of area 18 joining the callosal zone in area 19. For counting purposes the lateral border of the callosal zone in areas 17 and 18 was extrapolated across these bridges as shown in Figure 8.

The boundary between areas 17 and 18 is notoriously difficult to determine precisely in the cat; therefore, separate counts of the callosal neurons in each area were not attempted.

In areas 17 and 18, callosal neurons are distributed in two radially separated, superposed laminae in layers III and IV (callosal subzone a) and layer VI (callosal subzone c) (Fig. 2). Neurons in the two subzones could be counted separately; the few neurons in layer II were attributed to subzone a and the few neurons in layer V to the nearest subzone.

Diagrams of the number of labeled neurons per coronal section share some features in different animals. The neurons decrease in number from caudal to rostral. Superimposed on this trend are one to three peaks that correspond approximately to the area centralis representation and less reliably to the bridges crossing area 18. These features are similar to those observed in a previous study (Innocenti and Frost, 1980; cf. their Fig. 5), although, here, the use of a more sensitive substrate for HRP visualization raised the average number of neurons per section from 45.38 to 183.9. Most of the neurons (157.4/section on average) are in subzone a. Cat PC 3 is excluded from these statistics (see below). Within each group of animals, with the exception of SDV, the number of labeled neurons per section showed fairly small individual variations; these may depend on small differences in the size and location of the injections. This explanation appears totally unconvincing for PC 3. This animal had an average of 555 (±148, SD) labeled neurons/section in subzone a, i.e., 3.5 times the average for the other normal adults and 2.6 times the figure for the second most heavily labeled animal in this group. The callosal zone was also relatively wider in this animal than in any other animal of this group, resembling that of previously studied strabismic animals (Innocenti and Frost, 1979): at all rostrocaudal levels callosal neurons extended well beyond the suprasplenial sulcus into parts of area 17 which are usually acallosal in normal adult cats. We have no record suggesting technical differences between this experiment and the others. The size and intensity of labeling of the injection site and the packing density of labeled neurons in the ipsilateral dLGN were similar to those in the other animals of this group. Furthermore, in this animal, the number of labeled neurons in subzone c was within the average of its group and actually lower than in PC2 (Fig. 7). This speaks strongly against differences in HRP uptake, transport, or visualization as a probable explanation for the extraordinary callosal connections of this animal. Rather, it appears that large individual variations may exist in the normal structure of callosal connections. These individual variations may in some cases be due to the occurrence of natural strabismus (von Grünau and Rauschecker, 1983).

Number of callosal neurons following deprivation

Inspection of corresponding sections from any animal in the CD or LDV groups discussed below and from any normal cat is sufficient to show the effect of visual deprivation (Fig. 2). Fewer callosal neurons are labeled in the deprived animals. The loss is restricted to subzone a. In both normal and experimental animals there is some variability in the number of labeled neurons. The significance of results must therefore be tested statistically (Table I). In all intergroup comparisons we have chosen to accept only highly significant differences such as those documented by a nonparametric test (Mann-Whitney *U* test) applied to the mean number of neurons per section per animal (x in Table I) and with n (Table I) expressing the number of animals. We will not describe subzone c results in detail but will summarize them in Table I and Figures 5 and 7. Taken together, these results are sufficient to state that subzone c neurons are not decreased in number by any of the rearing conditions we have used.

CD group

Experiments BD 18, 20, and 26. Statistical evaluation of the results using the Mann-Whitney *U* test shows a highly signifi-

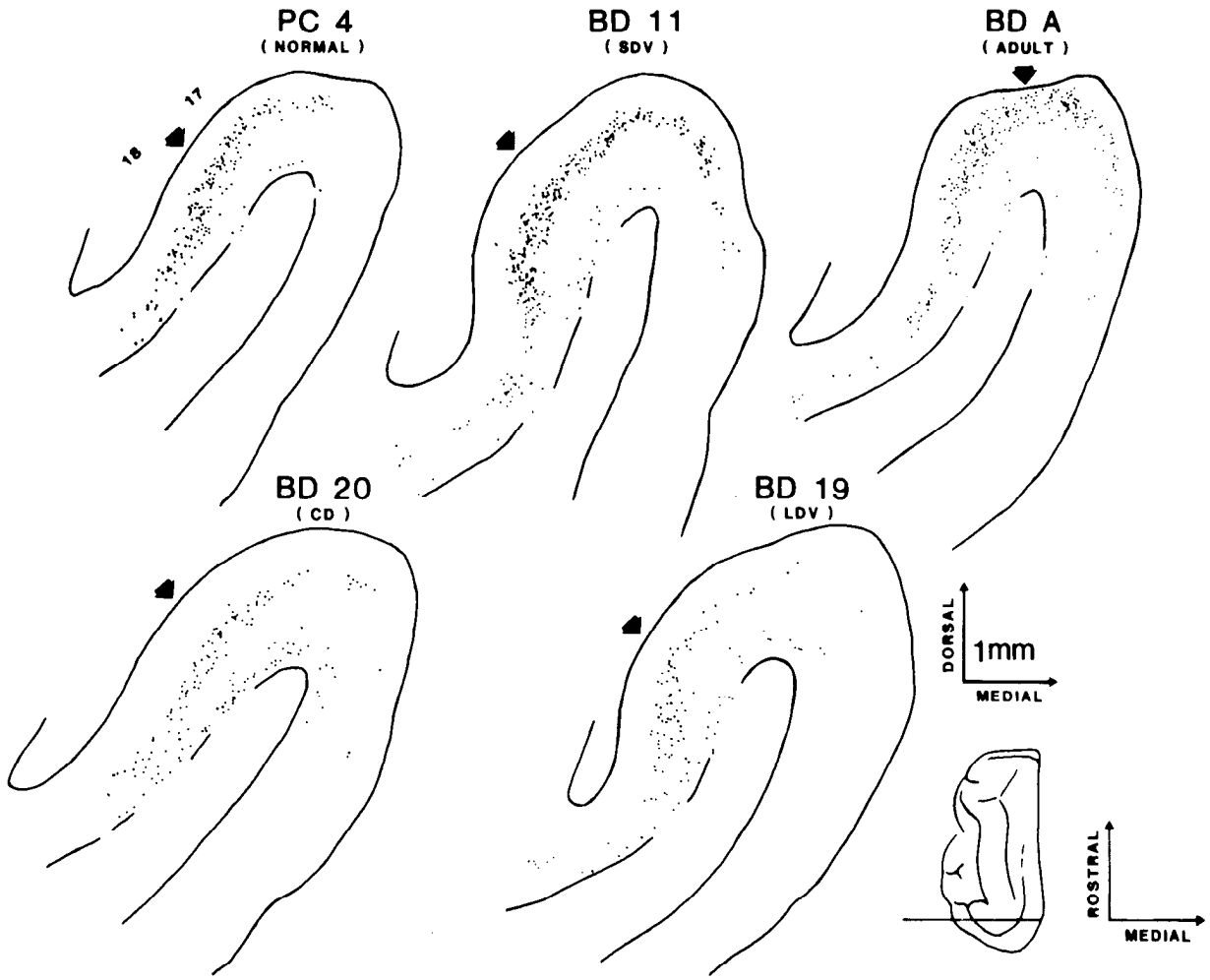

Figure 2. Computer-microscope plots of the distributions of HRP-labeled callosal neurons in TMB-reacted coronal sections through corresponding levels of areas 17 and 18 of five cats. Dorsal is up, lateral is to the left. *Arrows* indicate the 17/18 border as determined by cytoarchitectonic criteria in adjacent Nissl-stained sections. PC 4 is normal; BD 11 is from the SDV group; BDA was deprived as an adult; BD 20 is from the CD group; BD 19 is from the LDV group. The *inset* in the *lower right* shows a dorsal view of the left hemisphere of a cat brain (rostral is up; lateral is left); the *line* indicates the coronal level from which these sections are taken. *Scale* applies only to drawings of coronal sections.

TABLE I

Statistical evaluation of differences in number of HRP-labeled callosal neurons using the Mann-Whitney U test

	Group 1	Group 2	x_1[a]	x_2	n_1[b]	n_2	p
Subzone a	N	CD[c]	157.4	80.73	4	3	0.0000
	N	LDV	157.4	93.73	4	3	0.0000
	N	SDV	157.4	212.3	4	3	0.6286
	N	VLD[d]	157.4	173.7	4	6	0.4762
Subzone c	N	CD[c]	26.5	31.7	4	3	1.0
	N	LDV	26.5	46.1	4	3	0.2286
	N	SDV	26.5	20.5	4	3	0.8571
	N	VLD[d]	26.5	41.77	4	6	0.2571

[a] x, mean number of neurons per section per animal.
[b] n, number of animals.
[c] BD 16 was excluded.
[d] BDA was excluded.

cant reduction in the average number of subzone a callosal neurons per section in these three animals compared to normal adults (Table I; Figs. 3 and 7).

The loss of labeled neurons appears to be diffuse through subzone a. Figure 3 shows that there are fewer labeled neurons than normal at all rostrocaudal levels. However, we have not tested the possibility that, within subzone a, specific depths or neuronal types may be preferentially affected.

Experiment BD 16. This animal has more labeled neurons in subzone a than normal adult cats and the other, older CD cats have (Fig. 7). In this respect it appears very similar to the SDV cats (see below). As in two of the SDV animals, the labeled neurons are also more widely distributed than in normal or other CD cats (see below).

LDV group

Experiments BD 19, 21, and 22. The loss of labeled callosal neurons is similar to that found in CD cats (Table I; Figs. 4 and 7). Thus, the difference in the average number of labeled neurons per section (in subzone a) between the normal and the deprived groups is highly significant. It is difficult to compare the CD and LDV groups, given the variety of deprivation times in the former. Nevertheless, comparisons of the LDV group with BD 20 and 26 (deprived for longer intervals) showed no significant differences (not entered in Table I). As in CD animals, the loss appears diffuse throughout subzone a. Thus, normal visual experience following 3 months of deprivation

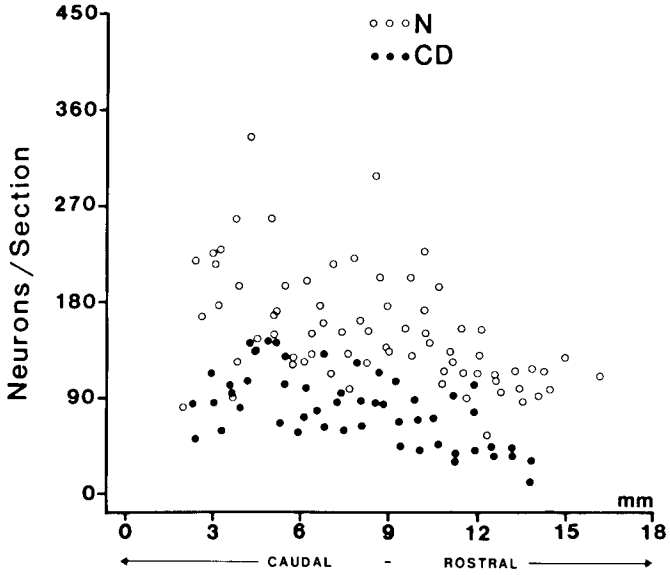

Figure 3. Rostrocaudal distributions of labeled callosal neurons in subzone a of normal adult cats (*N*; ○) and of binocularly deprived cats of the CD group (with the exception of BD 16; ●). Data are pooled from the members of each group. The *horizontal axis* indicates positions of coronal sections in millimeters rostral to the caudal extremity of the neocortex. The *vertical axis* indicates the number of HRP-labeled callosal neurons in subzone a of areas 17 and 18.

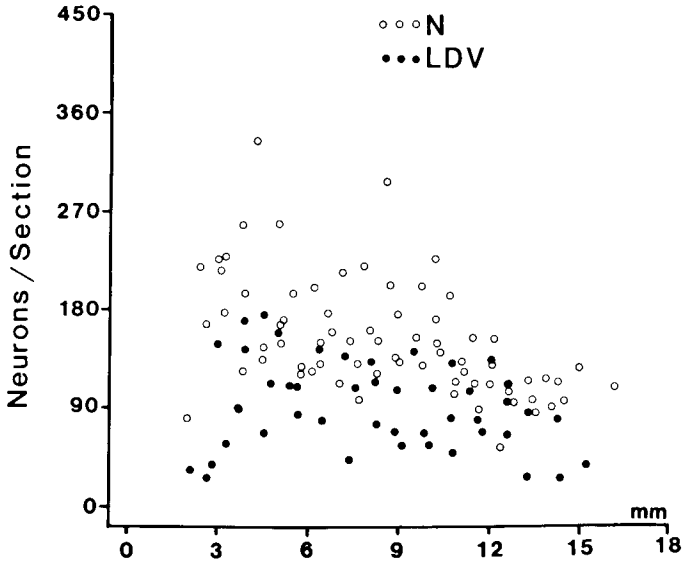

Figure 4. Rostrocaudal distributions of labeled callosal neurons in subzone a of normal cats (○) and of binocularly deprived cats of the LDV group (●). All other conventions are as for Figure 3.

apparently does not alter the effects of deprivation. Furthermore, the loss of callosal connections is probably fully established after 3 months of deprivation.

In summary, fewer subzone a callosal neurons can be labeled in cats which had been reared with their eyelids sutured for at least 3 months (Fig. 5) than in normally reared cats. Two months of vision following deprivation does not restitute a normal number of labeled callosal neurons. Continuing the deprivation beyond the third month increases the loss of labeled neurons modestly, if at all.

SDV group

Experiments BD 11, 12, and 27. Taken together, these animals have more neurons than do normal cats, but this difference

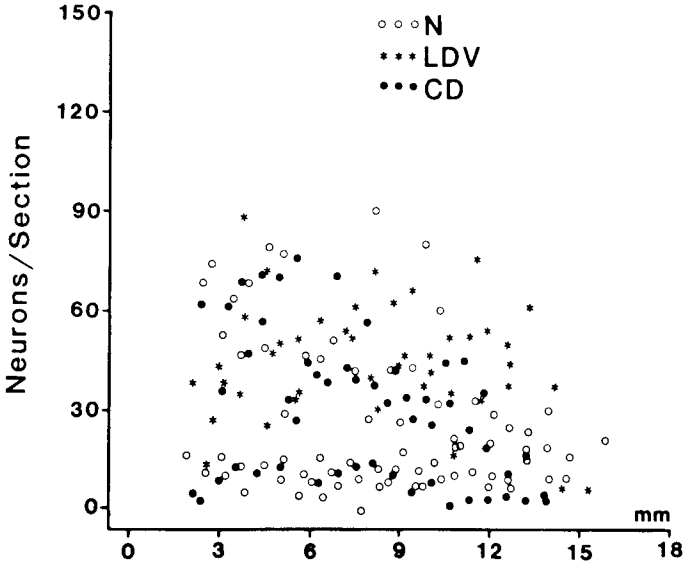

Figure 5. Rostrocaudal distributions of labeled callosal neurons in subzone c of normal cats (○), cats of the LDV group (*), and cats of the CD group (●). All other conventions are as for Figure 3.

is not statistically significant (Table I). In fact, they form a heterogeneous group (Fig. 7). The number of labeled callosal neurons in subzone a is higher in BD 11 and BD 12 than in any normal animal (with the exception of PC 3), whereas in BD 27 the number is in the range of that for the CD or LDV animals.

Two circumstantial observations suggest possible explanations for the variability observed in subzone a in these three animals. Judging from corneal reflection (Sherman, 1972) at the time of HRP injection, BD 11 (unfortunately, BD 12 was not tested) had a marked divergent strabismus (we did not try to quantify it) at the time of HRP injection. These two animals also had an abnormally wide callosal zone (see below), similar to that previously found in strabismic animals (Innocenti and Frost, 1979). Thus, strabismus may be responsible for the large number of callosal neurons in BD 11 (and possibly in BD 12).

In BD 27 we could not detect any strabismus using the corneal reflection; the tangential distribution of callosal neurons in area 17 was also normal. However, in the post-deprivation period, this animal never seemed to use its eyes and, indeed, had them constantly closed every time we checked its behavior in the colony. Thus, it is not impossible that this animal may have suffered binocular deprivation beyond the end of the eyelid suture period.

Although the results of the SDV experiments are clearly preliminary, they tend to suggest that 1 month of deprivation is not sufficient to cause an excessive loss of callosal connections. In this respect it is interesting that the number and distribution of neurons in BD 16 (deprived until day 42 and sacrificed) are similar to those found in the SDV animals (and higher than in normal cats; Fig. 7), suggesting that opening the eyes after 1 month of deprivation can stabilize the callosal connections which exist at that time (see below).

VLD group

Experiments BD 8, 13, 24, 25, 26A, and 28. The number of labeled neurons in subzone a varies among individuals of this group, a little more than among normal cats (Table I; Figs. 6 and 7). This variation cannot be related to the duration of either the deprivation or the preceding visual experience. Intragroup differences are not statistically significant when subgroups differing in length of deprivation (or of preceding vision) are compared.

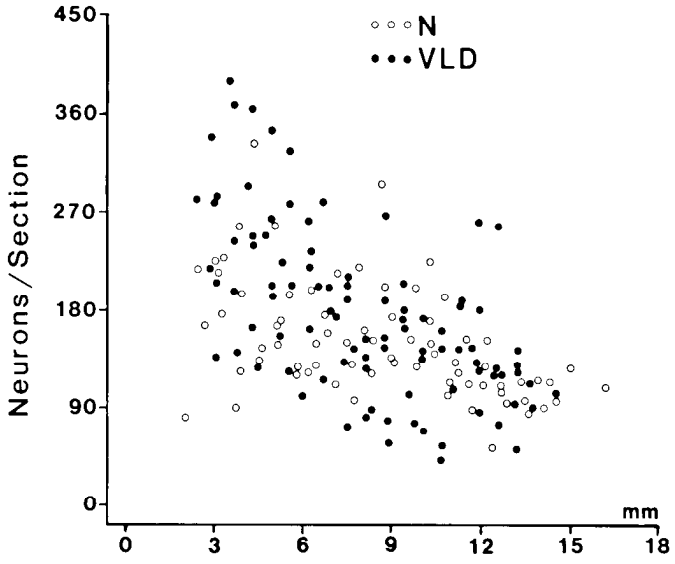

Figure 6. Rostrocaudal distributions of labeled callosal neurons in subzone a of normal cats (○) and of binocularly deprived cats of the VLD group (●). All other conventions are as for Figure 3.

Visual deprivation apparently did not produce a loss of subzone a callosal neurons in animals of the VLD group, which actually have a few more labeled neurons (but not a significant difference) than do normal cats (Table I). Subzone a is also wider than in normal cats (see below).

In keeping with the above results, no changes in neuronal number are found in subzone a of the BDA cat.

Thus, the loss of labeled callosal neurons induced by deprivation during the second and third postnatal months (Fig. 7) can be prevented by short periods of normal visual experience during the first postnatal month.

Tangential distribution of callosal neurons in normal and deprived animals

In normal adult cats, both the mediolateral width of subzone a and its position with respect to the crown of the lateral and postlateral gyri show important individual variations, as can be appreciated from Figure 8. The position of subzone a reflects that of the border between areas 17 and 18 as determined cytoarchitectonically. Callosal neurons rarely extend medially into area 17 as far as the suprasplenial sulcus, and this happens mainly rostrally and only in animals whose callosal zones (and 17/18 borders) are positioned most medially (cf. BDA in Fig. 8). The lateral border of subzone a is so individually variable that few generalizations apply to it except the presence of the previously mentioned bridges crossing area 18, whose number, thickness, and rostrocaudal position vary.

The mediolateral width of subzone a appears slightly decreased in animals of the CD and LDV groups (cf. Figs. 8 and 9). As in normal cats, the density of callosal neurons peaks near the 17/18 border and diminishes progressively with increasing distance from the border. The narrowing of subzone a in CD and LDV animals could be the consequence of the reduction in the number of callosal neurons.

In the VLD cats, as well as in BD 11 and 12 (SDV group), and in the two kittens sacrificed around the end of the first postnatal month (BD 16, binocularly deprived; D 33, normal visual experience), subzone a is markedly wider than in normal adult cats (cf. Figs. 8 and 9). Whereas in normal adult animals only a few callosal neurons are found in the medial part of area 17, in these cats, numerous callosal neurons are found (in subzone a) as far as the suprasplenial sulcus and, occasionally, below it. This distribution resembles that found in cats ren-

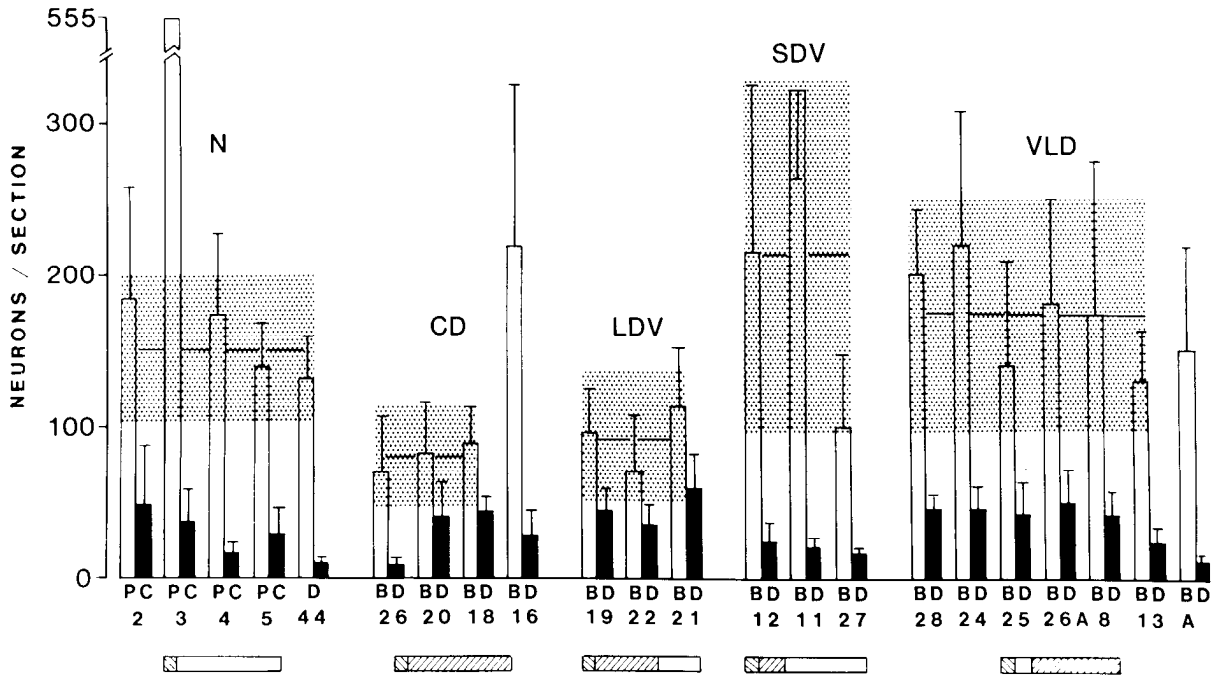

Figure 7. Histograms showing the mean number of HRP-labeled callosal neurons per section in areas 17 and 18 of each cat in this study. Cats are grouped along the *horizontal axis* according to the rearing paradigms, schematized by the *horizontal bars* using the conventions of Figure 1; each group is also designated by *letters* as in the text. *N*, normal adult cats. The *vertical axis* indicates the number of HRP-labeled neurons per section. *Open vertical bars* represent counts for subzone a; *solid vertical bars* represent counts for subzone c. *Vertical lines* represent 1 SD in the counts from individual cats (for PC 3 it was 148 in subzone a). *Horizontal lines* between the subzone a histograms of each group indicate the mean number of labeled subzone a callosal neurons per section for the group, while the *stippling* represents the region of ±1 SD from the mean (data from cats BDA and BD 16 were not used in calculating the statistics for their respective groups).

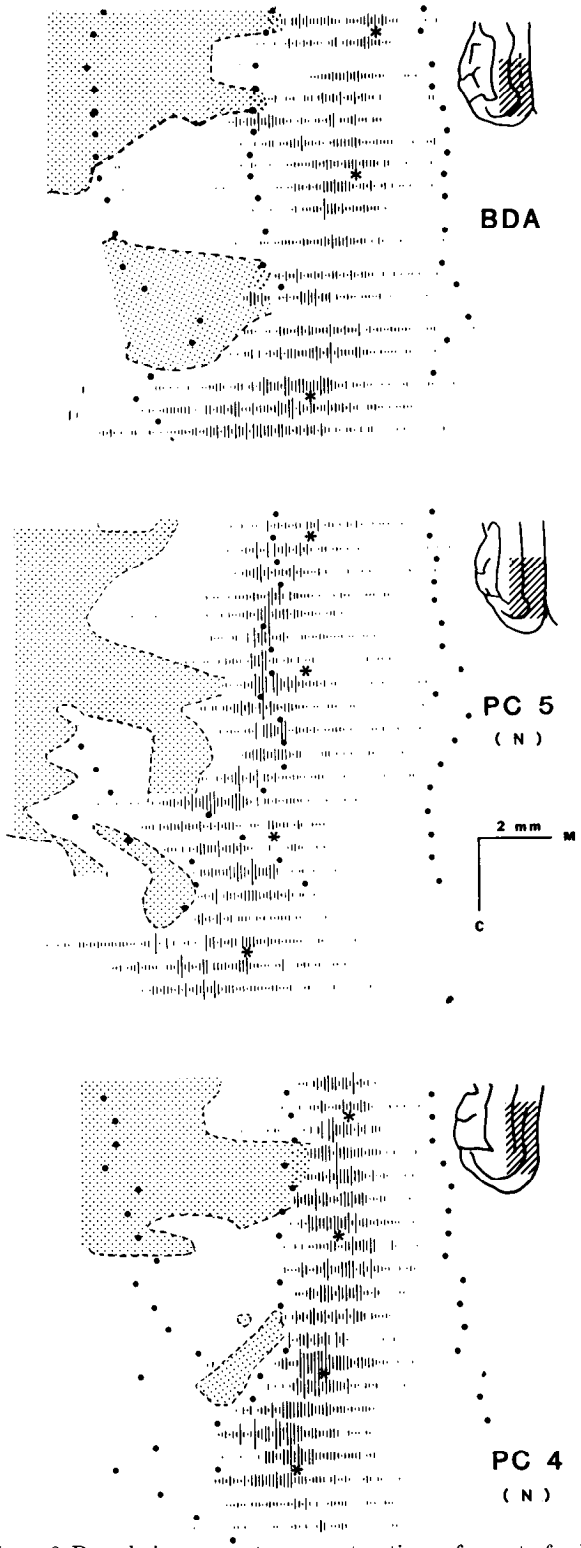

Figure 8. Dorsal view computer reconstructions of a part of callosal subzone a in two normal adult cats and in the binocularly deprived adult BDA. Flattened representations of postlateral and lateral gyri represent *hatched areas* in corresponding *insets* of dorsal views of brains (traced from photographs). *Dotted lines* represent, from lateral to medial, the fundi of the lateral, postlateral, and suprasplenial sulci. The *asterisks* mark the boundary between areas 17 (medially) and 18 (laterally). The neurons in subzone a of each section were projected onto a line running parallel to the pial surface and 400 μm deep; the line was divided into bins of 100 μm and the number of neurons in each bin was represented by a line segment whose length is proportional to the number of neurons in the bin; the number of labeled neurons represented by a unit of line segment height is the same in each case. Each row of line segments represents one section. *Stippling* indicates regions of areas 18 and 19 that are continuous with the reconstructed parts of the callosal zone, and within which there is a very high density of labeled callosal neurons; parts of these regions form the "bridges" described in the text and show a great deal of individual variability in both normal and deprived cats. Neuronal counts used for histograms and statistics exclude the stippled regions and correspond to the parts of subzone a reconstructed with line segments. *Scale lines* represent 2 mm. *M*, medial; *C*, caudal.

dered strabismic by early sectioning of their medial or lateral rectus muscles or raised with monocular deprivation or enucleation (Innocenti and Frost, 1979). The enlargement of the callosal zone could be related to loss of normal eye alignment for at least one of the SDV animals (BD 12). For the VLD animals, other explanations must be sought (see "Discussion").

Some of the animals with an enlarged subzone a also had more callosal neurons than normal in this subzone, but this was not always the case. For example, in BD 24 and 25, the subzones a were about equally enlarged compared to normal, although BD 25 was on the low end of the range in the normal number of callosal neurons, whereas BD 24 was on the high end (Fig. 7).

Anterograde labeling of terminating callosal afferents in normal and binocularly deprived cats

The TMB technique, unlike less sensitive methods for HRP histochemistry, visualizes in the hemisphere contralateral to the injection not only neuronal somata but also axon terminals or possibly preterminal processes. These appear as a diffuse, dusty precipitate interspersed between the labeled somata (Fig. 10).[5]

In normal cats, the bulk of the terminating axons is contained within a band, about 1 to 2 mm wide in the coronal plane and running rostrocaudally along the boundary between areas 17 and 18, i.e., within the region occupied by callosal neurons. In coronal sections the terminating axons are most dense in a radially oriented "column," about 0.5 mm wide, spanning all cortical layers, including layer I but with peak densities in layers III and VI. This column is in area 18. Labeled terminating axons extend away from the column, over another 500 μm or more, into area 17 where they become confined to the supragranular layers (often also to layer VI) while avoiding layers IV and V. The 17/18 border can be identified by cytoarchitectonic criteria, mainly the variation in the thickness of layers IV and III and the variation in the size of layer III pyramidal cells, and also by a sudden increase in the radial thickness of subzone a in area 18, due to the lowering of its inferior boundary.

The density of the anterograde labeling varies along the rostrocaudal direction, as can be appreciated in reconstructions from coronal sections as well as in sagittally sectioned brains. The heaviest labeling is reliably found 3 to 5 mm from the occipital pole, roughly corresponding to the peak density of retrogradely labeled neurons. The HRP-labeled telodendria span a narrower region than the somata of callosal neurons; their distribution is almost identical to that of terminating callosal axons traced with radioactive amino acids (Shatz, 1977) or with anterograde degeneration (Fisken et al., 1975). Therefore, it seems likely that the labeled terminal arbors are mostly those of anterogradely filled callosal axons. It cannot be excluded that the initial axon collaterals of retrogradely filled callosal axons may also have become labeled, but if so, these

[5] Anterogradely filled axons become labeled in the superior colliculus ipsilateral, and much less abundantly contralateral, to the injections.

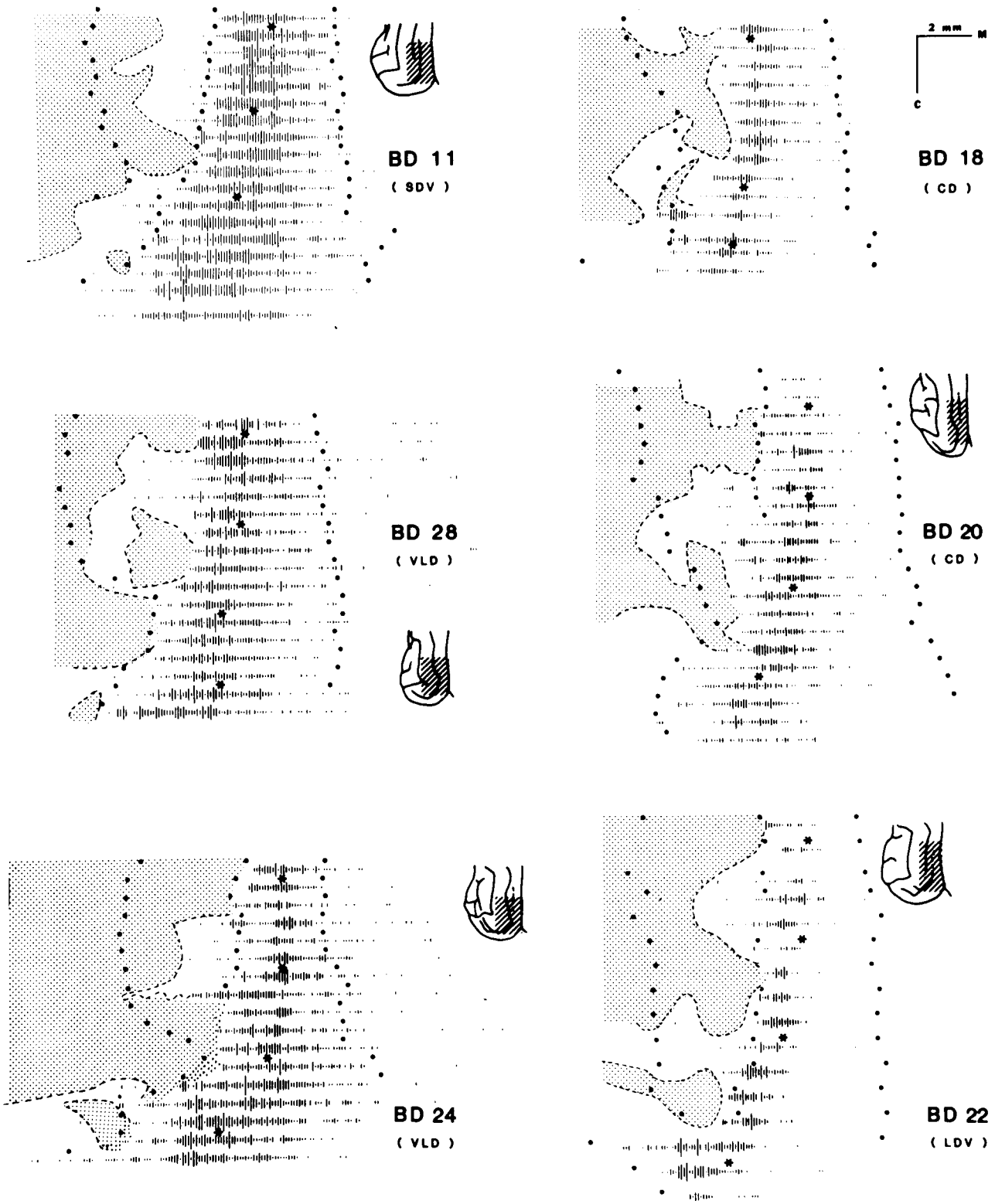

Figure 9. Dorsal view computer reconstructions of part of subzone a in six binocularly deprived cats from different experimental groups. All conventions are as in Figure 9.

collaterals must have the same distribution as callosal axons from the contralateral hemisphere.

Binocular deprivation decreases the density of anterograde labeling without affecting its overall distribution. However, because of the rostrocaudal inhomogeneity in the projection, the loss of labeled terminating axons can be detected only when sites of similar relative density are compared across animals. Figure 10 shows the outcome of this procedure for normal, LDV, and CD animals. One slide per animal was selected, containing the highest density of anterograde labeling in that brain. Slides from animals within each group were ranked in decreasing order of labeling density. This was achieved with some difficulty because animals of the same group are very similar. Finally, sections with similar ranking order were com-

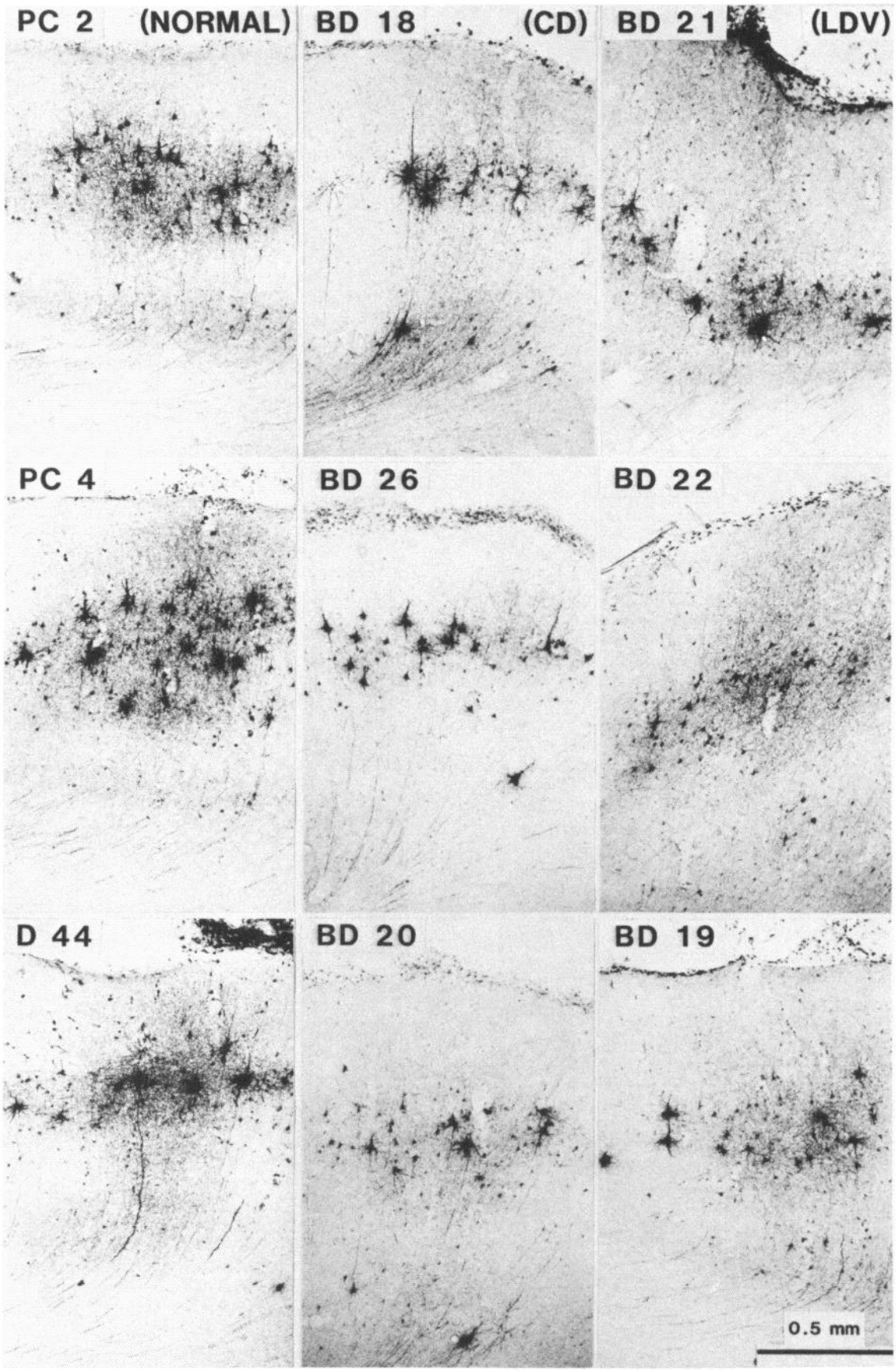

Figure 10. Lightfield photomicrographs of the region of the cytoarchitectonic 17/18 border in TMB-reacted coronal sections from the brains of three normal cats (*left*), three cats of the CD group (*middle*), and three cats of the LDV group (*right*). These cats had the densest anterograde labeling of terminating callosal axons in their respective groups; anterograde labeling is seen in these micrographs as a diffuse, dusty precipitate interspersed between the labeled somata. Each section was chosen because it contained the densest anterograde labeling in areas 17 and 18 of its brain. Cortical surface is up. *Bar scale* at bottom right applies to all micrographs.

pared across groups. The loss of terminating callosal afferents in the supragranular layers of the deprived animals is clear-cut even when the lightest labeled normal cat is compared with the densest labeled cat in each deprived group. The LDV animals also seem to have slightly more anterograde labeling than the CD ones.

Discussion

Reliability of results. The number, as well as the tangential distribution, of labeled callosal neurons in areas 17/18 of animals reared according to the same paradigm shows some variability.

Precautions were taken to minimize variability introduced by the injection, processing, and analysis procedures (see "Materials and Methods"). Briefly, each animal received the same amount of HRP distributed in an equal number of injections over a comparable part of cortex. The brains were processed in an identical manner and, indeed, the injection sites appeared very similar in their position, size, and intensity of labeling. Labeled cells were charted and counted with the same optics in similarly spaced sections distributed over a comparable rostrocaudal sector of cortex. Finally, by counting a few sections several times, we estimated our individual or interindividual counting error to be on the order of ±3%. We have discussed elsewhere our criteria for identifying labeled neurons and for differentiating them from labeled pericytes (Innocenti and Frost, 1980). The mean number of callosal neurons per section obtained in normal animals is nearly identical to that in a recent study from another laboratory (Berman and Payne, 1983) when correction for the different section thickness in the two studies is introduced. The standard deviation of our means is exaggerated by the inclusion of the rostrocaudal variations in number of callosal neurons within each animal (cf. Innocenti and Fiore, 1976).

There is little doubt that most of the intragroup variability in the number and distribution of callosal neurons is not due to technical factors. Visual callosal connections show important individual variations in normal cats (Figs. 7 and 8), as in rats (Cusick and Lund, 1981) and monkeys (Van Essen et al., 1982). In the cat, individual variations in the retinotopic maps in visual areas (Tusa et al., 1979) may correlate with, and possibly cause, the variations in number and distribution of callosal connections. Finally, we have observed important individual differences in the total number of callosal axons in a recent electron microscopic study in the cat (Koppel and Innocenti, 1983).

The individual variability in number and distribution of labeled callosal neurons does not conceal the main effects of the different rearing paradigms. (*i*) The number of callosal neurons which can be labeled in layers III and IV of areas 17/18 by HRP injected in the contralateral, lateral, and postlateral gyri is reduced to about 50% in cats raised for at least 3 months with their eyelids bilaterally sutured; the density of labeled callosal terminals in the same areas is also decreased. (*ii*) The effects seem to be irreversible. (*iii*) Ten days of normal vision preceding deprivation prevent the effects of the latter, but the labeled callosal neurons acquire a more widespread distribution than in normal cats.

Individual variability in the number of callosal neurons is greater among the SDV animals than in any other group. In our opinion, this variability reflects differences in the visual experience of these animals after eye opening, but does not preclude two conservative interpretations: (*i*) deprivation for 1 month is not sufficient to abnormally decrease the number of labeled callosal neurons, as is also supported by their high number in BD 16 (deprived until day 42); (*ii*) strabismus arising spontaneously after 1 month of binocular deprivation widens the tangential distribution of callosal neurons, as does strabismus surgically induced prior to vision (Innocenti and Frost, 1979) and short periods of visual experience followed by deprivation (VLD animals). It must be stressed that these are preliminary conclusions to be tested by further experiments.

Deprivation-dependent loss of callosal connections. As discussed previously (Innocenti and Frost, 1980), the effects of binocular deprivation seem related to the natural postnatal reshaping of callosal connections (Innocenti and Caminiti, 1980), i.e., the elimination of axons (Innocenti, 1981) that cortical neurons transitorily send through the corpus callosum. Binocular deprivation may exaggerate this normal elimination of callosal axons.

Three new findings support the above interpretations. First, binocular deprivation is effective approximately over the period when callosal connections are eliminated (see below). Second, only layers III and IV are affected and not layer VI; layers III and IV contribute most of the normal transitory callosal projection (Innocenti and Caminiti, 1980), hence they also undergo the most severe natural loss. Finally, the effects of binocular deprivation appear irreversible, as one would expect if they were due to the elimination of such a long axon. It must be stressed that none of the arguments above excludes the possibility that death of callosal neurons, rather than elimination of their axons, may be induced by the deprivation. In fact, there are no arguments against the possibility that even during normal development some (probably few) callosal neurons may die (Innocenti, 1981). For this reason we will henceforth call the effect of visual deprivation as shown by HRP: "loss of callosal efferents."

The deprivation-induced loss of callosal efferents probably underlies the reduction of terminating callosal afferents observed by us with anterogradely transported HRP and reported with the Fink-Heimer technique in dark-reared kittens (Lund and Mitchell, 1979). There are reasons to be prudent with this interpretation. Dark rearing and bilateral eyelid suture may have different effects on cortical morphology, as they apparently have on the functional properties of striate neurons (Mower et al., 1981). For example, the loss of labeled telodendria observed in dark-reared kittens could be due to a reduction in the amount of terminal arbor elaborated by each axon rather than to a loss of axons.

Comparison of previous (Innocenti and Frost, 1980) and present results shows an increase in the average number of labeled callosal neurons per section from 45.4 to 183.9 in normal cats and from 12.4 to 112.4 in CD cats (subzones a and c pooled as in the earlier series). The gain is probably due to the use of TMB for HRP visualization and is greater for deprived than for normal animals. The smaller relative loss of callosal neurons in this study has several possible explanations. (*i*) Individual variability in the number of callosal neurons within the various groups can affect the figures obtained from small series of animals. Indeed, the individual variations in the number of labeled neurons were much greater among our previous animals (normal or deprived). The present study would also show a substantially greater loss of callosal neurons had PC 3 not been excluded from the statistics. (*ii*) callosal neurons which normally can be visualized for some time after birth may decrease their uptake and/or transport of HRP (or metabolize it faster) as a consequence of binocular deprivation. This could explain the relatively greater increase in the number of labeled callosal neurons in deprived cats, when more sensitive techniques are used.

Although it appears unlikely that difficulties in HRP visualization would entirely account for the nearly 50% reduction in the number of labeled callosal neurons in BD animals, for the reduction in anterograde labeling and for the temporal and spatial (see below) specificity of these effects, this possibility cannot be fully dismissed (for further discussion see Innocenti

and Frost, 1980). The precise estimate of the deprivation-induced loss of callosal axons originating from areas 17/18 awaits direct axon counts in the corpus callosum itself.

Specificity of binocular deprivation effects. Only some (about 50%) callosal efferents in subzone a are lost due to long-lasting binocular deprivation. However, there is no deprivation-dependent loss of callosal efferents in subzone c.

It is not clear whether the differential sensitivity of callosal efferents is related to differences in other neuronal features, e.g., spontaneous activity, receptive field properties, field of termination, and so on. However, there may be special reasons why subzone c neurons (mostly neurons of layer VI) are not lost by deprivation. Layer VI is at the origin of intrinsic projections to layer IV and to the structures which project to the latter such as the dLGN and claustrum (Gilbert and Kelly, 1975; Carey et al., 1980; Baughman and Gilbert, 1981). In particular, the layer VI to dLGN projection seems to operate as part of a feedback loop enhancing the geniculate input to area 17 (for references see Singer, 1977). A similar loop involving layer VI may exist between the hemispheres and may be set up prenatally, independent of visual experience. Alternatively, binocular deprivation may have a stabilizing effect on this connection as an attempt to compensate for the reduction of visual input from the callosum.

The multiple injections into the lateral and postlateral gyri, such as those used here, selectively and reproducibly fill a region including three cytoarchitectonic areas (17, 18, and 19), each of which seems to receive from a different but spatially overlapping set of neurons at the 17/18 border (Innocenti and Clarke, 1983). We do not know whether these three efferent sets are equally affected by binocular deprivation.

A paradoxical critical period. One of our original goals was to determine whether callosal connections are affected by binocular deprivation over a definable, restricted period of an animal's life. Our approach to the critical period met some serious difficulties. We suspected that the critical period is over around the end of the third postnatal month since, at this time, the normal elimination of callosal axons seems to be complete (Innocenti and Caminiti, 1980) and the effects of binocular deprivation are completely and irreversibly expressed. This is also consistent with the critical period for the effect of monocular deprivation on the distribution of thalamocortical afferents and ocular dominance (for references see Wiesel, 1982). Since animals deprived for 38 days after birth and then allowed normal vision did not show any clear loss of callosal connections, we had to conclude that by day 38 the critical period had not yet begun or else the animals had not received enough visual deprivation. Thus, we hoped to define the beginning of the critical period for deprivation (and to confirm our estimate as to its end) by shifting the beginning of binocular deprivation. But animals allowed normal vision that was terminated well before the end of the first postnatal month were no longer affected by subsequent deprivation. Apparently, the limits of this critical period cannot be determined because the experimental manipulations needed to define these limits also eliminated the effects of the deprivation.

At the moment we see no simple way out of this paradox, but it may help to think of a critical period for the effects of vision rather than of deprivation. Vision-dependent processes seem to control which juvenile callosal axons are maintained and which ones are eliminated.

Nature of visual control of callosal development. Our previous experiments (Innocenti and Frost, 1978, 1979, 1980) provided evidence consistent with the notion (Changeux and Danchin, 1976) that vision acts by stabilizing a fraction of the juvenile callosal connections. Partial deprivation of visual experience by binocular eyelid suture or binocular enucleation led to abnormally high loss of callosal efferents. Other rearing conditions, namely, strabismus (also monocular enucleation and monocular eyelid suture, for which only preliminary data are available) led to the maintenance of a few of the efferents that would normally have been eliminated. It must be stressed that rearing paradigms that maintain efferents which would otherwise be eliminated cannot avoid loss of most of the original projection. On the other hand, even the severest form of visual deprivation (e.g. bilateral enucleation) cannot prevent maintenance of part of the juvenile projection.

The present experiments reveal a new role of vision and suggest that modifications of the earlier concepts may be necessary. The crucial, new finding is that the VLD animals which had short periods of visual experience during the first postnatal month do not suffer a deprivation-dependent loss of callosal efferents if their eyelids are subsequently sutured. More importantly, these animals retain callosal connections in parts of area 17 where they would have been lost had normal vision continued.

These results indicate that visual experience during the first postnatal month has a quick and potentially long-lasting, stabilizing influence on a fraction of the juvenile projections. However, this stabilization is reversible, at least over the following 1 or 2 months: if visual experience continues normally, it will provoke the elimination of some of the previously stabilized callosal projections, in particular, those from the more peripheral parts of area 17. This process progressively restricts callosal efferents to narrower portions of the visual field representation near the vertical meridian.

Two models can account for the vision-dependent[6] maturation of the callosal efferent zone in normal animals: (*i*) vision-dependent stabilization of callosal projections during the first postnatal month (nonstabilized connections are eliminated) followed during the second and possibly third months by vision-dependent destabilization of part of the same projections, and (*ii*) vision-dependent stabilization and destabilization both acting from eye opening but with the predominance of stabilization over progressively restricted portions of the visual areas during the first, second, and possibly third postnatal months.

In both models, the probability that vision will stabilize or destabilize a callosal axon depends on the tangential position of its cell body (but perhaps also of its termination). In both models, the destabilizing role of vision must be thought of as active. The alternative view that vision operates only by stabilizing and that elimination results simply from the lack of stabilization could explain the maturation of callosal projections during the first month (in model *i*) but not the wider tangential distribution of neurons in VLD than in normal cats.

At the moment there are no strong arguments in favor of one of the two models. However, the fact that SDV animals do not have an excessive loss of callosal efferents indicates a stabilizing effect of vision during the second postnatal month, thus supporting the second model.

In both models, vision may operate by biasing activity-dependent competition for maintenance between callosal and other axons. Vision-dependent stabilization of a callosal axon may occur when this axon is synergic with a thalamocortical one, for example, when the two fire synchronously a common target. Early postnatally, the poor optical qualities of the optic media and/or the immaturity of receptive fields could cause the stabilization of callosal connections over a wider part of the visual field representation than in the adult.

The later refinement of callosal connections could be deter-

[6] We will not consider vision-*independent* stabilization and/or destabilization that must also exist, as proven by the morphology of the callosal efferent zone in animals binocularly deprived for more than 3 months or binocularly enucleated at birth (Innocenti and Frost, 1980).

mined by the increase in response specificity during maturation of the visual system (see Buisseret and Imbert, 1976; Frégnac and Imbert, 1978). In this phase, a number of callosal axons, those originating and/or terminating in the most peripheral part of the visual field representation, may lose synergy of action with thalamocortical axons. These callosal axons would compete for targets with thalamocortical axons (or with the axons of neurons on which the latter impinge) and be eliminated. A similar explanation has been proposed for the effects of monocular deprivation in split chiasm kittens (Cynader et al., 1981).

The vision-dependent refinement of callosal connections seems to depend on normal binocular vision during the second to third postnatal month. Indeed, animals in which an enlarged distribution of callosal neurons was found, i.e., those rendered strabismic by surgery (Innocenti and Frost, 1979; Berman and Payne, 1983) or by previous deprivation (as in at least one of the SDV cats), as well as the VLD cats, all lacked normal binocular vision during the second postnatal month and seem to have kept the widespread distribution of callosal neurons which is normally found at the end of the first postnatal month (Innocenti and Caminiti, 1980; cf. D33 in this study).

Additional support for the notion that normal binocular vision is necessary for the late stages of the selection process may come from the confirmation (in progress) of our original data (Innocenti and Frost, 1979) suggesting enlargement of the callosal zone in monocularly deprived kittens. Monocular enucleation in cats and rats also produces enlargement of both the callosal zone and the callosal terminal territory (Innocenti and Frost, 1979; Rhoades and Dellacroce, 1980; Cusick and Lund, 1982; Rothblat and Hayes, 1982), although the interpretation of these findings is complicated by the possibility that enucleation may induce sprouting (or stabilization) of retinal axons from the remaining eye in parts of the dLGN which would normally be occupied by the enucleated eye (Hickey, 1975; Rakic, 1981).

The loss of callosal connections following early and uninterrupted binocular deprivation can be explained by assuming that this rearing condition may put callosal neurons at a severe competitive disadvantage with other neurons which are either spontaneously more active or more readily activated by retinal spontaneous activity or the poorly structured stimuli which reach the retina through the eyelids (Loop and Sherman, 1977; Spear et al., 1978). Spontaneous activity of retinal neurons or other neurons further along the visual pathway appears to be sufficient to sustain competition among axons at the cortical level: ocular dominance columns begin to segregate *in utero* in the monkey (Rakic, 1976; Hubel et al., 1977) and may segregate (although incompletely; Swindale, 1981) in dark-reared kittens, but not in kittens whose retinal activity is blocked by tetrodotoxin (Stryker, 1982). It is possible that, because of their higher spontaneous activity, less selective response properties, or greater sensitivity to retinal spontaneous activity, geniculate neurons have a competitive advantage over callosal neurons in conditions of bilateral eyelid suture. For similar reasons, some cortical neurons (forming intrinsic or ipsilateral corticocortical connections) could also be favored. The apparent paradox that bilaterally enucleated cats, undergoing a more severe form of deprivation, retain more callosal connections than do bilaterally lid-sutured ones (Innocenti and Frost, 1980) may be explained by the fact that enucleation removes via transneuronal degeneration some of the competitors of callosal axons. An alternative interpretation could be that enucleation abolishes evoked resonses from the retina as well as spontaneous activity of retinal origin. Thus, in enucleated cats, thalamocortical (or other competing) axons may lose the source of their normal competitive advantage over callosal axons. Dark rearing eliminates even poorly structured visual stimulation; therefore, it may have less severe effects than binocular lid suture on callosal development. This would suggest the use of opaque occluders in the period preceding correction of early cataract or corneal opacity.

Normal vision, binocular eyelid suture, dark rearing, and enucleation could all operate by differential biasing of competition among the different axonal systems impinging upon cortical neurons. Such biasing could be the only (or main) morphogenetic action of visual experience. This could explain the apparently modest and transitory changes in dendritic morphology, spine density, and synaptic density (e.g., Coleman and Riesen, 1968; Valverde, 1971; Vrensen, 1978; Winfield, 1981) in dark-reared or binocularly deprived animals.

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