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Selective Pruning of More Active Afferents When Cat Visual Cortex Is Pharmacologically Inhibited

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Summary

Activity-dependent competition is thought to guide the normal development of specific patterns of neural connections. Such competition generally favors more active inputs, making them larger and stronger, while less active inputs become smaller and weaker. We pharmacologically inhibited the activity of visual cortical cells and measured the three-dimensional structure of inputs serving the two eyes when one eye was occluded. The more active inputs serving the open eye actually became smaller than the deprived inputs from the occluded eye, which were similar to those in normal animals. These findings demonstrate in vivo that it is not the amount of afferent activity but the correlation between cortical and afferent activity that regulates the growth or retraction of these inputs.

Introduction

Activity-dependent mechanisms are thought to be important for the refinement of specific neural connections in the developing central nervous system. Activity influences and appears to guide the rearrangements of connections responsible for the normal development of the visual cortex (Antonini and Stryker, 1993a). These influences are more dramatic after occlusion of vision in one eye early in life, which causes most neurons in the visual cortex to lose their responses to the deprived eye and thereby to respond exclusively to the eye that has remained open (Wiesel and Hubel, 1963). Anatomically, the cortical territory serving the nondeprived eye is dramatically expanded, while the territory serving the deprived eye shows a compensatory shrinkage (Shatz and Stryker, 1978; LeVay et al., 1980), and afferents from the deprived eye lose half of their arbors in less than a week (Antonini and Stryker, 1993b, 1996). Such ocular dominance plasticity is thought to require neural activity in the visual cortex, because monocular deprivation does not induce a shift of cortical response toward the open eye when the activity of the cortical cells and their inputs are both blocked by tetrodotoxin infusion (Reiter et al., 1986).

Previous work has also demonstrated a role for the activity of the cortical cells themselves in controlling the direction of neural plasticity in vivo; when cortical cells were selectively inhibited pharmacologically by the $GABA_A$ receptor agonist muscimol, leaving the activity of geniculate afferents intact, the open eye became less effective than the deprived eye (Reiter and Stryker, 1988), and its territory was shrunken, while that of the deprived eye was expanded (Hata and Stryker, 1994). These findings suggest that the correlation between pre- and postsynaptic activity rather than the level of activity in afferent inputs per se plays an important role in ocular dominance plasticity. Indeed, identical patterns of input activity gave rise to opposite directions of plasticity, depending on whether the cortical target cells could respond to their inputs (Hata and Stryker, 1994). These results further suggest that there is a retrograde signal from target cells to presynaptic inputs and that this signal can be controlled by the target cells' activity. For example, in previous experiments, afferent inputs might have shown the opposite direction of plasticity, because the hypothesized retrograde signal had been shut down by inhibiting cortical cells.

The muscimol experiments, however, appeared to pose a difficulty for such possibilities. The difficulty was that if the retrograde signal were shut down by inhibiting the cortical target cells, the lack of signal ought to have effects on both the more active and less active inputs, because no messenger was available to either set of inputs. The two findings—that inhibiting cortical target cells using a drug that had no direct effect on the presynaptic afferent inputs caused a physiological plasticity in the direction opposite to normal, so that the more active input became less (rather than more) effective, while the less active input became relatively stronger, and that the more active inputs were confined to a smaller region of the cortex, while the overall territory covered by the less active inputs was larger than normal—raise the question of why the less active input pathway appeared to be spared the harm suffered by the more active input. They suggest that an additional mechanism modulates the effect of the signal from target cells, depending on the activity level of presynaptic inputs themselves.

However, we cannot make a conclusive statement on whether the two inputs from open and closed eyes really behaved differently in the inhibited cortex based on the earlier experiments, because we measured only relative, and not absolute, changes in the two inputs. The ocular dominance measured physiologically (Reiter et al., 1988) is a measure of the relative efficacy of the two eyes in driving cortical cells; the bulk transneuronal label following an eye injection (Hata and Stryker, 1994) showed the area over which the inputs spread but not their density. One could still maintain that both inputs were affected equally when trophic support from the target was curtailed but that the more active input was somewhat more severely affected and that these relative measures made it appear that the less active input was unharmed,

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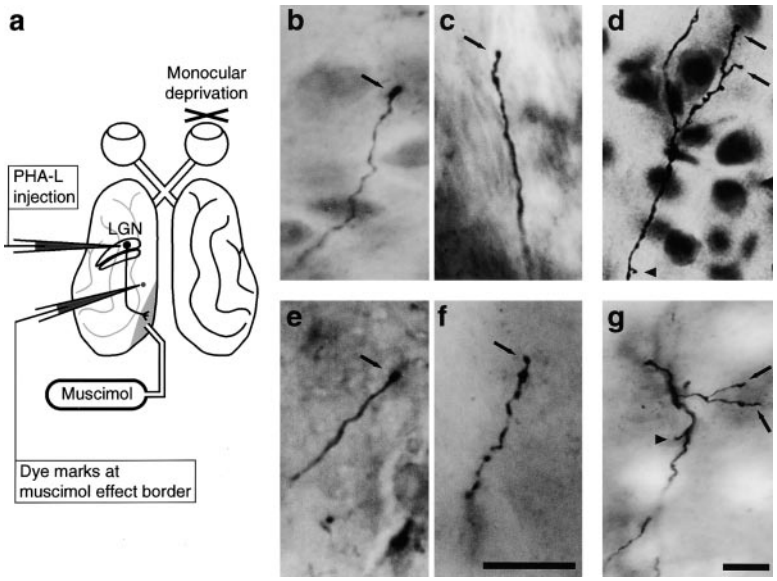

Figure 1. Labeling of LGN Afferents

(a) A schematic illustration showing experimental procedures.

(b–g) Examples of terminal arborization in each of six animals used in this study. The immunostaining procedure reveals that PHA-L is transported into the terminal structures, such as terminal swellings (arrows) and small varicosities on short stalks (arrowheads in [d] and [g]), equally well in the deprived eye arbors (b–d) and the nondeprived arbors (e–g). Scale bar, 20 μm.

despite being severely reduced. This explanation is not far fetched. For example, we know that earlier in normal development, afferents are very sparse but very widespread, occupying a territory much larger than they will later cover and being reasonably effective in driving cortical cells, despite their having few branches and synapses (Antonini and Stryker, 1993a). If in the muscimol experiments, the less active inputs became reduced but widespread when deprived of trophic support from the target, like the afferents in young normal animals, all of the earlier results might still be explained solely by a depletion of retrograde signal.

For this reason, it became essential to measure these plasticity effects by some absolute standard. Reconstruction of single afferent arbors is the only absolute standard available to us in this system, and ample data from normal animals and deprived animals with no drugs applied to the cortex are available for comparison. In the present experiments, we analyzed the morphology of individual geniculocortical arbors to obtain direct evidence of absolute rather than relative morphological changes during the reverse plasticity. Arbors serving the nondeprived eye in the pharmacologically suppressed cortex were much less elaborate in shape and much shorter in total length than those serving the deprived eye. Indeed, these open eye afferents were more severely shrunken than deprived eye arbors in untreated cortex. The branches of deprived eye afferents to suppressed cortex were similar in number to those of either eye's afferents in normal animals. These results establish that postsynaptic activity plays a fundamental role in the morphological plasticity of thalamocortical afferents and that this role is expressed in cooperation with presynaptic activity.

Results

We labeled the geniculocortical afferents in 4-week-old kittens by microinjection of the anterograde tracer Phaseolus lectin (PHA-L) within lamina A of the lateral geniculate nucleus (LGN) (Figure 1a) (Gerfen and Sawchenko, 1984; Antonini and Stryker, 1993a). One eye was deprived of vision by eyelid suture, while we suppressed the activity of visual cortical neurons by continuous infusion of the $GABA_A$ receptor agonist muscimol. After two weeks, which is long enough to cause visual cortical plasticity in the reverse direction (Hata and Stryker, 1994), the cortical region inactivated by muscimol was delineated physiologically by mapping cortical cells' activity. Dye marks made at the border of the inactivated region showed that all of the arbors in the present study were within the inactivated region of the cortex. The animals were perfused, and serial sections of visual cortex were stained immunohistochemically for anatomical demonstration of the geniculocortical arbors. Each single arbor was reconstructed in three dimensions.

Geniculocortical arbors were labeled well in the pharmacologically suppressed cortex, so that the terminal structures, such as terminal swellings and small varicosities on short stalks, could be observed clearly as shown in Figures 1b–1g. We found no difference in the quality of labeling between the arbors serving the deprived and the nondeprived eyes or between these and afferents published earlier (Antonini and Stryker, 1993a, 1993b, 1996).

Ten deprived eye arbors from three animals and ten nondeprived eye arbors from three animals were reconstructed in three dimensions in the present study. Five examples of arbors from each group are presented in Figure 2. The terminal arborizations of geniculocortical axons in the pharmacologically suppressed cortex were mainly localized in layer IV as in the normal cortex, consistent with the pattern observed in the previous transneuronal labeling experiments (Hata and Stryker, 1994). After monocular deprivation for 2 weeks, a period twice as long as that needed to achieve full shrinkage of deprived eye afferents in untreated cortex (Antonini and Stryker, 1996), the geniculocortical arbors serving the nondeprived eye showed a striking reduction in the complexity of the terminal arborization compared with the arbors serving the deprived eye. Furthermore, pial views of the arbors demonstrate that the cortical area covered by each axon serving the nondeprived eye is much less

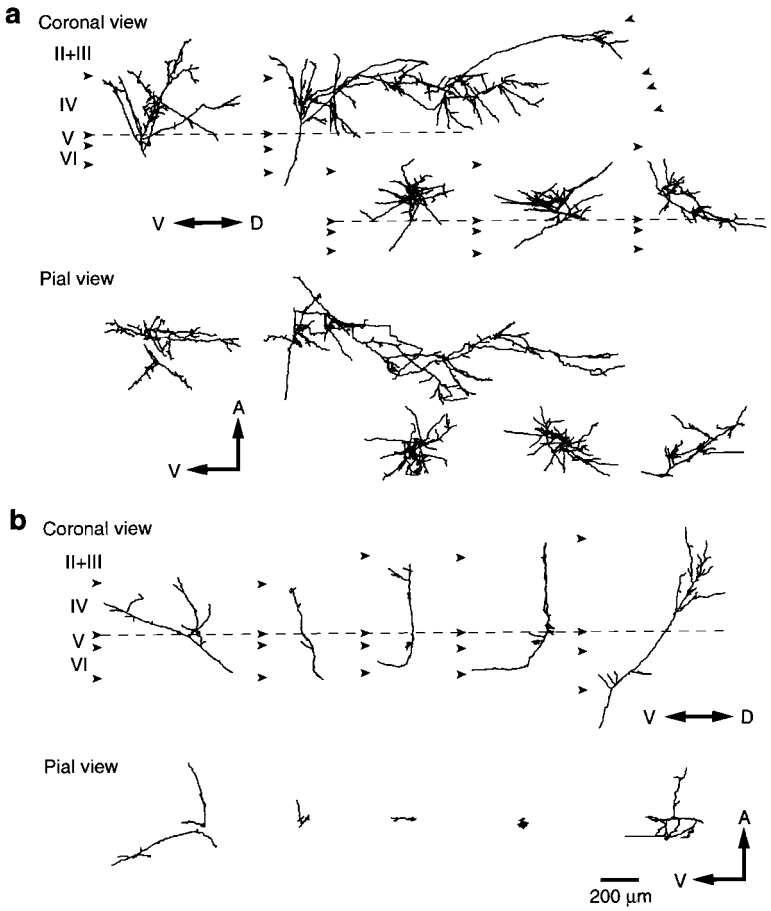

Figure 2. Examples of Reconstructed Arbors

Nondeprived eye arbors were less elaborate in shape and shorter in total length compared with the deprived eye arbors. (a) and (b) show examples of the computer reconstruction of deprived eye arbors (a) and nondeprived eye arbors (b). Arbors were originally reconstructed in the coronal plane. Pial view images were calculated on the computer by rotating the arbors 90° along the axis parallel to the cortical surface as indicated by a dotted line. Only the portions above the border between layers IV and V are shown as pial view images.

than that of the axons serving the deprived eye. This plasticity in axonal morphology is opposite in direction to the plasticity observed after monocular deprivation in normal animals (Antonini and Stryker, 1993b, 1996) and consistent with the plasticity of ocular dominance columns shown by transneuronal labeling (Hata and Stryker, 1994).

We measured three parameters (total length, number of branch points, and coverage area) for each arbor that are the estimates of size, shape, and projection area of the arbor, respectively (Figure 3). These measurements quantified the impression from visual inspection and allowed comparison of the present results with the published data (Antonini and Stryker, 1993a, 1996) for the arbors in animals of various experimental conditions (Table 1). The arbors serving the nondeprived eye in the muscimol-treated animals were highly significantly shorter in total length and had fewer branch points and narrower coverage areas than those serving the deprived eye. Comparing the arbors in the muscimol-treated animals with arbors in the other groups, nondeprived eye arbors in the muscimol-treated animals were shorter in total length than those found in all other groups; that is, arbors in normal kittens at postnatal day (P) 30 and 40 raised without any visual deprivation, deprived eye and nondeprived eye arbors in kittens that were monocularly deprived for 6 or 7 days, and deprived eye and nondeprived eye arbors in kittens that were monocularly deprived from eye opening to the day of perfusion. Furthermore, nondeprived eye arbors in muscimol-treated animals had fewer branch points and narrower coverage area than the arbors of all other groups.

Arbors serving the deprived eye in muscimol-treated animals were also somewhat shorter in total length than those in normal P30 and P40 animals and open eye arbors in monocularly deprived animals. If the lengths of deprived eye arbors in muscimol-treated animals are corrected for the approximate 33% $\pm$ 6% (SEM) overall shrinkage of the cortex in which they were embedded (measured in other animals), then they would not be significantly different in length from the open eye arbors in monocularly deprived animals that had not been treated with muscimol. However, they had a number of branch points similar to arbors in normal P30 and P40 animals. They also had significantly more branch points than deprived eye arbors in untreated cortex but fewer branch points than nondeprived eye arbors. The coverage area was similar to that in normal P30 animals but was significantly narrower than in normal P40 arbors and in open eye arbors in the long-term deprived animals.

Discussion

Individual geniculocortical arbors showed morphological plasticity in the reverse direction following monocular deprivation during inhibition of the cortex by an

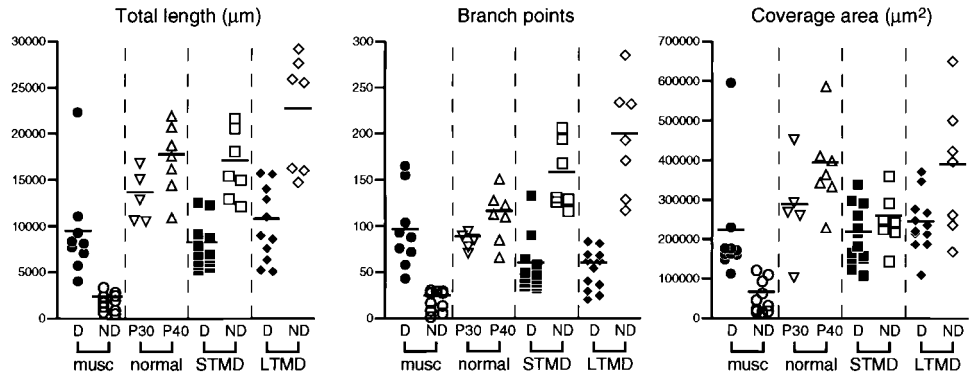

Figure 3. Quantitative Analysis of Size and Shape of Arbors

Scattergrams plot three measures of all individual arbors in the present study, together with the measures in normal and monocularly deprived animals published previously (Antonini and Stryker, 1993a, 1996). Short horizontal lines are their mean values. Only the portion of the arbor located in layers III and IV was considered for this analysis, so the axonal trunk and its bifurcations were clipped just below layer IV. Total length is the total linear length of the arborization obtained by the addition of the lengths of all of the branches constituting the terminal field of an arbor. Branch points are the total number of axonal bifurcations. The coverage area was evaluated from the pial view of the arbor, as the area that was within 50 μm from any branch of the arbor. Abbreviations for arbor and animal groups: D, deprived eye arbors; ND, nondeprived eye arbors; musc, kittens treated with muscimol; normal, kittens raised without any visual deprivation at P30 and P40, which correspond to the times of the beginning of muscimol infusion and the terminal perfusion in the present study, respectively; STMD, kittens that were monocularly deprived for 6 or 7 days before perfusion at about P40; and LTMD, kittens that were monocularly deprived from eye opening to the day of perfusion at about P40.

infusion of muscimol, consistent with the previous findings from extracellular recording (Reiter and Stryker, 1988) and transneuronal labeling experiments (Hata and Stryker, 1994). The quantitative analysis of arbor morphology in the present study further showed that the arbors serving the open eye were highly significantly smaller than those in the age-matched normal animals and the deprived eye arbors in the monocularly deprived animals that did not receive a cortical infusion of muscimol. On the other hand, arbors serving the deprived eye in muscimol-treated animals were somewhat shorter in total length than those in normal animals, but they had a similar number of branch points. This shrinkage of the deprived eye arbors in length may have nothing to do with plasticity mechanisms but may be due instead to the overall shrinkage of the cortex that is always observed in the area affected by the muscimol (Reiter and Stryker, 1988). Such an overall shrinkage of the brain tissue in which the arbors were embedded would necessarily reduce their total length but would not affect the number of branch points, exactly as observed. Therefore, we consider that the deprived eye arbors in the muscimol-treated animals may be similar to those in the normal animals.

Any discussion about activity-dependent plasticity in the muscimol-treated visual cortex assumes that muscimol acted selectively to suppress activity of the postsynaptic cells and did not have direct presynaptic effects. Pharmacological studies show that muscimol binds selectively to $GABA_A$ receptors that are located only on the postsynaptic sites (Hill and Bowery, 1981; Needler et al., 1984). In addition, if muscimol had had an effect on the viability of presynaptic fibers or on neurotransmitter release, such an effect should have caused a degradation of presynaptic afferents for both eyes or should have disrupted all plasticity, as was observed in cortex treated with tetrodotoxin (Reiter et al., 1986). The present finding that the plasticity of afferents in muscimol-treated cortex depended on their own activity as well argues strongly against these possibilities.

Table 1. Quantitative Analysis of Geniculocortical Arbors

	Arbor Groups						
	musc-ND	P30	P40	STMD-D	STMD-ND	LTMD-D	LTMD-ND
Total length							
musc-D	0.0002 ↑	0.0275 ↓	0.0084 ↓	ns	0.0063 ↓	ns	0.0018 ↓
musc-ND	—	0.0022 ↓	0.0006 ↓	<0.0001 ↓	0.0006 ↓	<0.0001 ↓	0.0006 ↓
Major branch points							
musc-D	0.0002 ↑	ns	ns	0.007 ↑	0.0084 ↓	0.0147 ↑	0.0025 ↓
musc-ND	—	0.0022 ↓	0.0006 ↓	<0.0001 ↓	0.0006 ↓	0.0016 ↓	0.0006 ↓
Coverage area							
musc-D	0.0002 ↑	ns	0.0084 ↓	ns	ns	ns	0.0147 ↓
musc-ND	—	0.0048 ↓	0.0006 ↓	<0.0001 ↓	0.0006 ↓	0.0001 ↓	0.0006 ↓

Statistical comparisons among arbor groups are provided as *p* values (Mann-Whitney U test) (ns, not significant).
Arrows by the p values indicate whether each of the three measures of musc-D and musc-ND groups shown at left were larger (↑) or smaller (↓) than those of the arbor groups shown at the top.

The difference in morphology between geniculocortical arbors serving the deprived eye and those serving the nondeprived eye might reflect the difference in the strength of some artifactual general effect of muscimol on them rather than the effect of visual deprivation on an unresponsive cortex. In this case, we would expect to find smaller arbors in the region of cortex closer to the muscimol infusing cannula, where such a hypothetical side effect of muscimol would have been stronger. This is not the case, however, because the distance from the cannula to the deprived arbors (mean $\pm$ SD, 1863.7 $\pm$ 1622.6 μm) was not significantly different from the distance to the nondeprived arbors (1391.1 $\pm$ 602.1 μm, $p = 0.76$, Mann-Whitney U test).

The present finding that nondeprived eye arbors showed a significant shrinkage in the inactivated cortex rules out simple versions of several theories of visual cortical plasticity. For example, a simple version of the Hebb rule, in which plastic changes in the efficacy of inputs are controlled by their temporal relationship to the action potentials of the postsynaptic cells, cannot distinguish between more active and less active inputs when, as in the present case, the postsynaptic cells never spike. The simplest version of the trophic factor hypothesis assumes that presynaptic inputs can get trophic support from their target neurons when they successfully activate the targets (Bonhoeffer, 1996; reviewed by Katz and Shatz, 1996). Theories of this type are based on the strengthening of a specific active pathway and a rather general or nonspecific weakening of the other inactive pathways. The present results, however, are difficult for such a theory to explain, and they suggest that another mode of plasticity that allows specific weakening of particular active pathways operates in the developing visual cortex, in addition to a strengthening mechanism.

On the other hand, the deprived eye arbors in the inactivated cortex were comparable in complexity to the arbors in the normal animals and did not show signs of expansion. Previous work showed that the nondeprived eye arbors in normal cortex expanded after monocular deprivation for 4 weeks, while 1 week of deprivation failed to cause such expansion (Antonini and Stryker, 1996). Therefore, the 2 weeks of monocular deprivation used in the present experiments might not have been sufficiently long for the deprived eye arbors in the inactivated cortex to expand, although it is also possible that only shrinkage or maintenance of afferent arbors can take place in the inactive cortex.

The present findings implicate a mechanism by which suppression of cortical activity can cause a retraction of presynaptic afferents, depending on their activity levels. The abundant evidence for involvement of neurotrophins in cortical plasticity (reviewed by Thoenen, 1995; Katz and Shatz, 1996; Pizzorusso and Maffei, 1996) makes a more complex trophic model, in which the less active presynaptic afferents serving the closed eye have a lower requirement for trophic support than the more active afferents, very attractive. The low requirement of the less active afferents might be met in full by constitutive release of a neurotrophin, if it were to take place on the dendrites that received these inputs (Blochl and Thoenen, 1995, 1996), while the more active afferents might require the activity-dependent release that would be absent in the presence of muscimol. Under this hypothesis, however, it is difficult to account for the reverse plasticity observed by Galuske et al. (1996) after exposure to exogenous brain-derived neurotrophic factor. A related possibility is the existence of an "activity threshold" in the presynaptic afferents for expressing structural changes in response to the signal for retraction, which could be a depletion of trophic factors or the supply of some negative substance. It is interesting in this connection that the influence of neurotrophins and depolarization interact strongly on some cells in model systems studied in vitro (Ghosh et al., 1994), such that neurotrophin effects are gated by the cells' own depolarization (McAllister et al., 1996; Shen et al., 1997, Soc. Neurosci., abstract); a similar gating in geniculocortical afferent terminals would constitute an activity threshold like that required for this hypothesis.

Alternatively, it is possible that there is a specific signal for retraction that is delivered only to the active afferents as a result of local synaptic interaction with target neurons, because muscimol treatment inhibits cortical neurons but does not prevent synaptic transmission itself. A modified Hebb rule based on local postsynaptic responses rather than action potentials and incorporating a postsynaptic plasticity mechanism with two separate thresholds for decreasing and increasing synaptic strength, like the abstract "sliding threshold" model (Bienenstock et al., 1982) or the concrete hypothesis put forward to explain long-term depression and potentiation (Bear and Malenka, 1994), could also be tuned to be consistent with the present findings, though such a model still requires retrograde messengers to communicate the reduced efficacy to the presynaptic afferent and cause it to retract. It is worth pointing out that inhibition of activity in the target neurons was shown to lead to suppression of synaptic transmission of the activated pathway in the visual system (Tamura et al, 1992; Kato and Yoshimura, 1993). In experiments using whole animals or visual cortical slices, electric stimulation given to afferent inputs, which usually induces long-term potentiation of evoked field potentials, caused a depression of field potential when neurons were inhibited pharmacologically during the conditioning period. Such a depression of physiological response may trigger a process leading to a shrinkage in morphology as observed in the present experiments. In any case, the cellular transaction responsible for activity-dependent plasticity involves signaling that is effectively bidirectional, and while it could well be mediated by trophic factors (e.g., von Bartheld et al., 1996), it uses them to steer the process rather than to fuel the engine that maintains synapses. These conclusions could not be drawn from the relative measurements in the earlier work on this system (Hata and Stryker, 1994).

Experimental Procedures

Surgery

All kittens in the present study were born in the breeding colony at Osaka University Medical School or the University of California, San Francisco. Figure 1a illustrates experimental procedures in the present study. A 30G stainless steel cannula connected to an osmotic minipump (Alzet 2002, Alza) was implanted in one hemisphere

of the primary visual cortex (stereotaxic location: A, −2.0 mm; L, 2.0 mm; depth from cortical surface: 2.0 mm) in 4-week-old kittens (P28–P31), and muscimol solution was infused continuously until terminal perfusion at P41–P45 (30 mM in saline, 0.5 μl/hr, 2 weeks). Before PHA-L injection, tungsten microelectrode penetrations were made between +3.0 and +5.0 mm (A) and 7.0 and 8.5 mm (L) to find a clear visual response from lamina A in the LGN, which is ipsilateral to the muscimol infusing cannula. Then, the microelectrode was withdrawn and substituted with a glass pipette (10–15 μm tip diameter) filled with 2.5% PHA-L solution (2.5% in phosphate-buffered saline [PBS], pH 8, Vector). The lectin was iontophoretically injected (pipette positive current of 5–10 μA, 2 s pulse, 1000 μA s in total) at the stereotaxic coordinates previously identified by the metal recording electrode, with verification of the depth by recording from the lectin electrode. Usually, four lectin injections were made in each LGN. Two days after the PHA-L injection surgery, one eye was deprived of vision by eyelid suture. All of the surgical procedures were performed using sterile procedures and N_2O:O_2 (2:1) combined with isoflurane (1.5% to 3.5%) for anesthesia. All of the incisions were infiltrated with xylocaine. Animals were given antimicrobial agent (enrofloxacin, 5 mg/kg) every day after the surgery.

Perfusion and Immunohistochemistry

Two weeks after the surgery, the cortical region inactivated by muscimol was delineated physiologically by mapping cortical cells' activity with a tungsten microelectrode at various distances from the cannula infusing muscimol solution. Animals were anesthetized with N_2O:O_2 (2:1) and Nembutal (2–4 mg/kg/hr) during recording experiments. Pontamine sky blue was injected iontophoretically to make dye marks at the border of the inactivated region. Then, the animals were sacrificed with an overdose of Nembutal (100 mg/kg, iv) and perfused transcardially with (in succession) ice-cold PBS; 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4); and 4% paraformaldehyde, 3.42 g/l L-lysine, 0.55 g/l sodium m-periodate, and 10% sucrose in PB. The brain was removed and postfixed overnight. Blocks containing the LGN and the entire caudal pole were embedded in gelatin albumin and cut (70 or 80 μm thickness) at the frozen microtome in the frontal plane. All sections were collected in potassium-buffered saline (KPBS, pH 7.4) and processed for standard indirect immunohistochemistry. Briefly, sections were incubated overnight at 4°C in a blocking solution composed of 2.5% bovine serum albumin (BSA), 2% normal rabbit serum (NRS), and 0.7% Triton X-100. They were then transferred into a solution of 2.5% BSA and 2% NRS containing the primary antibody (goat anti-PHA-L, Vector) at a dilution of 1:1000 and kept at 4°C for 72 hr. Following three washes in KPBS (10 min each), sections were incubated overnight at 4°C in a solution containing rabbit anti-goat biotinylated secondary antibody (Vector) in 2.5% BSA, 2% NRS, and 0.3% Triton X-100. After three washes in KPBS (10 min each), the sections were transferred for 3–4 hr into an avidin–horseradish peroxidase complex (Vector), washed for at least 1 hr, and finally reacted with a solution of 0.05% diaminobenzidine hydrochloride (DAB), 0.7% nitroammonium sulfate, and 0.3% hydrogen peroxide. All sections were mounted on gelatinized slides, dehydrated in graded series of ethyl alcohol, cleared in xylene, and coverslipped. Selected sections containing the visual cortex or LGN were stained with cresyl-violet for localization of the layer boundaries or injection sites.

Axonal Arbor Analysis

We limited our analysis to axons with well filled arbors that could be followed up to their thinnest terminals. PHA-L-filled arbors were reconstructed at ×1000 from serial sections in three dimensions with the aid of a computer graphic system, the Neurolucida system (Microbrightfield). Axonal reconstructions are shown in two views: in the coronal plane (as reconstructed) and after rotation about the ventro-dorsal axis, providing in this case a view tangential to the pial surface.

For each arbor, we made three measurements as described in detail in a previous paper (Antonini and Stryker, 1993a, 1996). First, the total linear length of the arborization obtained by the addition of the lengths of all of the branches constituting the terminal field of an arbor. Only the portion of the arbor located in layers III and IV was considered for this analysis; the axonal trunk and its bifurcations were thus clipped just below layer IV. Second, the number of branch points of the terminal arborization in layer IV obtained as described above. Third, the coverage area of the terminal arborization in layer IV evaluated from the pial view of the arbor. The terminal arborization was considered to be compressed along an axis perpendicular to the pial surface and to lie in a single plane. The coverage area of the arbor was calculated as the area that was within 50 μm from any branch of the arbor.

In these analyses, very short endings <5 μm (such as those indicated by arrowheads in Figures 1d and 1g) were measured but were omitted from all analyses. Arbors from previous experiments were analyzed again in the same way as the present data.

Acknowledgments

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