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Axonal sprouting accompanies functional reorganization in adult cat striate cortex

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REMOVAL of sensory input from a focal region of adult neocortex can lead to a large reorganization of cortical topography within the deprived area during subsequent months[1–9]. Although this form of functional recovery is now well documented across several sensory systems, the underlying cellular mechanisms remain elusive. Weeks after binocular retinal lesions silence a corresponding portion of striate cortex in the adult cat, this cortex again becomes responsive, this time to retinal loci immediately outside the scotoma. Earlier findings showed a lack of reorganization in the lateral geniculate nucleus and an inadequate spread of geniculocortical afferents to account for the cortical reorganization, suggesting the involvement of intrinsic cortical connections[4,10]. We investigated the possibility that intracortical axonal sprouting mediates long-term reorganization of cortical functional architecture. The anterograde label biocytin was used to compare the density of lateral projections into reorganized and non-deprived cortex. We report here that structural changes in the form of axonal sprouting of long-range laterally projecting neurons accompany topographic remodelling of the visual cortex.

To investigate the presence of sprouting of laterally projecting cortical neurons in functionally reorganized cortex, a comparison was made between axon fibres within normal and reorganized cortex. Receptive field (RF) maps were first documented before, immediately following and 3–9 months after making retinal lesions (Fig. 1 and Table 1). The cortical reorganization observed over this time period extended over an area 7–8 mm in diameter (Fig. 1). Because we were able to conclude from previous work (refs 4, 10 and C.D.-S. and C.D.G., manuscript in preparation) that changes occur largely at the cortical level, the present study focused on intracortical circuitry, particularly the horizontally projecting plexus of cortical neurons[11–13]. These connections extend laterally for 6–8 mm, and are thought to play a modulatory role in integrating visual information across neighbouring receptive fields. Although the horizontal connections can exhibit use-dependent changes in synaptic weights[14], these changes can occur within seconds and minutes. The reorganization described here, however, takes shape over several months. Our findings indicate these connections may provide the substrate through which visual information is transferred from normal to deprived cortical areas, and through which cortical circuitry is functionally and structurally redefined.

Intrinsic axonal projections were examined by placing extracellular injections of biocytin into cortex just outside the original boundary of the deprived region (cortical scotoma). This allowed labelled axons to be followed laterally from the same injection sites many millimetres into both normal and reorganized cortex. In this instance 'normal' refers to cortical areas representing unlesioned parts of retina, but because there could be distant effects in these areas as well, we also included controls in unlesioned animals. Three-dimensional reconstructions of axons (Fig. 2) projecting through layer III into reorganized cortex revealed a greater exuberance of terminal branching than seen in published examples of horizontally projecting neurons[11–13], and suggested that terminal sprouting might accompany functional reorganization. To examine this quantitatively, a comparison of fibre density was made between normal and reorganized cortex as documented physiologically. Fibre density was defined as the sum of the lengths of all fibre fragments found within the sampled volume of cortex. The selected sample regions were positioned at equal distances from the injection strip (see insert in Figs 2 and 3), and extended through the full cortical depth.

Axon fibres were always denser within reorganized than within normal cortex. In three of the four hemispheres analysed, the differences were highly significant (Fig. 3 and Table 1), with fibre densities 57-88% greater in reorganized than in normal cortex. Although in the fourth hemisphere the difference was not statistically significant, the trend was nonetheless similar, and there was a significant increase in density of axonal boutons

TABLE 1 Experimental details of the six hemispheres used

Cat number	Post-lesion survival	Δ Fibre density ($P<0.05$)	Scotoma size (°)	Distance from injections (mm)	Reorganization extent (mm)	Sample size (mm)
1	8 months	+66%, $P=0.008$	R = 8 × 16 L = 9 × 17	3.0	R ~ 2.9 L ~ 3.0	3.5 × 0.5
2	14 weeks	+16%, $P=0.133$	R = 10 × 17 L = 10 × 17	2.0	R ~ 2.5 L ~ 2.5	2.5 × 1.0
3R	8.5 months	+88%, $P=0.000$	R = 15 × 20 L = 15 × 22	1.0	R ~ 2.0 L ~ 2.2	2.5 × 1.0
3L	8.5 months	+57%, $P=0.003$	R = 15 × 20 L = 15 × 22	1.0	R ~ 2.0 L ~ 2.2	2.5 × 1.0
4R	Control no lesions	+13%, $P=0.443$	—	1.3	—	3.0 × 1.0
4L	Control no lesions	+6%, $P=0.692$	—	1.3	—	3.0 × 1.0

A paired sample *t*-test was used to compare the fibre densities in sample areas (dimensions in furthest right column), through the full sequence of sections cut tangentially from pia matter to white matter. Δ Fibre density is percentage difference between the summed fibre length (across 11–16 sections) in reorganized and normal sample cortex. Analogous sample regions were used for the control hemispheres. R and L, right and left hemispheres in column 1 and left and right eyes in column 4.

in the reorganized cortex of that animal (mean = 28 μm axon per bouton in reorganized compared with 46 μm in normal, $P < 0.05$). Preliminary counts indicate that bouton density is at least as great in lesioned animals with longer recovery periods (>8 months) as in unlesioned animals, suggesting that increased fibre density is associated with increased synapse number. Histogram profiles of the horizontal plexus showed a laminar distribution. When reorganized cortex was sampled 2–3 mm from the injection sites, excluding shorter range intrinsic neurons and thalamic afferents, profiles were hourglass in shape (black bars, Fig. 3*a*), with greatest densities found in supragranular and deep layers. Normal cortex, in comparison, showed bell-shaped profiles of the horizontal plexus (grey bars, Fig. 3*a*, *b*; grey and black bars, Fig. 3*c*), indicating that the greatest change with reorganization occurred within the superficial layers. In all cases, axons never extended further than 4–4.3 mm radially from the injection sites, other than in layer I. Therefore, growth occurred within the normal physical reach of the pre-existing fibre plexus.

The results indicate that axonal sprouting and presumably synaptic proliferation accompanies topographic reorganization of the striate cortex weeks and months after bilateral retinal lesions have been made. Certain advantages of the extracellular biocytin technique help strengthen the evidence for this. The lesion model allowed us to delineate precisely the boundary between normal and reorganized cortex and to differentiate fibre patterns between these two areas. Unlesioned cats were used as an additional control to allow for naturally occurring asymmetries in the horizontal projections at different visuotopic eccentricities within the striate cortex. An additional advantage gained by using extracellular injections is that the entire horizontal plexus of axonal fibres projecting into reorganized cortex was labelled, without the sampling bias associated with intracellular labelling studies.

Results from this and earlier work[3,4,6,10,15] suggest a possible sequence of mechanisms involved in and accompanying different phases of cortical reorganization. In the initial phase[4], occurring within minutes of making the retinal lesions, there is an expansion of RFs of cells located close to the border of the deprived cortex. This may represent the unmasking of subthreshold excitatory inputs, to a suprathreshold driving control, either by suppressing inhibitory or potentiating excitatory connections. The second phase, marked by the gradual expansion of the representation of perilesion retina into the intially silenced region of cortex, takes place over weeks and months. It is during this extended period that axonal growth and synaptogenesis occur. It may be that these morphological responses/adaptations to sustained sensory loss allow the cortical circuit to adjust to a new level around which rapid synaptic modulation could occur. Importantly, the morphological changes described respect the limits set by the pre-existing fibre plexus, resulting in the enrichment of fibre clusters rather than an apparent extension of fibres

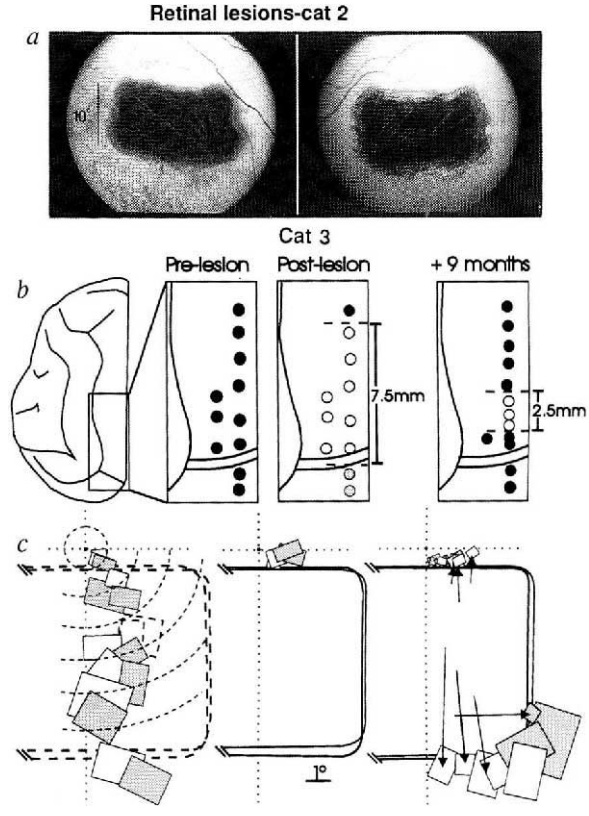

FIG. 1 Topographic reorganization of striate cortex after binocular retinal lesions. RFs were mapped from cells within a series of vertical electrode penetrations in the superficial layers, placed rostrocaudally through the striate cortex in adult cats. The example shown is from cat number 3 (*b*, *c*). Maps were made at corresponding sites before, immediately after and months after lesioning the eyes. Cortical vasculature and dural scarring served as fiduciary landmarks. In all cats, matching focal retinal lesions were made in the inferior visual hemifield, abutting but outside the area centralis (*a*, *c*). The lesions were photographed with a fundus camera (Zeiss FF4, *a*). Post-mortem histology showed that the pigment epithelium and photoreceptor layer were destroyed by the lesion, leaving the ganglion cell layer and vasculature intact. Retinal lesions in the two eyes were about 10° × 17° (see Table 1), traversed the vertical meridian to involve the two hemifields, and typically overlapped within visual space to an accuracy of 0.5° arc. Immediately after lesions were made, visually driven activity could not be elicited from cells within 7.5 mm of cortex, or 15° of visual space. Nine months later, however, 5 mm of the original cortical scotoma recovered visually driven activity, this time responding to stimuli presented immediately outside the retinal lesions. In visual space, RFs had shifted 6°-7° from their original positions inside the scotoma, to lie outside it (see arrows *c*).

METHODS. Retinal lesions, electrophysiological recording and iontophoretic injections of biocytin were made in anaesthetized, paralysed cats[4]. Lesions were made with multiple flashes fron a diode laser (Iris Instruments, 800 ms × 800 mW each). During the terminal experiment and after RFs had been mapped for the final time, a mediolateral strip of 3 biocytin injections (separated by about 1 mm and spanning the full depth of cortex) were placed just behind the original border of the deprived region of cortex, and the animal was perfused one day later. This border was localized according to pial vasculature patterns and dural scarring, recorded in photographs taken during earlier experiments. It was then possible to compare the densities of axons projecting from normal cortex into both reorganized and normal cortex. Axon fibres were reconstructed within sample regions at equal distances either side of the same injections (Fig. 3), and for sequential sections cut tangentially through the full thickness of cortex. The use of three injections of 300–800 μm diameter in each hemisphere and the reconstruction of fibre fragments within large sample areas lessened artefactual bias towards orientation or ocular dominance columns[31]. Only hemispheres with injection sites confined to grey matter were used. Injections of 4% Biocytin (Sigma) in saline were delivered iontophoretically (15 μm tip diam) using 2.5–3.5 μA positive current pulsed at 8-s intervals for 3–4 min. Two injections were made within each vertical penetration. Animals were perfused, the tissue was cut tangentially to the dorsal surface at 70–80 μm, and then processed using ABC reagent (Vector) and DAB as the chromagen. Retrogradely labelled neurons were rarely observed, and were pale in appearance when compared to anterogradely labelled cells[32]. Only anterogradely labelled axons were then reconstructed from sequential sections (Eutectics 3D Tracing System). Total length of fibre fragments and number of axonal boutons were documented within the reorganized and normal sample regions of each section, and paired according to depth from the pial surface. A dependent sample *t*-test was used for all calculations, for the full set of sections, reconstructed from pial surface to white matter. Results are listed in Table 1 and plotted in Fig. 3.

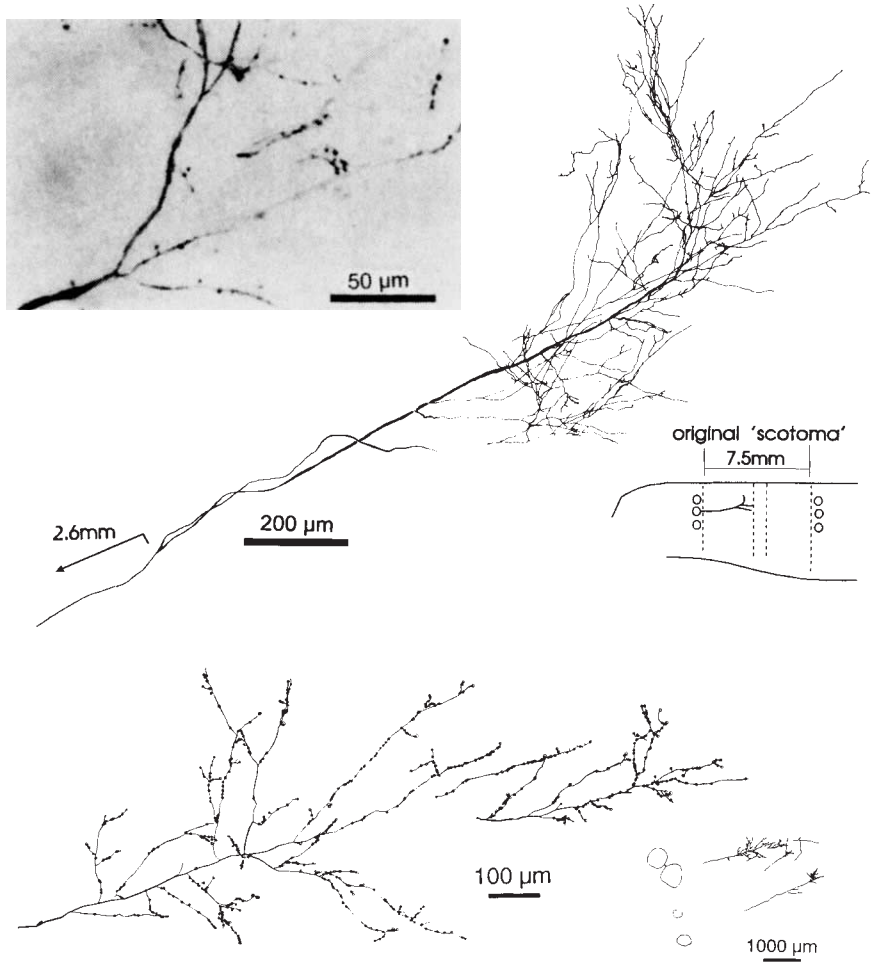

FIG. 2 Reconstructions of axon terminal tufts projecting from injections placed outside the original border of deprived cortex, into the reorganized cortical area. Both axons were reconstructed from layer III in the right hemisphere of cat number 1 (not used in the present analysis). Primary axons could be traced within two adjacent tangential sections for several mm back to injection sites but not to cell somata. The physical extent and richness of arborization contrasted with previously published accounts of long-range laterally projecting neurons in striate cortex[11–13].

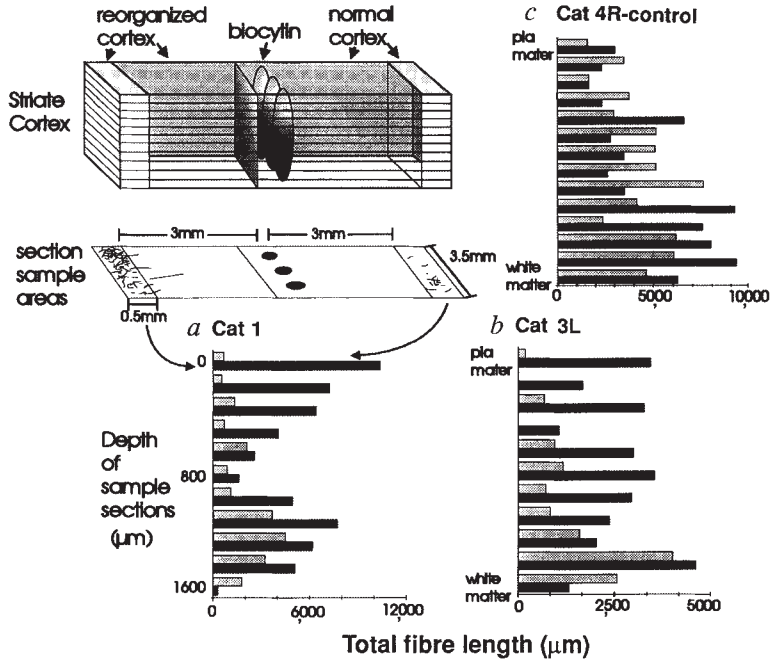

FIG. 3 Schematic of experimental model, showing placement of biocytin injections relative to cortical scotoma; and typical fibre density histogram profile for two lesioned (*a*, *b*) and one control (*c*) cat. Total fibre length (fibre density) was calculated as the length of fibre fragments summed within sample areas positioned at equal and constant distances (within any one hemisphere) either side of injection strips. Vertical abscissa (0–1,600 µm) indicates standardized depth of sections from pia to white matter; differences in the scale of the horizontal abscissae reflect differences in the size of injections within each hemisphere and thus the numbers of fibres labelled. Grey and black bars refer to analogous sample locations within striate cortex, in both reorganized and control hemispheres. Details of lesion dimensions, sample sizes and duration of recovery can be found in Table 1. Although axon fibre densities were significantly greater in reorganized cortex than in normal cortex (compare black with grey bars in *a*, *b*), similar differences were not observed within the control hemisphere (compare black with grey in *c*). Histogram profiles of the horizontal plexus showed a laminar distribution. In the reorganized cortex of lesioned cats (*a*, *b*), the greatest changes occurred consistently within the superficial layers. In cats where cortex was sampled 2 or 3 mm distant to the injection sites (for example, *a*), the profile was hourglass in shape, with lowest fibre densities occurring within layer 4, where thalamic afferents predominantly terminate. Density profiles for normal cortex (grey in *a*, *b*; grey and black in *c*) in both reorganized and control hemispheres were typically bell-shaped with greatest densities found within infragranular layers.

beyond the normal. This observation implies an upper limit for the physical extent of cortical reorganization and is consistent with findings in other sensory systems[8,16,17].

Synaptogenesis and sprouting have previously been affiliated with functional remodelling of Aplysia[18], and in a variety of structures in the adult mammalian central nervous system, including the hippocampal formation[19], ascending and descending pathways of the spinal cord[20-24] (M. Galea and I. Darian-Smith, manuscript in preparation), in the brainstem[22,25], thalamus[26], and most recently in motor cortex after cerebellar lesions[27] and electrical stimulation[28,29]. The findings presented here may be distinguished by a combination of features. Axonal sprouting is occurring within adult sensory neocortex, is directly related to functional changes, and is occurring in response to lesions made at the sensory periphery, without cortical deafferentation[4,10] or synaptic vacancies being left by retracted connections. Although these results show sprouting of intrinsic connections as an important element in cortical reorganization, they do not exclude potential additional influences by distant cortical and subcortical areas. It is tempting to suggest that sprouting and synaptogenesis accounts for some of the topographic reorganization reported in other sensory systems, following peripheral manipulations and even training[1-9,29,30]. Our findings raise the possibility that terminal sprouting and synaptic proliferation represents part of the normal response of the adult cortex to extended modification of sensory experience. □

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A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation

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PHARMACOLOGICAL studies of long-term potentiation (LTP) in the hippocampus are starting to provide a molecular understanding of synaptic plastic processes which are believed to be important for learning and memory in vertebrates[1]. In the CA1 region of the hippocampus, the synaptic activation of glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype is necessary for the induction of LTP under most experimental conditions[2,3]. The synaptic activation of metabotropic glutamate receptors (mGluRs) is also needed for the induction of LTP[4,5]. We now show that the role of mGluRs in the induction of LTP is fundamentally different from that of NMDA receptors. NMDA receptors initiate a molecular event that needs to be triggered each time a tetanus is delivered to induce LTP. In contrast, mGluRs activate a molecular switch which then negates the need for mGluR stimulation during the induction of LTP. This mGluR-activated switch is input-specific and can be turned off by a train of low-frequency stimulation. The molecular switch is a new feature of LTP which has fundamental consequences for our understanding of synaptic plastic mechanisms.

The specific mGluR antagonist (±)α-methyl-4-carboxyphenylglycine ((±)MCPG)[6] blocks the induction of LTP, but not short-term potentiation (STP), in the Schaffer collateral–commissural pathway[4,5]. The activity of MCPG as a mGluR antagonist resides solely in the (+) isomer[7]. Consistent with the block by MCPG being due to antagonism at these receptors, (+)MCPG, but not (−)MCPG, blocked the induction of LTP when tested sequentially on three slices (data not shown). We therefore used (+)MCPG throughout. We tested 200 μM MCPG on 39 slices that had not received any other treatments (naive slices). In 33 of these slices, the induction of LTP was blocked (the response measured 60 min following a tetanus was 101 ± 1% control; range 95–108%). Thus, tetanic stimulation in the presence of MCPG induced STP but not LTP and after washout of MCPG tetanic stimulation induced LTP. In 6 slices, however, the induction of LTP was not prevented by MCPG (the response measured 60 min following a tetanus was 133 ± 2% control; range 130–142%; data not shown).

mGluRs can initiate transient signals, such as the formation of inositol phosphates, and sustained signals, such as phosphorylation through the activation of protein kinase C[8,9]. We reasoned that if mGluRs initiate a persistent change, then mGluRs might not need to be activated during each tetanus. We therefore established that MCPG blocked the induction of LTP; after washout of MCPG we induced LTP and then stimulus-matched the potentiated response to control levels. Finally, we tested the ability of MCPG (200 μM) to block the induction of further LTP. Invariably MCPG failed to prevent the induction of further LTP ($n = 5$; Fig. 1*a*, *b*). In case 200 μM was insufficient to block the induction of further LTP, after LTP had been induced, we repeated the experiment with a fivefold higher concentration of MCPG. In each slice tested, MCPG (1 mM) failed to prevent the induction of further LTP ($n = 5$; Fig. 1*c*).

These experiments show that there is a conditioning effect of an LTP-inducing tetanus which negates the need for the synaptic activation of mGluRs for the induction of further LTP. We have assumed that this conditioning process is initiated by the synaptic activation of mGluRs during the LTP-inducing tetanus.