rate was restricted to codons that code for the same amino acid in all three taxa.

Method (1). For each pair of sequences (i and j), the rates of synonymous transition ($K_{ts4(i,j)}$) and transversion ($K_{tv(i,j)}$) mutations were estimated separately at fourfold degenerate sites using the equations of Ina[29] for Kimura's two-parameter method[22]. The rate of transition substitution at twofold degenerate sites ($K_{ts2(i,j)}$) was estimated as $K_{ts2(i,j)} = -\frac{1}{2}\ln(1-2P_{s(i,j)})$, where $P_{s(i,j)}$ is the proportion of sites that show a difference between sites. The overall synonymous transition rate (K_{ts}) was calculated as a weighted (by number of sites) average of the twofold and fourfold rates. The non-synonymous substitution rate per codon was calculated as $K_{n(i,j)} = -\ln(1-P_{n(i,j)})$, where $P_{n(i,j)}$ is the proportion of amino acids that differed between sequences. The distances along each branch were calculated using Fitch and Margoliash's method; for example, $K_{ts(i)} = (K_{ts(i,j)} + K_{ts(i,k)} - K_{ts(j,k)})/2$.

Method (2). Primate sequences are sufficiently similar that parsimony can also be used to estimate substitution rates. We estimated the numbers of synonymous transition and transversion and amino-acid substitutions for the human and chimpanzee lineages. Dividing these numbers by the relevant number of sites gave the substitution rate per site (as above). By reconstructing the sequence ancestral to the human/chimpanzee divide, we could also separately estimate the transition rate at CpG dinucleotides and incorporate this rate into the calculation of mutation rates. All methods gave quantitatively similar results.

Estimates of non-synonymous and deleterious mutation rates and constraint. Rates of non-synonymous (M) and deleterious (U) mutation were estimated using weighted (by length) and unweighted substitution-rate estimates. Weighted method: $M = Z\Sigma(L(K_{ts}N_{ts} + K_{tv}N_{tv}))/\Sigma L$, $U = M - Z\Sigma(LK_n/3)/\Sigma L$; unweighted: $M = Z(\overline{K_{ts}N_{ts}} + \overline{K_{tv}N_{tv}})$, $U = M - Z\overline{K_n}/3$; where Z is a constant that incorporates the number and length of genes and the generation time; for example, for humans $Z = 2(\text{genomes}) \times 60,000(\text{genes}) \times 1,523(\text{base pairs}) \times 25(\text{years})/6 \times 10^6$ (years), and all summations were across genes.

As the estimate of constraint is subject to large sampling error, we produced a joint estimate of the level of constraint in hominid protein-coding genes in the following manner. Data sets for all genes with homologues in humans and chimpanzees ($n = 53$), humans and gorillas (30), chimpanzees and gorillas (28), humans and orang-utans (25) and chimpanzees and orang-utans (25) were compiled and rates of substitution were measured as in method (1). We then estimated the number of non-synonymous substitutions predicted to occur in all sequences should all non-synonymous mutations be neutral from:

$$X = \sum_{\text{data sets}} \sum_{\text{genes}} L(K_{ts}N_{ts} + K_{tv}N_{tv})$$

and the number of non-synonymous substitutions that have occurred from:

$$Y = \sum_{\text{data sets}} \sum_{\text{genes}} LK_n/3.$$

Constraint was then estimated as $C = 1 - Y/X$.

All estimates of standard error were obtained by bootstrapping the data, by gene, 1,000 times.

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Monocular deprivation induces homosynaptic long-term depression in visual cortex

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Brief monocular deprivation during early postnatal development can lead to a depression of synaptic transmission that renders visual cortical neurons unresponsive to subsequent visual stimulation through the deprived eye. The Bienenstock–Cooper–Munro (BCM) theory[1] proposes that homosynaptic mechanisms of long-term depression (LTD) account for the deprivation effects[2,3]. Homosynaptic depression, by definition, occurs only at active synapses. Thus, in contrast to the commonly held view that the synaptic depression caused by monocular deprivation is simply a result of retinal inactivity, this theoretical framework indicates that the synaptic depression may actually be driven by the residual activity in the visually deprived retina[4]. Here we examine the validity of this idea by comparing the consequences of brief monocular deprivation by lid suture with those of monocular inactivation by intra-ocular treatment with tetrodotoxin. Lid suture leaves the retina spontaneously active, whereas tetrodotoxin eliminates all activity. In agreement with the BCM theory, our results show that monocular lid suture causes a significantly greater depression of deprived-eye responses in kitten visual cortex than does treatment with tetrodotoxin. These findings have important implications for mechanisms of experience-dependent plasticity in the neocortex.

Previous work has shown that monocular inactivation with tetrodotoxin (TTX), like monocular lid suture, shifts the ocular dominance of cortical neurons strongly towards the non-deprived eye[5,6]. However, those studies used prolonged TTX treatment

(~1 week) in animals at postnatal days 23–34 (P23–34), when shifts in ocular dominance occur very rapidly (over periods of hours to days)[7]. Thus, differences in the rate of the ocular dominance shift in the monocular-inactivation and monocular-suture groups might have been obscured because the deprivation-induced synaptic depression was completely saturated at the time that ocular dominance was measured. For our experiments, therefore, we decided to use short periods of deprivation in slightly older animals. The experimental design is illustrated in Fig. 1a. Kittens were reared normally until P45–61, at which time they were briefly anaesthetized and received a monocular intravitreal injection of either TTX (monocular-inactivation group) or the same volume of saline (monocular-suture group). The experimenters were 'blind' to the contents of the injection syringe (see Methods). Following the injection, the lid of the injected eye was sutured closed. After two days of monocular visual experience, the animals were placed in a darkroom for two additional days to allow the effects of TTX to wear off. The animals were then anaesthetized and assayed for changes in cortical ocular dominance.

The ocular-dominance assay consists of recording from multiple cortical sites and, for each site, comparing the activity evoked by stimulation of the right (non-deprived) and left (deprived) eyes. We recorded multi-unit activity every 100 μm along electrode penetrations that ran tangentially through striate cortex, down the medial bank of the lateral gyrus. Recordings were always made from the hemisphere ipsilateral to the non-deprived eye. Visual responses were evoked with moving sinusoidal gratings, presented at each of 16 evenly spaced orientations. Spikes evoked by each stimulus, and those occurring spontaneously when the screen was blank, were discriminated and stored on a computer. The peak firing rate in response to the optimal stimulus (orientation and direction) was determined for each eye, and an ocular-dominance score was calculated (Fig. 1b). A score of −1 means that the unit responds exclusively to stimulation of the left (deprived) eye; a score of +1 means that the unit responds only to the right (non-deprived) eye; a score of zero means that right and left eye responses are equal.

Figure 2a shows the cumulative probability distribution of the ocular-dominance scores for the 273 sites recorded in ten animals in the monocular-suture group (black line) compared with the distribution of the scores for the 238 sites recorded in ten animals in the monocular-inactivation group (grey line). As expected[7], the distribution in the monocular-suture group is clearly skewed towards the non-deprived eye (66% of cells have ocular-dominance values of >0). In contrast, the distribution in the monocular-inactivation group shows roughly equal numbers of units with responses dominated by the open and deprived eyes. The two distributions are significantly different at $P < 0.005$ (Kolmogorov–Smirnov test). Other measures of cortical-response properties, such as the degree of orientation selectivity and the response modulation to the grating stimuli, did not differ significantly between groups (data not shown).

Assays of ocular dominance are often done using subjective determinations of the magnitude of responses to stimulation of the two eyes. In these cases, units are assigned to discrete ocular-dominance

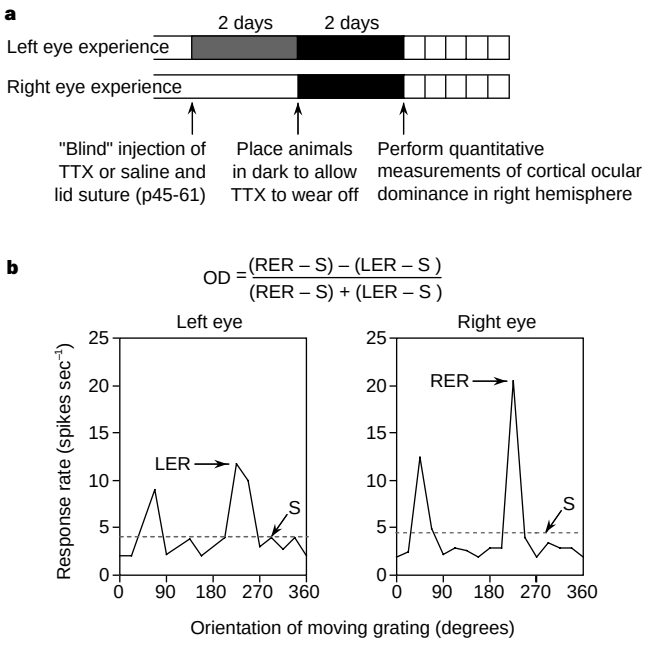

Figure 1 Experimental design. **a**, Kittens (P45–61) received intravitreal injections of TTX (monocular-inactivation (MI) group) or saline (monocular deprivation by lid suture (MS) group) into the left eye. The lids of the injected eye were sutured closed. The animals had two days of monocular visual experience (12 h/12 h light/dark cycle), and were then placed in the dark for a further two days to allow the effects of the TTX to wear off. The animals were then anaesthetized and a quantitative ocular-dominance assay was performed in area 17 contralateral to the deprived eye. **b**, At each recording site in the cortex ($n = 273$ for the MS group; $n = 238$ for the MI group), response rates were determined for moving grating stimuli presented at 16 different orientations/directions. These data were used to construct tuning curves. The peak responses to stimulation of the right (right-eye response, RER) and left (left-eye response, LER) eyes above spontaneous activity (S) were used to calculate an ocular-dominance score. A score of 1 means that the activity was driven solely by the right (non-deprived) eye; a score of –1 means that the activity was driven solely by the left (deprived) eye; a score of 0 means the activity was driven equally by stimulation of either eye. The ocular-dominance (OD) score for this example cell was 0.39.

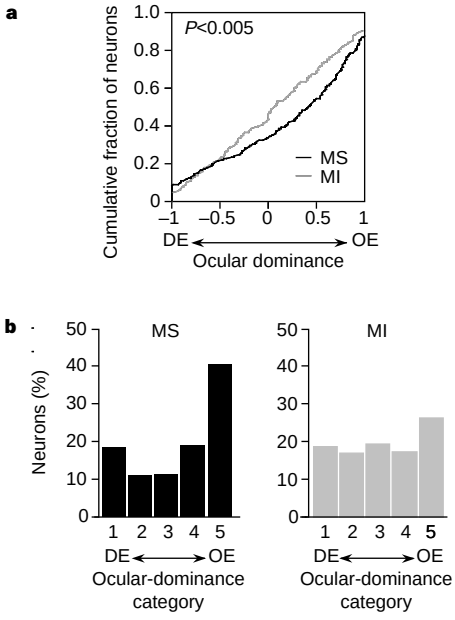

Figure 2 Analysis of the ocular-dominance data pooled from all animals in each group. **a**, The cumulative fraction of the total number of units with each ocular-dominance score recorded in MS animals ($n = 273$; black line) and MI animals ($n = 238$; grey line) is plotted against the units' ocular-dominance scores. The MS and MI distributions are significantly different ($P < 0.005$; Kolmogorov–Smirnov test). Unlike the MI distribution, the MS distribution is skewed towards ocular-dominance values of >1, which indicates a shift in ocular dominance towards the open (non-deprived) eye (OE). DE, deprived eye. **b**, The ocular-dominance data from **a** were divided into five equally spaced ocular-dominance categories, such that cells with ocular-dominance values of –1 to –0.6 were assigned to category 1, cells with ocular-dominance values of –0.59 to –0.2 were assigned to category 2, and so on. The ocular-dominance distribution of the MS units (left; black histogram) is shifted towards category 5 (dominated by the open eye); the ocular-dominance distribution of the MI units (right; grey histogram) is flat in comparison.

categories. To aid the comparison with such studies, we binned the quantitative ocular-dominance data and plotted them in histograms (Fig. 2b). The ocular-dominance histogram constructed from units recorded in animals of the monocular-suture group shows the expected shift towards the open eye. In contrast, the histogram from animals in the monocular-inactivation group is flat. Taken together, these data show that a larger fraction of the cortical neurons have lost responses to the deprived eye in the monocular-suture group than in animals that had been monocularly inactivated.

Ocular-dominance data pooled from many animals can be biased towards those individual animals in which the largest number of units were studied. Therefore, we next analysed the results by case (Fig. 3). Cumulative probability distributions of ocular-dominance scores for each animal reveal a clear tendency for animals of the monocular-suture group to have a larger proportion of positive ocular-dominance scores (that is, more cells dominated by the open eye) than monocularly inactivated animals (Fig. 3a, b). To quantify this difference, we calculated an open-eye dominance (OED) score, which reflects the percentage of neurons with ocular-dominance scores of >0.5, for each case. Figure 3c shows the cumulative probability distribution of the OED values for the individual animals in each group. The two groups are significantly different at $P < 0.02$ (Mann–Whitney U-test).

Ocular-dominance plasticity declines with age[7,8]. It is possible that there might have been a systematic bias in our study, such that the animals of the monocular-inactivation group were older than those in the monocular-suture group. However, age differences cannot account for the differences between the groups (Fig. 3c): the average age of animals in the monocular-inactivation group was 53.6 ± 1.8 days, compared with 54.4 ± 1.4 days in the monocular-suture group.

Despite the overall difference in ocular dominance between groups, inspection of the data in Fig. 3 revealed that two of the ten animals in the monocular-inactivation group showed large

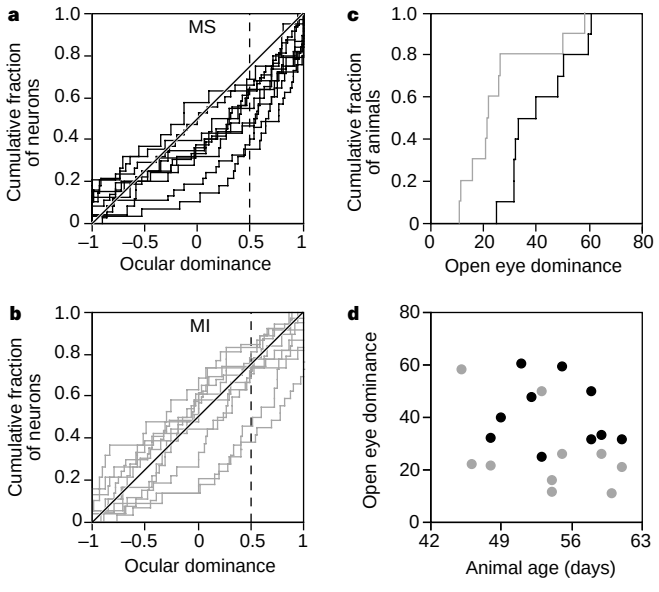

Figure 3 Analysis by case. **a**, **b**, Cumulative probability distributions for the units recorded in each MS (**a**) and MI (**b**) animal. The fraction of cells in each case with ocular-dominance scores of >0.5 (dashed vertical line) was designated as the open-eye-dominance (OED) value for that case. **c**, Cumulative probability distributions of the OED values for the ten MS animals (black line) and the ten MI animals (grey line). The MS group has significantly larger OED values than the MI group ($P < 0.02$; Mann-Whitney U-test). **d**, Individual OED values (MS animals, black symbols; MI animals, grey symbols) as a function of the animals' ages. A systematic age difference cannot account for the difference in OED between groups.

shifts in ocular dominance towards the open eye. Possible explanations for these cases, other than biological variability, are that the TTX injections failed to block retinal activity in these animals (thus making them monocularly sutured animals), or that TTX had not been completely cleared from the eyes at the time at which recordings were made from cortex (thus making it difficult to drive activity from the injected eye). However, even taking into account these apparent outliers, the two groups of animals are significantly different. We conclude that the synaptic depression induced in visual cortex by monocular deprivation is greater when the eyelid is simply sutured than when all retinal activity is eliminated.

The synaptic plasticity that underlies the shift in ocular dominance occurs in the striate cortex; the striate cortex receives retinal input indirectly through a relay in the lateral geniculate nucleus (LGN). We designed our experiment on the basis of the assumption that our manipulations of retinal activity translate fairly directly into alterations of LGN activity. To confirm the validity of this assumption, in five more experiments we recorded from the LGN as we manipulated retinal activity. In two animals we simply sampled activity in LGN neurons as the electrode was tracked up and down through lamina A and A1. In agreement with previous studies[9,10], we found that the average spontaneous activity with the eyelid closed (9.7 ± 1.5 spikes s^{-1}) was sharply reduced following TTX injection into the eye (2.0 ± 0.4 spikes s^{-1}). In the remaining three animals, we followed the activity in single LGN neurons as retinal activity was varied. These experiments yielded a similar conclusion, namely that retinal TTX treatment reduces spontaneous activity by ~80%. Thus, the crucial difference between animals in the monocular-suture and monocular-inactivation groups is the amount of activity in the visually deprived afferent neurons that lead to the cortex; the greater synaptic depression in the cortex is associated with the inputs that are more active.

Previous studies have shown that presynaptic activity can lead to a depression of synaptic strength in visual cortex if postsynaptic activity is blocked completely by intracortical infusion of a GABA (γ-aminobutyric acid)-receptor agonist[11] or a glutamate-receptor antagonist[12]. Our results show that presynaptically driven weakening of synapses also occurs under natural conditions. How does this mechanism contribute to the shift in ocular dominance during monocular deprivation? Theoretical analysis and simulation[4] indicate that afferent inputs leading from deprived eyes lose synaptic strength when they are active at the same time that the activity of the postsynaptic cortical neuron is less than a threshold value. For stimulus-selective neurons in visual cortex, weak postsynaptic responses occur whenever the neuron's receptive field is not stimulated by its preferred visual pattern viewed through the open eye. It is interesting in this context that when cortical stimulus selectivity is broadened by intracortical infusion of a GABA-receptor antagonist, thus making the postsynaptic cells respond strongly more often, the ocular-dominance shift is prevented[13].

It is possible to recreate experimentally *in vitro* the conditions that theoretically should produce synaptic depression (that is, presynaptic activity correlated with weak postsynaptic responses). Such experiments have confirmed that homosynaptic LTD results from this type of stimulation of synapses throughout the cerebral cortex[14,15]. Our results add experimental support to the theoretical suggestion that the mechanisms of homosynaptic LTD could account for aspects of ocular-dominance plasticity[2]. Indeed, like the synaptic depression caused by monocular deprivation[8,12,16], LTD in visual cortex is regulated by age[17,18], NMDA (*N*-methyl-D-aspartate)-receptor activity[19] and neuromodulators (serotonin[20], acetylcholine and norepinephrine[21]). Taken together, these data indicate that homosynaptic LTD and naturally occurring synaptic depression in visual cortex may share common molecular mechanisms.

Prolonged monocular inactivation does produce a shift in ocular dominance in P23–34 animals[5,6]. If homosynaptic mechanisms are

responsible for ocular-dominance plasticity, why is there ever any shift in ocular dominance after monocular inactivation? There are several possible reasons why plasticity can be observed with prolonged monocular inactivation in younger animals. First, some level of noise almost certainly converges on cortical neurons even after retinal TTX treatment. Monocular inactivation does not completely eliminate activity in the LGN afferents serving the deprived eye, and even spontaneous presynaptic release of glutamate can activate NMDA-receptor-mediated postsynaptic currents at some synapses[22]. It is possible that such weak presynaptic activity could cause synaptic depression, given enough time (especially in young animals, in which the mechanisms of LTD are highly expressed[17,18]). Second, because ocular dominance is a measurement of relative response magnitude, part of the shift could be accounted for by homosynaptic potentiation of the non-deprived afferents[4]. Finally, it is possible that the mechanisms of ocular-dominance plasticity are different in animals of less than five weeks in age.

The synaptic depression induced by visual deprivation is more pronounced when one eye is deprived (monocular deprivation) than when both eyes are deprived (binocular deprivation)[23]. This observation led to the early suggestion[24,25] that *heterosynaptic* LTD occurs in visual cortex (that is, a depression of inactive deprived-eye synapses that is triggered by the strong activation of the post-synaptic neuron by the non-deprived eye). The BCM theory offers an alternative explanation for the difference between the effects of monocular and binocular deprivation. According to this theory, the amount of homosynaptic depression induced by presynaptic activity varies depending on the average level of cortical activity, which is higher during monocular deprivation than during binocular deprivation[4]. Indeed, in agreement with the theory, it has been shown experimentally that homosynaptic LTD is significantly reduced in binocularly deprived cortex[26].

The occurrence of homosynaptic depression may, therefore, be sufficient to account for the initial loss of cortical responsiveness to visual inputs to an eye that was deprived of normal vision for a period of time. If this hypothesis is correct, we could be on the threshold of unprecedented insight into the detailed molecular mechanisms of one form of visual cortical plasticity. □

Methods

Injection and lid suture. Kittens were anaesthetized by continuous administration of isoflurane gas (2–3% in 100% O_2 at 11 min^{-1}). Ten animals received an injection of TTX (4 μl of 1.25 mM TTX in 5% citrate buffer; Calbiochem) into the vitreous humour of the left eye; ten others received an injection of 4 μl saline. Injections were performed without experimenter knowledge of the contents of the injection syringe; experimenters remained blind until the analysis of all experiments had been completed. Following the injection, the margins of the upper and lower lids of the injected eye were trimmed and sutured together. The entire procedure was completed in less than 20 min and the kittens then recovered rapidly. Previous work[10] and pilot studies in our laboratory indicated that the TTX inactivates the retina completely for two days, after which the retina recovers gradually over another two days.

Electrophysiology and visual stimulation. Animals were prepared for electrophysiology and visual stimulation as described[27]. Multiunit activity was recorded using glass-covered tungsten electrodes with an impedance of 0.8–1.5 MΩ. The visual stimulation used to quantify responses consisted of high-contrast, drifting sinusoidal gratings with a spatial frequency of 1 cycle per degree and a temporal frequency of 1 Hz. These stimulus parameters were chosen because, in our experience, they elicit a response from most cortical cells. The grating stimuli were 19° tall × 28° wide, and extended well beyond the receptive fields of both eyes. Gratings were presented at 16 different orientations/directions that varied by 22.5° around a full 360°. Each grating orientation/direction was presented 5 times for 5 s each. Data were also collected during 5 blank-screen trials (5 sec each) to measure spontaneous activity. To ensure that we did not oversample from the first ocular-dominance column, the electrode penetrations were highly oblique, beginning medial to the crown of the lateral gyrus and running parallel to the cortical surface, down the medial wall of the hemisphere. The tracks were always long enough to sample complete ipsi-contra eye cycles of ocular dominance.

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In vivo regulation of axon extension and pathfinding by growth-cone calcium transients

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Growth cones at the tips of extending neurites migrate through complex environments in the developing nervous system and guide axons to appropriate target regions using local cues[1,2]. The intracellular calcium concentration ($[Ca^{2+}]_i$) of growth cones correlates with motility *in vitro*[3–7], but the physiological links