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 \( (K_{ts}(i,j)) \) and transversion \( (K_{tv}(i,j)) \) mutations were estimated separately at fourfold degenerate sites using the equations of Ina\(^2\) for Kimura’s two-parameter method\(^2\). The rate of transition substitution at twofold degenerate sites \( (K_{ts}(i,j)) \) was estimated as \( K_{ts}(i,j) = -2\ln(1 - P_{ts}(i,j)) \), where \( P_{ts}(i,j) \) is the proportion of sites that show a difference between sites. The overall synonymous transition rate \( (K_s) \) 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 \( K_{ns}(i,j) = -\ln(1 - P_{ns}(i,j)) \), where \( P_{ns}(i,j) \) 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, \( K_{ts}(i,j) = (K_{ts}(i,k) + K_{ts}(k,j) - K_{ts}(i,j))/2 \).

**Method (2).** Prime 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-rate estimates. Weighted method: \( M = Z\Sigma(I(K_{ts}(\text{unw}) + K_{ts}(\text{w}))/\Sigma I), \quad U = M - 2Z\Sigma L(K_{ts}/3)/\Sigma I; \) unweighted: \( M = Z(K_{ts}(\text{unw}) + K_{ts}(\text{w})), \quad U = M - ZK_{ts}/3 \); where \( Z \) is a constant that incorporates the number and length of genes and the generation time; for example, for humans \( Z = 2 \text{ (genomes)} \times 60,000 \text{ (genes)} \times 1,523 \text{ (base pairs)} \times 25 \text{ (years)} / 6 \times 10^9 \text{ (years)} \), 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 (\( n = 53 \)) and 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:

\[
X = \sum_{\text{data sets genes}} \sum_{\text{sites}} I(K_{ts}(\text{unw}) + K_{ts}(\text{w}))
\]

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

\[
Y = \sum_{\text{data sets genes}} \sum_{\text{sites}} LK_{ts}/3\beta.
\]

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.

Received 16 October; accepted 1 December 1998.


Correspondence and requests for materials should be addressed to A.E.-W. (e-mail: A.C.Eyre-Walker@susx.ac.uk).

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**Monocular deprivation induces homosynaptic long-term depression in visual cortex**

Cynthia D. Rittenhouse\(^*\),†, Harel Z. Shouval\(^*\), Michael A. Paradiso\(^†\) & Mark F. Bear\(^†\)

\(*\) Howard Hughes Medical Institute and † Department of Neuroscience, Brown University, Providence, Rhode Island 02912, USA

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\(^2\). 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\(^5\). 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)\(^2\). Thus, differences in the rate of the ocular dominance shift in the monocular-inactivating 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\(^2\), 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 categories. The cumulative fraction of the total number of units with each ocular-dominance score recorded in an animal is plotted against the units’ ocular-dominance scores. The MS and MI distributions are significantly different at \(P < 0.005\) (Kolmogorov–Smirnov test). Unlike the MI distribution, the MS distribution is 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).

Figure 2b shows the distribution of ocular-dominance categories for the 273 sites recorded in ten animals in the monocular-suture group and the 238 sites recorded in ten animals in the monocular-inactivation group. As expected\(^2\), 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).

$$\text{OD} = \frac{(RER - S) - (LER - S)}{(RER - S) + (LER - S)}$$

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 lenses 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. At each recording site in the cortex (\(n = 273\); black line) or MI animals (\(n = 238\); grey line) were evoked with moving sinusoidal gratings, presented at each of 16 orientations and directions. These data were used to construct tuning curves. The peak responses to stimulation of the 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 that the activity was driven equally by stimulation of either eye. The ocular-dominance (OD) score for this example cell was 0.39.
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 monocural-suture group shows the expected shift towards the open eye. In contrast, the histogram from animals in the monocural-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 monocural-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 monocural-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. It is possible that there might have been a systematic bias in our study, such that the animals of the monocural-inactivation group were older than those in the monocural-suture group. However, age differences cannot account for the differences between the groups (Fig. 3c): the average age of animals in the monocural-inactivation group was 53.6 ± 1.8 days, compared with 54.4 ± 1.4 days in the monocural-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 monocural-inactivation group showed large 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,[4,5] 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 monocural-suture and monocural-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[13] 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.[14]

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.[4,15] 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, the synaptic depression caused by monocular deprivation[6,12,14,15] LID in visual cortex is regulated by age[7,16] NMDA (N-methyl-D-aspartate)–receptor activity[17] and neuromodulators (serotonin[18], acetylcholine and norepinephrine[19]). 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
Methods

Injection and lid suture. Kittens were anesthetized by continuous administration of isoflurane gas (2–3% in 100% O2 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\(^6\) and pilot studies in our laboratory indicated that the TTX inactivates the retina completely for two hours, after which the retina recovers gradually over another two days.

Electrophysiology and visual stimulation. Animals were prepared for electrophysiology and visual stimulation as described.\(^2\) 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\(^\times\)19 pixels with a size of 0.8°\times\times\times\times0.8°, with a period of 1.75°. 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 eyes of ocular dominance.

Received 27 October, accepted 1 December 1998.

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Acknowledgements. This work was supported by the Howard Hughes Medical Institute, the NIH and the Dana Foundation.

Correspondence and requests for materials should be addressed to M.F.B. (e-mail: mmbear@brown.edu).

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

Timothy M. Gomez & Nicholas C. Spitzer

Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92039-0357, USA

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. The intracellular calcium concentration ([Ca\(^{2+}\)]) of growth cones correlates with motility in vitro\(^a\), but the physiological links...