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Functional Masking of Deprived Eye Responses by Callosal Input during Ocular Dominance Plasticity

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SUMMARY

Monocular deprivation (MD) is a well-known paradigm of experience-dependent plasticity in which cortical neurons exhibit a shift of ocular dominance (OD) toward the open eye. The mechanisms underlying this form of plasticity are incompletely understood. Here we demonstrate the involvement of callosal connections in the synaptic modifications occurring during MD. Rats at the peak of the critical period were deprived for 7 days, resulting in the expected OD shift toward the open eye. Acute microinjection of the activity blocker muscimol into the visual cortex contralateral to the recording site restored binocularity of cortical cells. Continuous silencing of callosal input throughout the period of MD also resulted in substantial attenuation of the OD shift. Blockade of interhemispheric communication selectively enhanced deprived eye responses with no effect on open eye-driven activity. We conclude that callosal inputs play a key role in functional weakening of less active connections during OD plasticity.

INTRODUCTION

The corpus callosum is the largest white matter structure in the brain and mediates most of inter-hemispheric communication (Gazzaniga, 2005). Callosal fibers are involved in many cortical functions requiring either integration of information across hemispheres or independent function of the two sides of the brain (Bloom and Hynd, 2005; Paul et al., 2007). In particular, in the visual cortex, commissural connections serve to bind together the separate representations of the two halves of the visual field in the two hemispheres (Innocenti, 1980; Houzel and Milleret, 1999). Consistent with this function, cell bodies and axonal terminals of callosal neurons in cat visual cortex are densely packed along the representation of the vertical meridian, i.e., at the border between areas 17 and 18 (Innocenti, 1980; Payne, 1994). In rodents, callosal cells occupy the entire mediolateral extent of striate cortex (Olavarria and Van Sluyters, 1983; Lewis and Olavarria, 1995); however, their terminals are still particularly concentrated in a quite narrow stripe at the area 17/18 border (Jacobson, 1970; Cusick and Lund, 1981; Mizuno et al., 2007). The majority of commissural fibers derive from neurons in superficial layers and make synapses mostly in layers II, III, and V of the contralateral hemisphere (Jacobson and Trojanowski, 1974; Aggoun-Aouaoui et al., 1996; Mizuno et al., 2007).

We have previously shown that callosal connections are crucial for the developmental maturation of primary visual cortex during the sensitive period (Caleo et al., 2007). Here we probe the involvement of callosal inputs in visual cortex plasticity using the well established paradigm of monocular deprivation (MD) in young rats. MD by eyelid suture induces a dramatic loss of visual responsiveness to the closed eye, resulting in a shift of ocular dominance (OD) toward the open eye (Wiesel and Hubel, 1963). The mechanisms underlying this effect are only partly understood (Frenkel and Bear, 2004; Hensch, 2005; Mrsic-Flogel et al., 2007). Previous studies revealed anatomical changes in the transcallosal pathway following visual deprivation (Innocenti and Frost, 1979; Cusick and Lund, 1982; Innocenti et al., 1985; Innocenti and Price, 2005). In particular, monocular visual deprivation produces an anatomical expansion of the callosal pathway (Innocenti and Frost, 1979; Cusick and Lund, 1982). Changes in callosal output were demonstrated in split-chiasm kittens subjected to MD (Cynader et al., 1981). However, there is no evidence supporting the requirement for callosal inputs in the ocularity changes induced by MD. Here we demonstrate that callosal afferents play a key role in the weakening of deprived eye inputs during MD.

RESULTS

Callosal Connections Contribute to Normal Binocularity

We first asked whether interhemispheric connections are involved in cortical binocularity. Experiments were performed in naive rats during the developmental critical period [postnatal day (P) 26–P30]. We compared binocularity of cortical cells before and after silencing of the visual cortex contralateral to the recording site (Figure 1A). The spiking activity of cortical neurons was recorded extracellularly in two-three penetrations in one hemisphere. Activity was recorded from superficial layers and in correspondence with the cortical representation of the vertical meridian (i.e., at the border between area 17 and 18), where callosal inputs terminate most densely (Cusick and Lund, 1981; Mizuno et al., 2007). After recording, we injected either the $GABA_A$ agonist muscimol (1 μl; 30 mM solution) or saline into the contralateral hemisphere. Muscimol blocked activity in the infused side within 30 min (Figure 1B). After this period we started to record from single units again in the opposite cortex (Figure 1A).

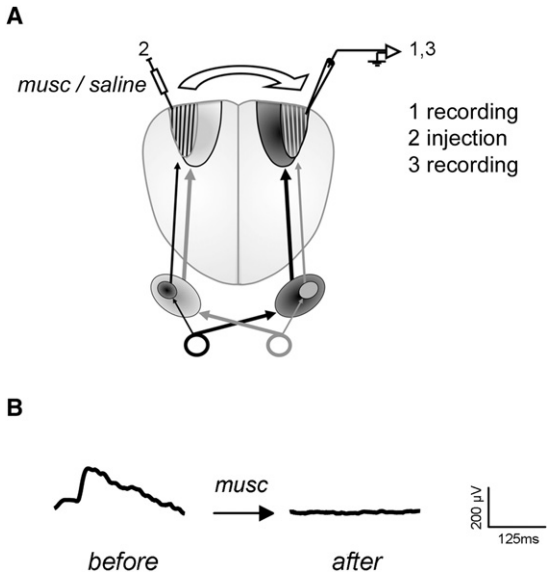

Figure 1. Experimental Protocol

(A) Schematic diagram of the rat visual system and description of the experimental protocol. The striped areas indicate the binocular portion of the primary visual cortex in both hemispheres. Binocularity was recorded before and after injection of either saline or muscimol (musc) into the contralateral cortex.

(B) Representative examples of VEP recordings demonstrating blockade of injected visual cortex after muscimol (musc) delivery.

We found that the OD distribution of cortical neurons significantly shifted toward the contralateral eye following muscimol, but not saline, injection. OD was quantitatively assigned to each unit according to a five point scale (Maffei et al., 1992; Lodovichi et al., 2000) and was based on the computer-calculated peak firing rate in response to stimulation of each eye with a light bar drifting into the receptive field (RF; Mandolesi et al., 2005; Caleo et al., 2007). Saline infusion into the opposite side had no effect on binocularity, as shown by analysis of both OD distributions (χ^2 test, naive before saline versus after saline, $p = 0.48$; Figure 2A) and contralateral bias index (CBI; post ANOVA Holm-Sidak test, $p = 0.82$; Figure 2C). Conversely, muscimol injection increased the proportion of class 1 cells and led to a corresponding decrease of binocular units (χ^2 test, naive before muscimol versus after muscimol, $p < 0.001$; Figure 2B). Accordingly, CBIs were significantly higher following muscimol (one-way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, $p < 0.001$; Figure 2C). We conclude that acute silencing of callosal input affects cortical OD.

This change in binocularity could be due to an increase in contralateral eye strength or to a decreased visual drive through the ipsilateral eye. To define the mechanism, we analyzed the peak discharge rates of cortical units following stimulation of each eye, before and after muscimol (or saline) administration. Injection of saline impacted neither contralateral nor ipsilateral eye responses (one-way ANOVA on ranks followed by Dunn's test, $p > 0.05$ for both comparisons; data not shown). Following muscimol infusion, we found a consistent reduction of responses through the ipsilateral eye (one-way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, IPSI before versus IPSI after, $p < 0.01$; Figure 2D). Thus, callosal connections contribute ipsilateral eye inputs to cortical neurons, and when interhemispheric communication is silenced, OD shifts toward the contralateral eye (Figures 2B and 2C).

Callosal Effect on Binocularity Is Restricted to the Vertical Meridian

Since contralateral bias is graded across the mediolateral extent of the visual cortex in rodents (Drager, 1975; Gordon and Stryker, 1996; Caleo et al., 1999), it was of interest to determine how the muscimol effect varies with mediolateral position in the cortex. Penetrations were made at different sites (4.0–4.9 mm lateral from lambda) to sample the whole binocular hemifield before and after muscimol administration to the opposite side. The relationship between OD and RF position is shown in Figure 3, which reports the CBI as a function of RF center azimuth. As previously described (Gordon and Stryker, 1996; Caleo et al., 1999), binocularity was high within the central 20° of the visual hemifield and contralateral dominance increased at more peripheral locations (20°–30° from the vertical meridian; Figure 3). Muscimol selectively increased the contralateral bias of cells with RFs close to the vertical meridian and the effect was no longer apparent at more medial locations in the cortex (two-way ANOVA followed by Holm-Sidak test, 0°–10° before versus after, $p = 0.005$; Figure 3). This is consistent with callosal projections being particularly concentrated in the lateral aspect of primary visual cortex (Cusick and Lund, 1981; Mizuno et al., 2007).

Acute Silencing of Callosal Inputs Restores Binocularity in Monocularly Deprived Animals

Since interhemispheric connections play a role in binocularity, we studied their involvement in the plastic shift of OD after MD. Rats at the peak of the critical period (age P20–P23) were monocularly deprived for 7 days, and extracellular recordings of neuron activity were performed from the cortex contralateral to the occluded eye before and after injection of muscimol (or saline as control) into the opposite hemisphere. This protocol allows the dissection of the effect of MD under acute deprivation of callosal input, i.e., the OD shift that would be measured via the sole geniculocortical pathway.

All recordings were made in correspondence with the vertical meridian. Seven days of MD produced the expected change in eye preference of cortical neurons and the OD distribution was skewed to the open, ipsilateral eye (Figures 4A and 4B). Injection of saline into the opposite side produced no changes in eye preference, as indicated by statistical analysis of OD distributions (χ^2 test, MD before saline versus after saline, $p = 0.29$; Figure 4A) and CBIs (one-way ANOVA followed by Holm-Sidak test, before saline versus after saline, $p = 0.86$; Figure 4C).

In contrast, muscimol infusion had a dramatic impact on the OD histogram. There was a consistent reduction of class 7 cells and a corresponding rise in the proportion of closed eye-driven units (Figure 4B). Statistical analysis of OD histograms indicated that the OD shift was robustly attenuated after muscimol (χ^2 test, MD before muscimol versus after muscimol, $p < 0.001$). Analysis of CBIs of single animals strengthened the conclusions obtained from the pooled OD distributions. Deprived animals recorded before muscimol showed a consistent drop in CBI values as

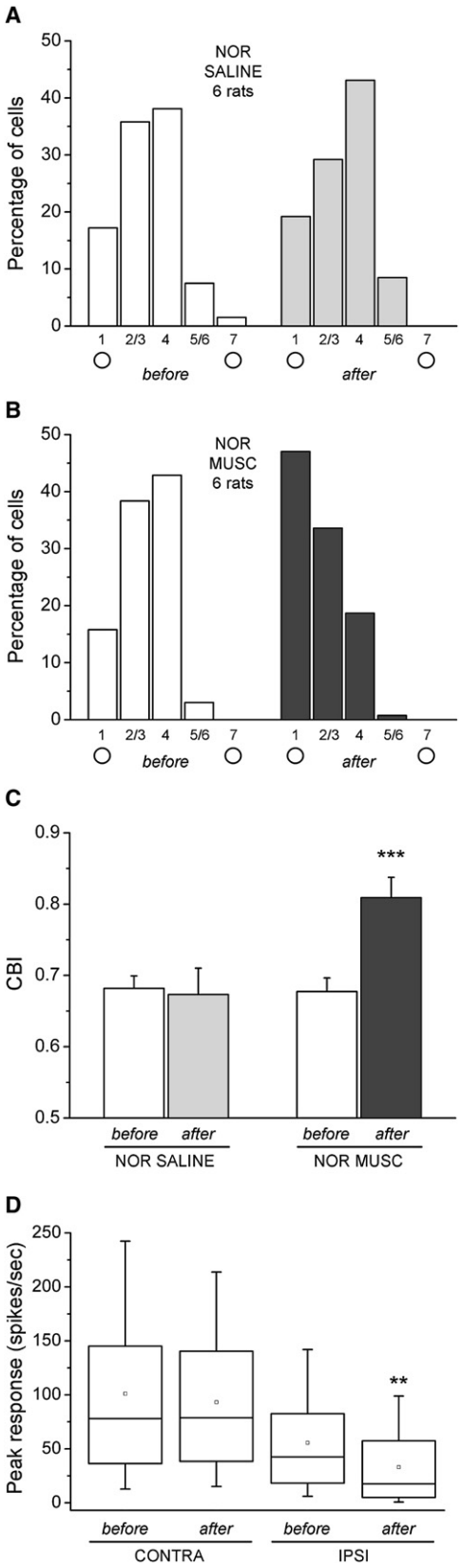

Figure 2. Contribution of Callosal Inputs to Cortical Binocularity

(A and B) OD distributions of normal (NOR) young rats (age P26–P30) before and after injection of saline (A) or muscimol (MUSC; B) into the opposite hemisphere. Saline has no effect on binocularity (χ^2 test, $p = 0.48$), whereas rats injected with muscimol show a clear increase in the number of units driven exclusively by the contralateral eye (χ^2 test, $p < 0.001$). Number of animals as indicated. Before saline, n = 135 cells; after saline, n = 130 cells; before muscimol, n = 133 cells; after muscimol, n = 134 cells.

(C) CBIs of all treated animals. Data are mean ± standard error. There is a significant enhancement of contralateral bias after muscimol (one-way ANOVA followed by Holm-Sidak test, CBI before muscimol versus after muscimol, $p < 0.001$). Saline, n = 6 rats; muscimol, n = 6 rats. ***$p < 0.001$.

(D) Box chart showing peak firing rates of visual cortical neurons in naive rats injected with muscimol. There is a very significant reduction of the responses evoked by the ipsilateral (IPSI) eye following muscimol administration into the opposite side (post ANOVA Dunn's test, ipsilateral eye, before versus after muscimol, $p < 0.01$). There is no significant effect on contralateral (CONTRA) eye responses. The horizontal lines in each box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbols denote the mean of the column of data. Before muscimol, n = 133 cells; after muscimol, n = 134 cells. **$p < 0.01$.

compared to naive animals (one-way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, MD before muscimol versus naive, $p < 0.001$). Binocularity was recovered to a large extent by blocking the opposite hemisphere (one-way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, MD after muscimol versus MD before muscimol, $p < 0.001$; Figure 4C). It is noteworthy that the change in eye preference induced by acute muscimol injection in MD animals was much greater than that obtained in naive rats (t test, $p < 0.001$; Figure 4D). Thus, the ipsi-shifted OD bias contralateral to the deprived eye can be reversed by acute silencing of callosal inputs.

Acute Silencing of Callosal Afferents Restores Binocularity after Long-Term MD

We next asked whether a role for callosal inputs in OD plasticity is maintained after long-term MD. Rats were monocularly deprived for 15–20 days starting at P16–P18 and then recorded contralateral to the closed eye. OD histograms and CBI values indicated a very dramatic shift of eye preference toward the open eye (see Figure S1, available online, and Figure 4C). Acute silencing of the opposite hemisphere with muscimol produced a consistent return of OD toward normal values in all recorded animals (OD histograms, before versus after muscimol, χ^2 test, $p < 0.001$; CBI values, before versus after muscimol, t test, $p < 0.001$; Figures S1 and 4C). The OD change triggered by acute muscimol infusion was similar for both 7 days and long-term MD (t test, $p = 0.65$; see Figure 4C). Thus, an involvement of the callosum in OD plasticity is still detected following prolonged MD.

Acute Removal of Callosal Input Affects OD in the Hemisphere Ipsilateral to the Deprived Eye

We also examined the effects of callosal afferents on the OD shift in the hemisphere ipsilateral to the deprived eye. We found that visual responses were strongly dominated by the contralateral, open eye following 7 days of MD (Figure S2). Infusion of saline into the opposite side had no significant effect on OD (χ^2 test, before saline versus after saline, $p = 0.275$; data not shown). Notably, injection of muscimol produced a clear recovery of

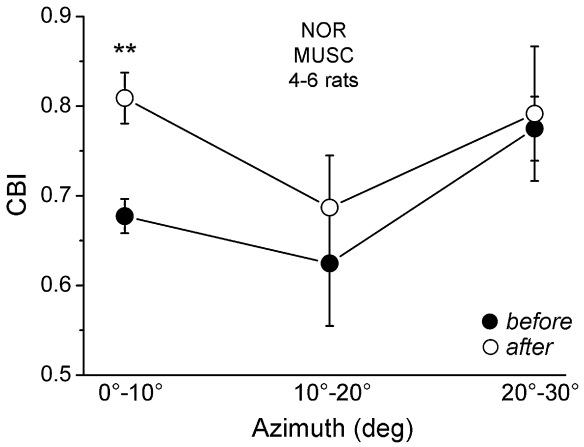

Figure 3. Acute Blockade of Callosal Input Alters the Eye Preference of Cells with RFs Close to the Vertical Meridian

Relationship between OD and RF location in the visual cortex before/after muscimol infusion into the contralateral hemisphere. CBI was calculated from the OD distributions of cells with their RF center azimuths located between 0 and 10°, 10° and 20°, and 20° and 30° from the vertical meridian. Data are mean ± standard error. Muscimol significantly increases the contralateral bias within the central 10° (two-way ANOVA followed by Holm-Sidak test, 0°–10° before versus after, $p = 0.005$). For each point, n = 115–194 cells from four to six rats, except for 20°–30° after muscimol, where n = 80 cells. **$p < 0.01$.

binocularity. There was a substantial increase in the proportion of class 4 cells and a parallel decrease in the number of monocular units (Figure S2A). CBIs were also significantly changed following muscimol (t test, $p < 0.01$; Figure S2B). Thus, acute silencing of callosal inputs consistently alleviates the OD shift also in the hemisphere ipsilateral to the closed eye.

Role of the Callosum in Adult MD

The data shown above demonstrate that acute removal of transcallosal influences restores binocularity after a period of MD in young rats. To determine whether this enhancement of binocular responses is restricted to the critical period, we analyzed the effects of unilateral muscimol treatment in adult MD. Rats older than P80 were monocularly deprived for 7 days and recordings were performed contralateral to the occluded eye. We found that adult MD produced no significant alteration of the normal OD histogram (χ^2 test, normal adult versus MD adult, $p > 0.05$; Figures 5A and 5B), consistent with previous reports (Pizzorusso et al., 2002; Caleo et al., 2007). Acute muscimol infusion slightly increased the contralateral eye bias in these animals (χ^2 test, before muscimol versus after muscimol, $p = 0.007$; Figure 5B; t test, $p < 0.05$; Figure 5C), an effect that is reminiscent of that observed in young undeprived rats (see Figure 2B). Thus, callosal inputs change their effect on OD as a specific result of critical period plasticity.

Requirement of Callosal Inputs in Generating the Monocular Bias During MD

So far we have shown a role for callosal inputs in OD plasticity based on acute silencing of interhemispheric communication after a period of MD. To demonstrate that the callosal pathway is required for the shift of eye preference during MD, we performed experiments in which callosal input activity is blocked continuously throughout the period of sensory deprivation. Young (P20–P23) rats were monocularly deprived for 7 days and muscimol (10–30 mM solution) or saline were concurrently infused by osmotic minipumps into the visual cortex ipsilateral to the closed eye. At the end of the deprivation period, minipumps were removed and the animals were placed in complete darkness for 26–48 hr to allow washout of muscimol. In a series of experiments, we determined that minipump delivery of muscimol efficiently blocked activity in the infused side and that visual responses were restored by 26 hr after minipump removal (data not shown). OD was measured in the cortex contralateral to the occluded eye (i.e., contralateral to minipump infusion). In animals treated with saline, OD histograms were strongly skewed in favor of the open, ipsilateral eye (Figure 6A). In contrast, we found a very significant attenuation of the OD shift in rats treated with muscimol (Figure 6B). Indeed, the OD distribution of muscimol-infused animals was significantly different from that of MD saline rats (χ^2 test, MD saline versus MD muscimol, $p < 0.001$). The mean CBI value of the MD muscimol rats was also significantly different from that of MD saline animals (t test, $p < 0.01$; Figure 6E). These data demonstrate that blockade of callosal input activity during MD consistently reduces the OD shift.

Intracortical infusion of muscimol paired with MD is known to lead to a paradoxical OD shift in favor of the deprived eye (Reiter and Stryker, 1988; Hata and Stryker, 1994). We have confirmed this finding by recording eye preference in the muscimol-infused cortex (i.e., ipsilateral to the closed eye) in a group of rats (Figure S3). Thus, one possible explanation of the lack of plasticity observed contralateral to the minipump infusion (see Figure 6B) might be that callosal fibers transmit a strong deprived eye input that counteracts the expected OD shift in the opposite hemisphere. If this were the case, acute muscimol injection again into the muscimol-treated side should reveal an OD shift toward the open eye in the other hemisphere. We performed this experiment (see Figure 6C) in a subset (n = 6) of the deprived animals with minipump infusion of muscimol. We found that injection of muscimol again into the treated cortex had absolutely no effect on the OD histogram, which remained highly binocular (MD minipump MUSC, before versus after MUSC, χ^2 test, $p = 0.16$; compare Figures 6B and 6D). Analysis of CBI values yielded the same result (MD minipump MUSC, before versus after MUSC, t test, $p = 0.24$; Figure 6E). Thus, reduction of the OD shift in the rats with minipump infusion of muscimol does not depend on a deprived eye input provided by callosal afferents from the muscimol hemisphere.

Callosal Inputs Suppress Deprived Eye Responses

The data shown above demonstrate a role for interhemispheric connections in plasticity based on OD, which represents a measure of relative responsiveness of the visual cortex to stimulation of each eye. It was important to determine whether the recovery of binocularity observed after muscimol infusion in monocularly deprived animals depends on an enhancement of deprived eye inputs, on a reduction of open eye responses, or both. We analyzed the peak firing rates of cortical neurons

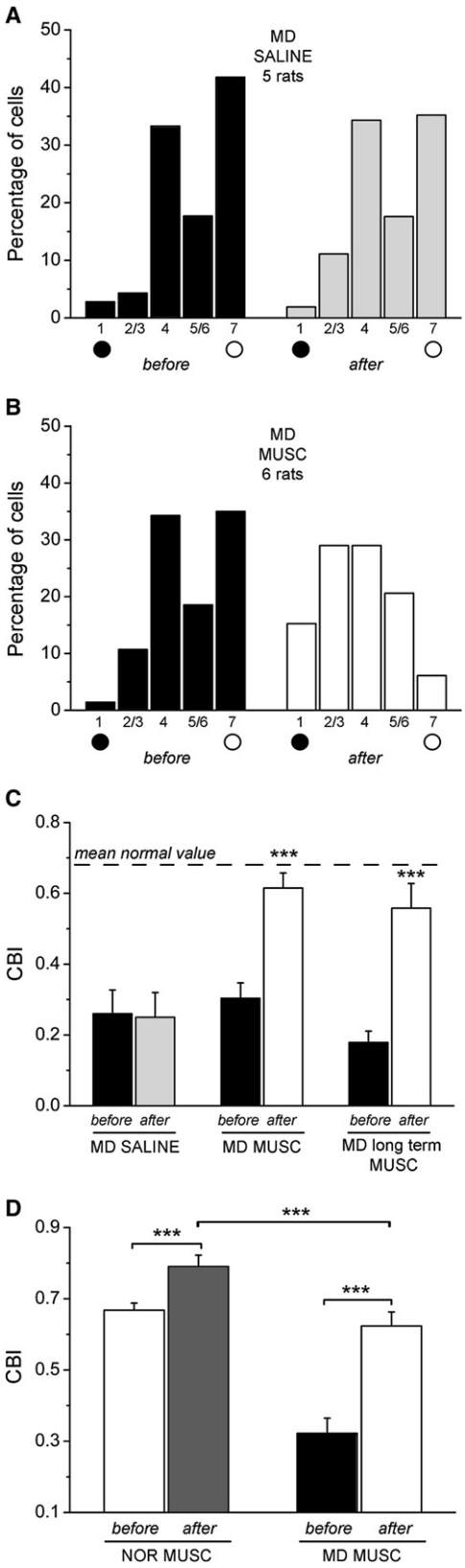

Figure 4. Recovery of Binocularity in MD Rats after Acute Blockade of Callosal Input

(A and B) OD distributions of rats monocularly deprived for 7 days, before and after injection of saline (A) or muscimol (MUSC; B) into the opposite side. Recordings were performed in the visual cortex contralateral to the closed eye (filled circle). Following injection of saline, OD remains shifted toward the undeprived eye (open circle; χ^2 test, $p = 0.29$; A). Muscimol causes a reduction of the cells driven exclusively by the open eye and a corresponding increase in the proportion of units controlled by the contralateral, deprived eye (χ^2 test, $p < 0.001$; B). Number of animals as indicated. Before saline, n = 141 cells; after saline, n = 108 cells; before muscimol, n = 140 cells; after muscimol, n = 131 cells.

(C) CBIs of all treated animals. Data are mean ± standard error. There is a substantial change in OD following muscimol, but not saline, infusion in rats monocularly deprived for either 7 days (MD) or >15 days (long-term MD) (one-way ANOVA followed by Holm-Sidak test, CBI before muscimol versus after muscimol, $p < 0.001$ for both comparisons). A very similar OD change is triggered by acute muscimol in both groups of deprived animals (t test, $p = 0.65$). MD SALINE, n = 5 rats; MD MUSC, n = 6 rats; MD long term MUSC, n = 6. ***$p < 0.001$.

(D) Comparison of the effects of acute callosal silencing in naive (NOR) versus monocularly deprived (MD) rats. Data are mean CBIs ± standard error.

following stimulation of each eye, before and after acute microinjection of muscimol (or saline as control) into the opposite side. We first examined the recordings contralateral to the deprivation. We found that acute saline infusion into the other hemisphere had no effect on visual responses (ANOVA on ranks followed by Dunn's test, $p > 0.05$; data not shown). Acute muscimol delivery affected firing rates in an eye-specific manner. There was a slight, nonsignificant decrease in the peak response of cells after stimulation of the open, ipsilateral eye (one-way ANOVA on ranks followed by Dunn's test, IPSI before muscimol versus after muscimol, $p > 0.05$; Figure 7A). In contrast, we found a highly consistent enhancement of deprived, contralateral eye responses following muscimol (one-way ANOVA on ranks followed by Dunn's test, CONTRA before muscimol versus after muscimol, $p < 0.01$; Figure 7A). Acute removal of callosal input increased strength of the closed eye also in the hemisphere ipsilateral to the deprivation (one-way ANOVA on ranks followed by Dunn's test, $p < 0.01$). Thus, acute silencing of the callosal pathway alleviates the effect of MD by elevating the strength of inputs from the deprived eye.

We also compared peak firing rates of cortical units in the minipump-implanted animals. MD produced a clear reduction of responsiveness of the contralateral, deprived eye, and muscimol delivery throughout the deprivation blocked this decrease (peak discharge rates, CONTRA eye, Mann-Whitney rank sum test, minipump muscimol versus minipump saline, $p < 0.001$). These data are consistent with the idea that callosal afferents act to suppress closed eye inputs during MD.

It is worth noting that callosal inhibition of closed eye afferents differs from the naive situation where the opposite hemisphere merely supplies ipsilateral eye input (see Figure 2). To examine whether MD produces alterations in the neurochemical phenotype of transcallosal cells, we performed retrograde labeling of callosal neurons [by cholera toxin B subunit (CTB) injection] combined with GABA immunostaining in naive and monocularly deprived rats (Figure S4). Colocalization of CTB and GABA was examined at the confocal microscope. We found that the

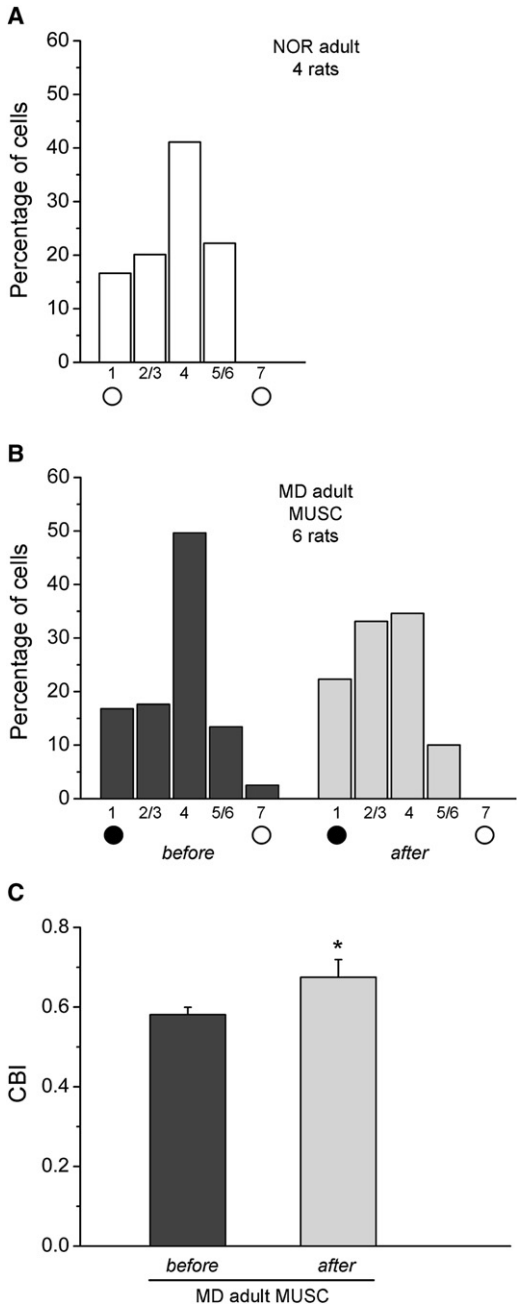

Figure 5. Acute Silencing of Callosal Input Slightly Increases Contralateral Bias Following Adult MD

(A) OD distribution of normal (NOR) adult rats (n = 90 cells). Number of animals as indicated.

(B) OD distributions of monocularly deprived (MD) adult rats before and after inactivation of the opposite hemisphere with muscimol (MUSC). Recordings were performed contralateral to the occluded eye. Note the small enhancement of contralateral bias following muscimol infusion (χ^2 test, p = 0.007). Number of animals as indicated. Before muscimol, n = 120 cells; after muscimol, n = 130 cells.

(C) CBIs of monocularly deprived adult rats (n = 6). Data are mean ± standard error. There is a small increase of contralateral bias following muscimol (t test, $p < 0.05$). *$p < 0.05$.

percentage of callosal GABAergic neurons was extremely low in both normal and deprived animals (normal: 5 out of 497 cells; MD: 4 out of 325 cells; t test, p = 0.89; Figure S4), ruling out that inhibition of closed eye input is exerted directly by callosal afferents.

To further address the mechanisms of recovery of binocularity, we performed visual evoked potential (VEP) recordings in animals deprived from P20–P23 for 7 days, before and after acute injection of muscimol (or saline as control) into the opposite hemisphere. VEPs represent the integrated synaptic response of cortical cells to visual stimulation and are commonly used to evaluate alterations in binocularity (Porciatti et al., 1999; Frenkel and Bear, 2004; Maya Vetencourt et al., 2008). VEPs evoked by each eye were recorded from superficial layers contralateral to the deprivation. As expected (Frenkel and Bear, 2004), we found that after monocular occlusion open eye inputs were dominant and responses from the contralateral, deprived eye were weak (Figure 7B). The electrode was left in place and muscimol (or saline) was delivered to the opposite hemisphere. Saline had no effect on either contralateral or ipsilateral eye VEP amplitudes (paired t test, $p > 0.12$ for both comparisons; data not shown). Notably, we found that muscimol delivery selectively elevated deprived eye responses (paired t test, p = 0.002; Figures 7B and 7C). There was no effect on the amplitude of the field potential evoked by stimulating the ipsilateral, open eye (paired t test, p = 0.57; Figures 8B and 8C). Responses through the contralateral, deprived eye were elevated throughout the low spatial frequency range (0.1–0.3 c/deg; Figure S5A). However, visual acuity of the closed eye was not improved by muscimol injection (Figure S5B).

Finally, we examined the time course of the muscimol effect. Figure 7D reports deprived eye VEP amplitudes before muscimol and at different times following injection of the blocker. The statistical analysis indicated that potentiation of VEP responses occurred as soon as 30 min after muscimol infusion (the earlier time of recording) and persisted unaltered for the following 2 hr (one-way ANOVA on ranks followed by Dunn's test; before muscimol differs from all other groups, $p < 0.01$; the after muscimol groups do not differ from each other, $p > 0.05$; Figure 7D). Identical results were obtained by plotting the time course of the recovery of the peak responses of single units (one-way ANOVA on ranks followed by Dunn's test; before muscimol differs from all other groups, $p < 0.05$; the after muscimol groups do not differ from each other, $p > 0.05$; data not shown). Thus, elevation of deprived eye strength is apparent right after muscimol, pointing to the removal of functional inhibition onto closed eye afferents.

DISCUSSION

This study describes the contribution of callosal connections to normal OD in rat visual cortex, as well as to the OD shift that follows MD. In normal rats, we found that acute functional blockade of callosal input by muscimol injection into the opposite hemisphere shifted OD toward the contralateral eye. This enhancement of contralateral bias was due to a reduction in the response to the ipsilateral eye. Thus, in normal animals a substantial fraction of the influence of the ipsilateral eye on cortical responses arrives via callosal connections from the

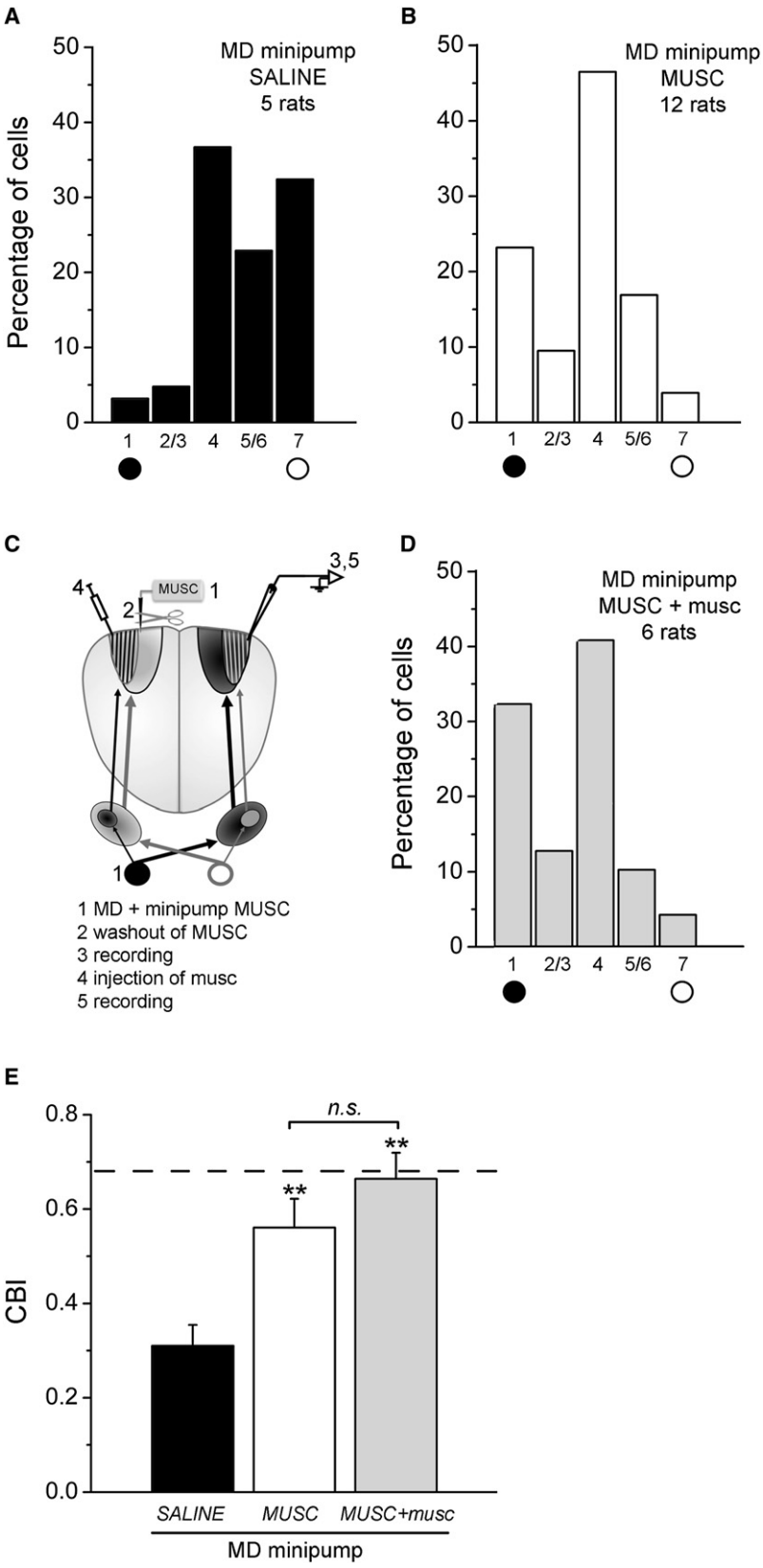

Figure 6. Continuous Blockade of Callosal Input during MD Reduces the OD Shift

(A and B) OD distributions of monocularly deprived rats that received minipump infusions of saline (A) or muscimol (MUSC; B) into the visual cortex ipsilateral to the deprivation. Recordings were performed contralateral to the occluded eye (filled circle). Note the substantial reduction of the OD shift in muscimol-treated rats (χ^2 test, MD saline versus MD muscimol, $p < 0.001$). Number of animals as indicated. SALINE, n = 188 cells; MUSC, n = 284 cells.

(C and D) These panels refer to a subset of animals that received acute muscimol again into the muscimol-treated hemisphere (MUSC + musc; see schematic description of the experimental protocol in C). Binocularity remains unaffected following acute muscimol again into the minipump-infused side (χ^2 test, $p = 0.16$; compare B and D). Number of animals as indicated. MUSC + musc, n = 118 cells.

(E) CBIs of the minipump-implanted animals. Data are mean ± standard error. CBIs of muscimol-infused rats is significantly higher than in saline controls (t test, $p < 0.01$). SALINE, n = 5 rats; MUSC, n = 12 rats; MUSC + musc, n = 6 rats. **$p < 0.01$; n.s., not significant.

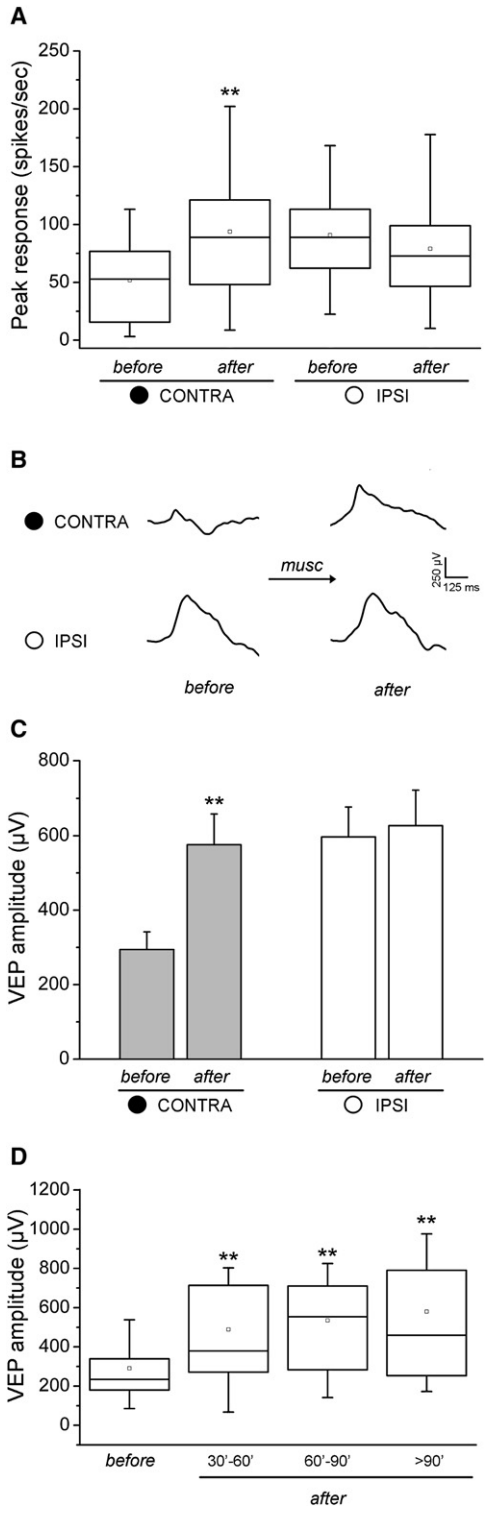

Figure 7. Acute Silencing of Callosal Input Rapidly Unmasks Deprived Eye Responses

(A) Box chart showing peak firing rates of visual cortical neurons in monocularly deprived rats injected with muscimol. Contralateral (CONTRA), deprived eye responses are significantly increased after muscimol (post ANOVA Dunn's test, contralateral eye, before versus after muscimol, $p < 0.01$), while ipsilateral (IPSI), open eye responses are unaffected (post ANOVA Dunn's test, ipsilateral eye, before versus after muscimol, $p > 0.05$). The horizontal lines in each box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbols denote the mean of the column of data. Before muscimol, n = 140 cells; after muscimol, n = 131 cells. **$p < 0.01$.

(B) Representative examples of VEP responses for both eyes, before and after muscimol (musc) administration to the opposite side. Visual stimulus: square-wave grating alternating at 0.5 Hz, spatial frequency 0.07 c/deg, contrast 90%. CONTRA, contralateral deprived eye; IPSI, ipsilateral open eye.

(C) VEP amplitudes for the deprived, contralateral eye (CONTRA; gray bars) and the ipsilateral, open eye (IPSI; white bars), before and after muscimol delivery. Data are mean ± standard error. Deprived eye responses increase consistently after blockade of callosal input (paired t test, $p < 0.01$; n = 8 rats). **$p < 0.01$.

(D) Time course of the recovery of deprived eye responses. Contralateral eye VEP amplitudes measured before muscimol and at different times following injection of the blocker. Deprived eye VEPs are increased by 30–60 min after muscimol and remain elevated for at least 2 hr (one-way ANOVA on ranks followed by Dunn's test; before muscimol differs from all other groups, $p < 0.01$; the after muscimol groups do not differ from each other, $p > 0.05$). The horizontal lines in each box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbols denote the mean of the column of data. **$p < 0.01$.

opposite hemisphere, where it is the dominant eye. A different situation was found in rats monocularly deprived at the peak of the critical period. After MD, acute silencing of callosal afferents consisted in an unexpected increase in the strength of the deprived eye. This elevation of closed eye responses was apparent both contralateral and ipsilateral to the deprivation. Thus, acute removal of callosal influence following MD unmasks deprived eye inputs. In keeping with this observation, continuous silencing of callosal input throughout the MD prevented the loss of responsiveness of the deprived eye, resulting in a dramatic reduction of the OD shift. These data indicate that callosal afferents act primarily to inhibit closed eye inputs under visual deprivation. Thus, transcallosal connections are crucially involved in the weakening of deprived eye responses during MD.

Previous studies in animals with section of the corpus callosum have yielded contradictory results concerning the role of interhemispheric connections in binocularity (Payne et al., 1980; Diao et al., 1983; Minciacchi and Antonini, 1984; Elberger and Smith, 1985). The discrepancies between these different reports likely arise as a consequence of technical aspects, including age at which the callosal section is performed and time elapsed between surgery and recording. In contrast to these previous reports, our experiments are based on an acute functional blockade of callosal input that allows comparison of binocularity in the same animal, with and without the contribution of callosal afferents. The data demonstrate that in the normal rat visual cortex, binocularity depends to a great degree on the function of callosal fibers. In rodents, retinogeniculate afferent input to the cortex from the contralateral eye is much stronger than that from the ipsilateral eye (indeed, over 95% of retinal fibers decussate at the chiasm). However, cells mapping the central part of the visual field are highly binocular, and our findings indicate that callosal afferents contribute to this binocularity by providing input from the ipsilateral eye.

Having established a role for callosal inputs in cortical binocularity, we reasoned that the shift in eye preference following

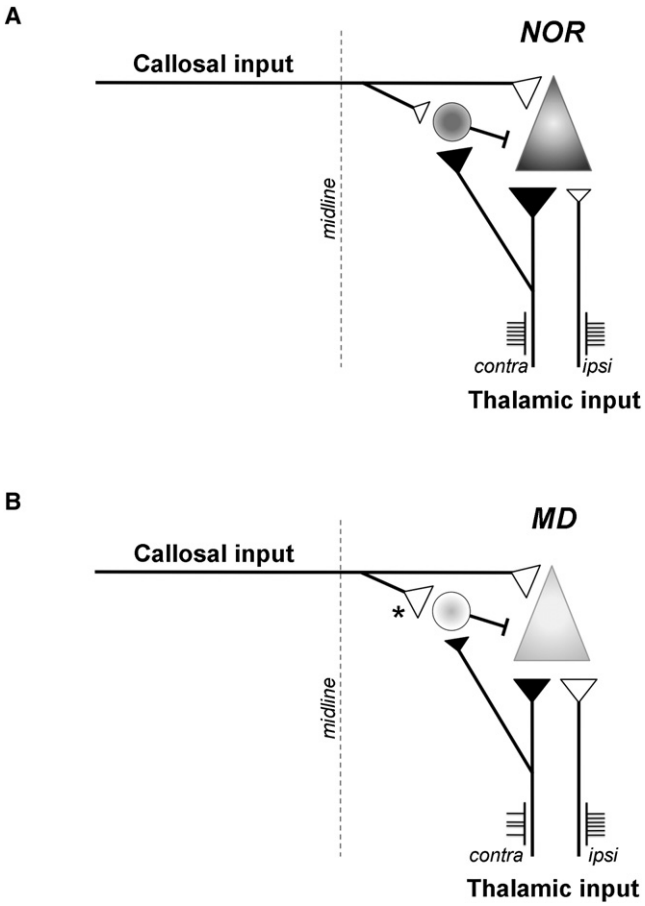

Figure 8. Simplified Model of Visual Cortical Circuitry in Naive and Monocularly Deprived Rats

Thalamic and callosal inputs to a principal neuron (triangle) and an inhibitory cell (circle) are shown. Contralateral eye- and ipsilateral eye-driven synaptic terminals are in black and white, respectively. Size of the terminals indicates relative synaptic strength.

(A) In normal animals, callosal afferents have a net excitatory action, contributing to ipsilateral eye responses.

(B) MD might result in strengthening of the synaptic connections (asterisk) between callosal afferents and inhibitory cells in the opposite hemisphere, thus masking weak inputs from the contralateral, deprived eye. Remodeling of callosal connections during MD would have no net effect on ipsilateral eye responses, since the increased inhibition via the callosum (asterisk) would be balanced by the normal transcallosal excitatory drive. Thus, acute silencing of interhemispheric communication might selectively unmask contralateral, deprived eye inputs with no impact on ipsilateral, open eye responses.

monocular occlusion might potentially derive either from changes in the direct thalamocortical pathway or from modifications in the transcallosal route. To assess the involvement of the callosal pathway in OD plasticity, in a first series of experiments we measured eye preference in MD rats before and after acute blockade of interhemispheric communication. This experimental protocol allows plasticity to proceed normally and probes the results of acute removal of callosal input, thus dissecting the OD shift that would be measured via the sole geniculocortical pathway. We found that silencing transcallosal input in animals that had undergone MD strongly alleviated the OD shift and produced a change in eye preference consistently greater than that obtained in normal animals. This is particularly clear from the data reported in Figure 4D, which summarize the OD changes triggered by acute muscimol in both normal and monocularly deprived rats. It is important to note that acute muscimol resulted in a remarkable recovery of binocularity after MD, but did not fully recovered OD. More specifically, the quota of MD effects that is due to rearrangements in the geniculocortical pathway can be computed by comparing the OD post muscimol in naive and MD rats (mean CBI post muscimol: 0.81 ± 0.03 in naive versus 0.61 ± 0.04 in MD animals; t test, $p < 0.001$; Figure 4D). Indeed, the post muscimol condition indicates the eye preference that is determined by the sole thalamocortical route, and the difference observed in naive versus deprived animals measures MD-induced alterations in this pathway. Thus, changes in both transcallosal and thalamocortical input contribute to MD effects.

We found that the recovery of binocularity after callosal silencing in MD rats was selectively due to an enhancement of deprived eye responses. This differs from the naive situation in which callosal afferents supply ipsilateral eye inputs (Figure 2). Deprived eye inputs were rapidly unmasked by muscimol injection into the opposite hemisphere, indicating that a mechanism of transcallosal inhibition is used to drive down the efficacy of afferents from the closed eye. Acute muscimol produced a similar OD change in both 7 days and long-term MD (see Figure 4C). It is known that the time course of MD effects includes an initial depression of deprived eye inputs followed by slower growth and potentiation mechanisms (Antonini et al., 1999; Taha and Stryker, 2002; Frenkel and Bear, 2004; Mrsic-Flogel et al., 2007). Thus, considerable depression of closed eye responses is still attributable to callosal afferents even after prolonged periods of MD, when potentiation events have taken place.

To further demonstrate that interhemispheric connections contribute to the competition process itself, we continuously blocked callosal input activity by contralateral muscimol infusion throughout the MD and then examined the OD properties in the absence of muscimol. This experiment silences callosal influences throughout the MD and allows competition to occur only via the thalamocortical route. The data were clear in indicating a substantial reduction of the OD shift, due to a blockade of the weakening of deprived eye responses.

We reasoned that the prevention of the OD shift observed in the animals with minipump infusion of muscimol could be due to two distinct mechanisms: (1) prevention of plasticity of callosal afferents themselves (since their cell bodies are silenced) or (2) transmission of a strong deprived eye input (due to paradoxical OD shift in the muscimol-treated hemisphere) via the callosal route. If this second hypothesis were true, acute muscimol injection again into the muscimol-infused side should unmask a shift toward the open eye. However, we found that further silencing of the muscimol hemisphere had no effect on OD in the opposite cortex (see Figures 6B and 6D). This can be easily explained by the fact that a prolonged silencing renders callosal afferents quite ineffective in driving cortical neurons in the opposite side. Thus, the prevention of the OD shift in the minipump animals is likely due to a lack of plastic rearrangements of the callosal pathway during MD. Altogether, these data indicate the requirement of callosal inputs in generating the monocular bias during MD.

Previous studies have indicated that inhibition is crucially involved in the effects of MD (for a review, see Hensch, 2005). For example, an adequate level of intracortical inhibition is required for OD plasticity to occur (Hensch et al., 1998). Visual deprivation potentiates inhibitory feedback between fast-spiking basket cells and star pyramidal neurons (Maffei et al., 2006). Microiontophoretic delivery of the $GABA_A$ antagonist bicuculline restores inputs from the deprived eye in the visual cortex of monocularly deprived cats (Duffy et al., 1976; Sillito et al., 1981; Mower and Christen, 1989). Thus, functional inhibition is one important factor determining abnormal eye preference following MD, but the source of this inhibition has remained unclear. One previous study of CRE-mediated gene transcription following MD suggested the idea of inhibitory influences from outside the primary visual cortex (Pham et al., 1999). The present findings clearly demonstrate that the callosal input is a major source of inhibition and a key determinant of the OD shift.

These experiments uncover a novel role for callosal inputs in OD plasticity. Specifically, we have shown that after monocular visual deprivation the transcallosal pathway changes from a mainly excitatory action (supplying ipsilateral eye input) to a predominantly inhibitory function (providing selective suppression of deprived eye afferents). Theoretically, this excitatory/inhibitory switch following MD might be due either to a change in the neurochemical phenotype of callosal neurons or to the recruitment of inhibitory circuits in the other hemisphere. We ruled out the first hypothesis by performing retrograde labeling of callosally projecting neurons combined with GABA immunostaining in normal and monocularly deprived rats. The percentage of commissural GABAergic cells remained extremely low (about 1%) in both cases, excluding the possibility of a neurochemical switch.

We therefore favor the idea that callosal axons recruit inhibitory neurons in the opposite hemisphere (Figure 8). It has been reported that callosal fibers mainly evoke a direct excitation of principal neurons in the opposite hemisphere (supplying ipsilateral eye input; present results), but can also produce a disynaptic inhibitory postsynaptic potential via a local GABAergic cell (Toyama et al., 1974; Innocenti, 1980). We suggest that connections between callosal fibers and inhibitory neurons may be strengthened during MD (Figure 8B). Under these conditions, responses from the contralateral deprived eye could be effectively suppressed by callosal afferents; no changes would be apparent for the ipsilateral eye, since enhanced GABAergic inhibition via the callosum would compensate the normal transcallosal direct excitation (Figure 8B). This scenario could explain how selective suppression of deprived eye inputs can be achieved during MD. Further studies with intracellular recordings are needed to corroborate this hypothesis.

Another possible mechanism of disinhibition following muscimol treatment might be a modification of the strength of thalamic inputs from the deprived eye via alterations of geniculocortical axon arborization. We consider this interpretation unlikely, based on the following lines of evidence. First, geniculocortical projections are only modestly affected by MD in rodents, and these effects require at least 20 days of deprivation (Antonini et al., 1999). Second, the fast and persistent elevation of deprived eye strength by acute muscimol application (Figure 7D) supports the removal of intracortical functional inhibition. Indeed, the rapid unmasking strongly favors functional versus anatomical rearrangements in the mechanisms of the OD shift.

Our data prompt a reconsideration of the mechanisms involved in OD plasticity. We demonstrate that transcallosal inhibition plays a key role in the loss of deprived eye inputs. The importance of this mechanism for OD plasticity in higher species such as monkeys and humans remains to be investigated. It is worth pointing out, however, that transcallosal inhibition has been demonstrated to participate in plastic events occurring during several pathological conditions of the human brain. For example, it has been shown in neglect patients that some of the behavioral symptoms are attributable to a pathological state of increased inhibition exerted onto the damaged parietal cortex by the contralateral, intact hemisphere (Fecteau et al., 2006; Fierro et al., 2006). Indeed, inactivation of the unaffected hemisphere by transcranial magnetic stimulation ameliorates visuospatial neglect (Fecteau et al., 2006; Fierro et al., 2006). It has also been reported that changes in transcallosal inhibition contribute to the occurrence of mirror movements in Parkinson's disease and ischemic patients (Cincotta et al., 2006; Li et al., 2007; Nair et al., 2007).

EXPERIMENTAL PROCEDURES

Animal Treatment and Surgical Procedures

Long-Evans hooded rats were used in this study. Animals were reared in a 12 hr light/dark cycle, with food and water available ad libitum. All experimental procedures conformed to the European Communities Council Directive number 86/609/EEC.

We used 42 naive animals during the critical period (age range: P26 – P30). An additional 90 rats were monocularly deprived for 7 days starting at P20–P23. Eight rats were monocularly deprived for 15–20 days starting from P16–P18. Ten adult rats (age greater than P80; naive, n = 4; monocularly deprived, n = 6) were also used. MD was performed by eyelid suture under isoflurane anesthesia. MD animals were carefully inspected every day to make sure that the lid suture remained intact. The deprived eye was reopened using thin scissors at the time of recording.

Cortical microinjection of muscimol (1 μl; 30 mM solution; Sigma-Aldrich) or saline was performed with a glass pipette (tip diameter, 40 μm) mounted on a micromanipulator. The solution was slowly delivered at a depth of 0.6–1 mm from the pial surface. The experimenters did not know whether muscimol or saline was injected and the animal code was broken only at the completion of the analysis.

Minipump implantation was performed as described previously (Lodovichi et al., 2000). Micro-osmotic pumps (Alzet 1007D; Alza, USA; pumping rate 0.5 μl/hr) were filled with muscimol (10–30 mM solution) or saline and connected with polyethylene tubing to 30 G stainless steel cannulae (Reiter and Stryker, 1988; Lodovichi et al., 2000). A small hole was made in the skull (3 mm lateral and in correspondence with lambda) and the cannula was lowered into the cortex. The minipump was positioned subcutaneously under the neck and the cannula was secured to the skull with acrylic cement.

Electrophysiology

Rats were anesthetized with urethane (7 ml/kg; 20% solution in saline, i.p.; Sigma-Aldrich) and placed in a stereotaxic apparatus. Both eyes were fixed by means of adjustable metal rings surrounding the external portion of the eye bulb, and optic disk locations were projected onto a tangent screen to determine the vertical meridian. Body temperature during the experiments was constantly monitored with a rectal probe and maintained at 37°C with a heating blanket. Electrocardiogram was also continuously monitored. In all of the animals, a portion of the skull overlying the binocular visual cortex was carefully drilled on both sides. A glass micropipette (2 MΩ) filled with NaCl (3 M) was mounted on a three axis motorized micromanipulator and

inserted into the binocular portion of visual cortex. Single units were recorded from two to three penetrations per animal in one side. The contralateral hemisphere was then injected with saline or muscimol. After a delay of 30 min, we started to record single units again. Care was taken to record at the same coordinates before and after injection in each animal.

Extracellular recordings of spiking activity were performed from supragranular layers (i.e., at a depth less than 800 μm from the cortical surface). The visual stimulus consisted of a computer-generated bar (contrast, 90%; thickness, 3°; speed, 28°/s) presented on a monitor (Sony; 40 × 30 cm; mean luminance 15 cd/m^2). Signals were amplified 25,000-fold, bandpass filtered (300–5000 Hz), and conveyed to a computer for storage and analysis. Action potentials were discriminated from background by a voltage threshold that was set as 4.5 times the standard deviation of noise, as described previously (Caleo et al., 2007). Spontaneous activity and peak response were determined from peristimulus time histograms (bin size = 33 ms) of the cell response to the stimulus, averaged over 20 consecutive stimulations as described previously (Lodovichi et al., 2000; Mandolesi et al., 2005; Caleo et al., 2007). Peak response was evaluated as the peak firing rate (spikes per second) in the cell response to the stimulus.

OD was evaluated according to the methods of Hubel and Wiesel (1962). Neurons in OD class 1 were driven exclusively by stimulation of the contralateral eye; neurons in OD class 2/3 were binocular and preferentially driven by the contralateral eye; neurons in OD class 4 were equally driven by the two eyes; neurons in OD class 5/6 were binocular and preferentially driven by the ipsilateral eye; neurons in OD class 7 were driven only by the ipsilateral eye. For each animal, the bias of the OD distribution toward the contralateral eye (CBI) was calculated as follows: CBI = $[(N_{(1)} - N_{(7)}) + 1/2\ (N_{(2/3)} - N_{(5/6)}) + N_{TOT}]/2N_{TOT}$; where $N_{(i)}$ is the number of cells in class (i) and N_{TOT} is the total number of recorded cells in a specific animal.

For VEP recordings, the electrode was typically positioned at a depth of 100 μm within the cortex. In some animals, recordings were also performed at a depth of 400 μm and yielded the same results, consistent with a single major VEP dipole source in the visual cortex (Pizzorusso et al., 1997; Porciatti et al., 1999; Liu et al., 2008). Transient VEPs were recorded in response to abrupt reversal (0.5 Hz) of a horizontal square wave grating (spatial frequency, 0.07–0.1 c/degree; contrast, 90%), generated by computer on a display (Sony; 40 × 30 cm; mean luminance 15 cd/m^2) by a VSG card (Cambridge Research System). The display was positioned in front of the rat's eyes to include the binocular visual field. Signals were amplified (10,000-fold), bandpass filtered (0.1–500 Hz), and fed to a computer for storage and analysis. At least 50 events were averaged in synchrony with the stimulus contrast reversal. VEP amplitude was quantified for each eye by measuring the peak to trough amplitude, as described previously (Porciatti et al., 1999; Pizzorusso et al., 2002). VEPs in response to a blank stimulus were also frequently recorded to estimate noise.

Retrograde Labeling of Callosal Cells

CTB (1% solution in water; Sigma-Aldrich) was injected intracortically in P28 rats (naive, n = 4; monocularly deprived, n = 4). In monocularly deprived animals, injections were contralateral to the occluded eye. Two days after CTB, rats were deeply anesthetized and perfused with 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and cut in the coronal plane with a freezing microtome. After a blocking step, coronal sections (40 μm thick) were reacted overnight with anti-CTB (goat polyclonal; Calbiochem; 1:3000 dilution) and anti-GABA (rabbit polyclonal; Sigma-Aldrich; 1:1,000) antibodies. Bound primary antibodies were revealed by donkey anti-goat Alexa 568 and donkey anti-rabbit Alexa 488 secondaries (Invitrogen; 1:400 dilution). We examined 80–135 CTB-positive cells (four to five sections) per animal with an Olympus confocal microscope. We restricted our analysis to cells located in superficial layers and at the border between area 17 and 18.

Statistical Analysis

Statistical analysis was performed with SigmaStat (version 3.1). Differences between two groups were assessed with t test. Differences between VEP amplitudes before and after muscimol (or saline) administration into the contralateral hemisphere were evaluated with a paired t test. Differences between three or more groups were evaluated with ANOVA followed by Holm-Sidak test for data normally distributed and with Kruskal-Wallis one-way ANOVA (ANOVA on ranks) with Dunn's post hoc test for data not normally distributed. Variation of CBI with eccentricity was evaluated with two-way ANOVA followed by Holm-Sidak test. Normality of distributions was assessed with Kolmogorov-Smirnov test. Differences between OD histograms were assessed using a χ^2 test (four degrees of freedom). Level of significance was $p < 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include five figures and can be found with this article online at http://www.cell.com/neuron/supplemental/S0896-6273(09)00849-6.

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