Supplementary Online Material

Materials and Methods

Benzodiazepine actions on GABAergic inhibitory postsynaptic currents (IPSCs) were confirmed in slices of mouse visual cortex (C57Bl/6) prepared and maintained as described previously (*S1, S2*). IPSCs were evoked in supragranular pyramidal cells by electrical stimulation to layer 4 in the presence of ionotropic glutamate receptor antagonists (10μM CNQX, 50μM D-APV). Whole-cell patch pipette solutions contained (in mM): 122.5 KGluconate, 17.5 KCl, 10 HEPES buffer, 0.2 EGTA, 8 NaCl, 2.0 Mg-ATP, 0.3 Na_3-GTP (3-8MΩ, pH 7.2, 290-300mOsm). After stable baseline IPSC recordings (V_h -50mV), benzodiazepines (diazepam, DMCM, or propylene glycol vehicle) were bath applied, followed by picrotoxin (100μM) to confirm the $GABA_A$ component of the response (Fig. S1). In some cases, drugs were kept at 37°C for 4 weeks to simulate in vivo conditions prior to in vitro testing.

Experiments were designed to begin before the onset of focused thalamocortical axon branching (*S3-S5*), but after the well-known conversion of depolarizing $GABA_A$-mediated currents into mature hyperpolarizing responses in vivo (P14-17) (*S6*). Cannulae (33 ga.) connected to osmotic minipumps (Alzet 2ML4, or paraffin-coated Alzet 2002, Alza) were implanted under sterile, anesthetized conditions into one

hemisphere of kitten primary visual cortex (Area 17; AP 0 interaural line, LM ±2mm) (*S7*). Cannulae bevels were designed to deliver only in the anterior direction and pumps were previously filled with either a benzodiazepine agonist (3.5 or 35 mM diazepam), inverse agonist (50 μM DMCM), or vehicle (20% propylene glycol) solution. After 4 weeks of normal visual experience and local infusion, animals were prepared for single-unit recordings, and in some cases optical imaging of intrinsic signals, using standard techniques (*S3, S7*). Single-unit responses isolated by window discriminator were assayed with a computer-based visual stimulation and data acquisition system pseudo-randomly presenting a moving high contrast bar at 16 different orientations (or no stimulus for measurements of spontaneous activity). Contralateral Bias (CBI) and Monocularity Indices (MI) were calculated for ocular dominance distributions as described (*S7*).

Units were scored for habituation and for responsivity on a zero (no habituation/sluggish responsivity) to five (prolonged and rapid habituation/brisk firing) scale (*S7*). We observed no significant change of maximal discharge, spontaneous activity or habituation despite trends consistent with enhanced or reduced inhibition for diazepam and DMCM, respectively. Strikingly, cortical direction selectivity was significantly reduced by manipulation of inhibition out of a balanced regime in either

direction (Figs. 1C, S2), consistent with its well-known sensitivity to inhibition both in theory and experiment (*S8*-*S12*). These results are consistent with the modest effects of benzodiazepines on cortical processing in somatosensory cortex (*S13*). The maturation of intracortical networks, thus, appears to be quite robust to severe quantitative alterations of visual experience (*S14*) and neuronal activity (*S15*).

Following electrophysiological characterization, animals were perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The caudal portion of cortex including primary visual cortex was unfolded and flattened between two glass slides, sunk in sucrose/PFA, mounted on a Leica freezing microtome and cut into serial, tangential sections (40 μm) through all cortical layers from pial surface down to the white matter. Monocular [^{3}H]-proline injections (2 mCi in 20 μl saline) 10 days prior to terminal physiology experiments revealed the pattern of ocular dominance columns in sections exposed to photoemulsion (Kodak) in the dark (6 weeks, 4°C) (*S16, S17*). Photomontages of the overall layout of labeling in layer 4 were made from several, serial tangential sections using Photoshop software (Adobe).

Reconstructions were judged to be uncompromised by prior electrophysiological recording in 6 of 8 animals treated with diazepam, all 3 animals receiving 50 μM DMCM, and 2 vehicle (20% propylene glycol) controls. Moreover, a large sample

(n=18) of flattened ocular dominance maps from the literature (courtesy of Drs. Y. Hata and S. Löwel) labeled with [^{3}H] proline or 2-deoxyglucose were analyzed similarly for comparison (*S18-S21*). For every autoradiograph, regions of interest (ROIs) were defined at varying distances in front and behind the cannula site (Fig. S3), as well as spanning the homologous extent of vehicle or untreated animals. This controlled for the fact that local column spacing typically exhibits systematic intraareal variation (*S22, S23*), with largest spacings along the representation of the horizontal meridian, and smallest spacings along the peripheral representation of the vertical meridian (*S21*).

To quantify column spacing, images were processed using two independent computer algorithms written in IDL. An image analysis technique based on the 'wavelet' transform extracted a two-dimensional map of local column spacings for each ROI, as described previously (*S21*). Similar results were independently obtained when labeled and unlabeled columns were reduced to their centers by a medial axis transform, and radial distances to the nearest column center were determined for each pixel in the skeletonized image (data not shown). To pool measurements across animals, spacing of individual experimental ROIs were normalized to the mean of control regions far from the infusion site within the same hemisphere. Variability about the mean was statistically compared between drug and control groups by unpaired Student's t-test.

The organization of intracortical excitatory-inhibitory balance is complex and sensitive to synaptic dynamics (*S24-S26*). We, therefore, tested whether simply varying inhibitory strength as a function of horizontal distance from a central point of excitation could explain the bidirectional changes of column spacing observed experimentally. Indeed, the Miller et al model (*S27*) predicts a broadening of columns when long-range inhibition is preferentially enhanced (Fig. S4). Inverse agonists acting similarly at a distance more than locally during development instead produce narrow columns. Taken together, these simulations strongly support a primary role for large-basket cell type connections in the refinement of visual cortical circuits (*S28*).

Fig. S1. Benzodiazepines specifically modulate postsynaptic $GABA_A$-mediated synaptic transmission in the visual cortex. IPSCs were evoked in supragranular pyramidal cells of mouse visual cortex by focal electrical stimulation in the presence of ionotropic glutamate receptor antagonists (50 μM D-APV, 10 μM CNQX). The benzodiazepine agonist diazepam (35 μM) kept for 4 weeks at body temperature (37°C) effectively doubled (left, arrow) and prolonged the decay kinetics of $GABA_A$ currents (middle, scaled trace). Picrotoxin (100 μM) was bath applied to confirm the $GABA_A$ receptor component of the IPSC (right, arrow). Scale, 100pA, 100msec.

Fig. S2. Responsiveness, habituation and direction selectivity in the presence of benzodiazepines. A slight trend for reduced responsiveness and increased habituation with diazepam was reversed for DMCM, but neither was statistically significant (vs. vehicle, all $p>0.2$). Instead, a striking loss of directionality was observed in the presence of either benzodiazepine agonist or inverse agonist. *$p<0.05$ vs. vehicle, t-test. Diazepam,143 cells; DMCM, 92 cells; vehicle, 56 cells.

Fig. S3. Unbiased sampling of ocular dominance column measurements across the overall extent of visual cortex. Regions traced in white indicate areas in which wavelet measurements of column spacing were made (E=experimental). Table lists corresponding region normalized to mean of all control (non-E) areas. These examples of the animals shown in Figs. 2 and 4 are typical.

Fig. S4 Predictions of benzodiazepine effects from Miller *et al* model (*S27*). Left, profiles of corticocortical interaction derived from a mixture of local excitation and two kinds of inhibition, local (20% wider than excitation) and long- range (3-fold wider than excitation). 'Control' cortex is shown in green. Diazepam treatment to selectively enhance the proportion of long-range inhibition is shown in red. DMCM treatment

to selectively attenuate the proportion of long-range inhibition is shown in blue. Right, prediction of ocular dominance column spacing from cortical interactions shown at left (S27): Control, 1 mm; Diazepam, 1.2 mm; DMCM, 0.83 mm.

References

S1. T.K. Hensch *et al. Science* **282**, 1504 (1998).

S2. T.K. Hensch *et al. J. Neurosci.* **18**, 2108 (1998).

S3. M.C. Crair, J.C. Horton, A. Antonini, M.P. Stryker, *J Comp Neurol*. **430**, 235 (2001).

S4. A. Antonini, M.P. Stryker, *J. Neurosci.* **13**, 3549 (1993).

S5. S. LeVay, M.P. Stryker, C.J. Shatz, *J. Comp. Neurol.* **179**, 223 (1978).

S6. M. Ikeda *et al*., *Brain Res*. **984**, 149 (2003).

S7. H.O. Reiter, D.M. Waitzman, M.P. Stryker, *Exp. Brain Res*. **65,** 182 (1986).

S8. A.M. Sillito, *J. Physiol. (Lond.)* **271**, 775 (1977).

S9. R. Maex, G.A. Orban, *Proc. Natl. Acad. Sci. USA* **88**, 3549 (1991).

S10. J.M. Crook, Z.F. Kisvarday, U.T. Eysel, *J. Neurophysiol.* **75**, 2071 (1996).

S11. J.M. Crook, Z.F. Kisvarday, U.T. Eysel, *Eur. J. Neurosci.* **10**, 2056 (1998).

S12. A. Murthy, A.L. Humphrey, *J. Neurophysiol.* **81**, 1212 (1999).

S13. J-I. Oka, T.P. Hicks, *Can. J. Physiol. Pharmacol.* **68**, 1025 (1990)

S14. MP Stryker, H Sherk, AG Leventhal, HVB Hirsch, *J Neurophysiol.* **41**, 896 (1978).

S15. D.C. Somers, S.B. Nelson, M. Sur, *J. Neurosci.* **15**, 5448 (1995).

S16. J. Olavarria, R.C. van Sluyters, *J. Neurosci. Methods* **15**, 191 (1985).

S17. P.A. Anderson, J. Olavarria, R.C. van Sluyters, *J. Neurosci.* **8**, 2183 (1988).

S18. Y. Hata, M.P. Stryker, *Science* **265**, 1732 (1994).

S19. S. Löwel, W. Singer, *Exp. Brain Res.* **68**, 661 (1987).

S20. S. Löwel, *J. Neurosci.* **14**, 7451 (1994).

S21. M. Kaschube *et al.*, *Eur. J. Neurosci.* **18**, 3251 (2003).

S22. J.C. Horton, D.R. Hocking, *J. Neurosci.* **16**, 7228 (1996).

S23. D.L. Adams, J.C. Horton, *Nat. Neurosci.* **6,**113 (2003).

S24. K. Kang, M. Shelley, H. Sompolinsky, *Proc. Natl. Acad. Sci. USA* **100**, 2848 (2003).

S25. M. Galaretta, S. Hestrin, *Nat. Neurosci.* **1**, 587 (1998).

S26. T.R. Tucker, L.C. Katz, *J. Neurophysiol.* **89**, 488 (2003).

S27. K.D. Miller, J.B. Keller, M.P. Stryker, *Science* **245**, 605 (1989).

S28. M. Fagiolini *et al.*, accompanying manuscript.

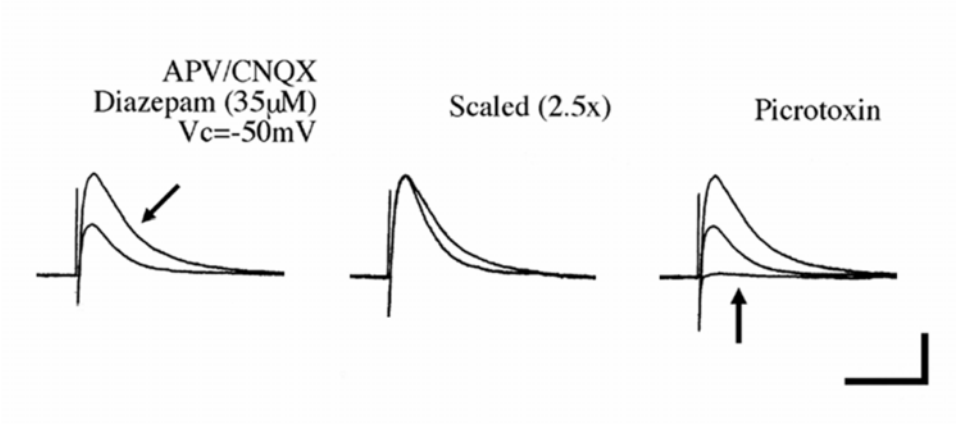
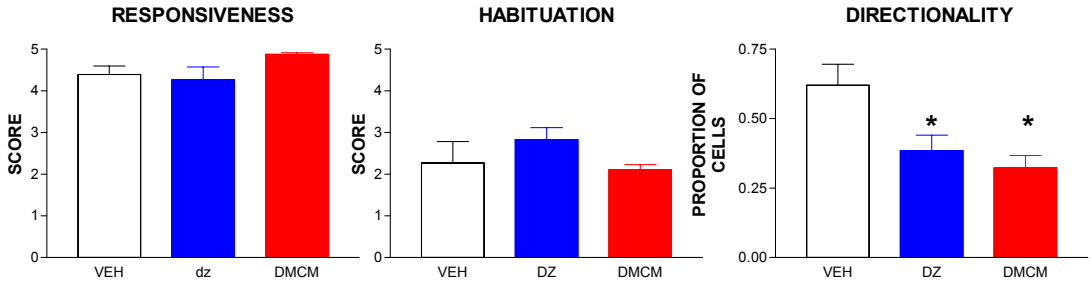


Fig. S1.


RESPONSIVENESS
HABITUATION
DIRECTIONALITY
SCORE
SCORE
PROPORTION OF CELLS
0
1
2
3
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0.25
0.50
0.75
VEH
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DMCM
VEH
DZ
DMCM
VEH
DZ
DMCM
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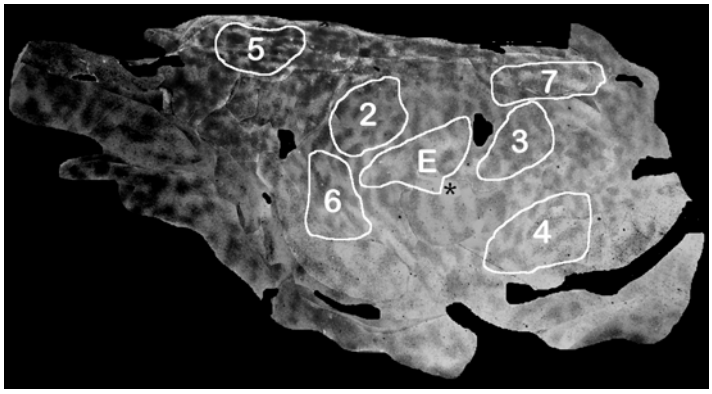
Fig. S2.

Diazepam example illustrated in Figure 2.

E	36%
2	-9%
3	-6%
4	0%
5	-6%
6	17%
7	4%



DMCM example illustrated in Figure 3.

E	-14%
2	10%
3	2%
4	-8%
5	0%
6	-4%

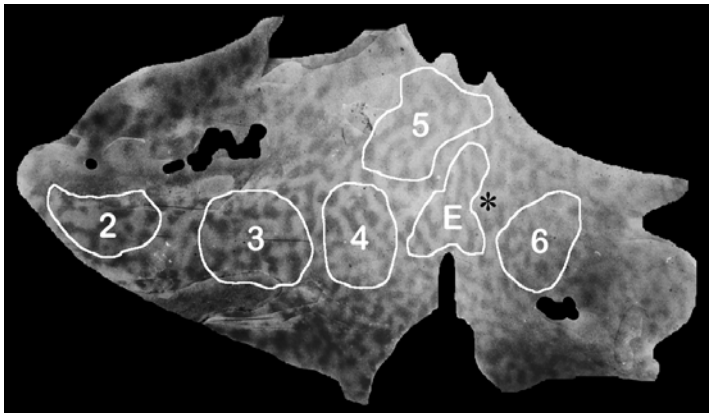


Fig S3.

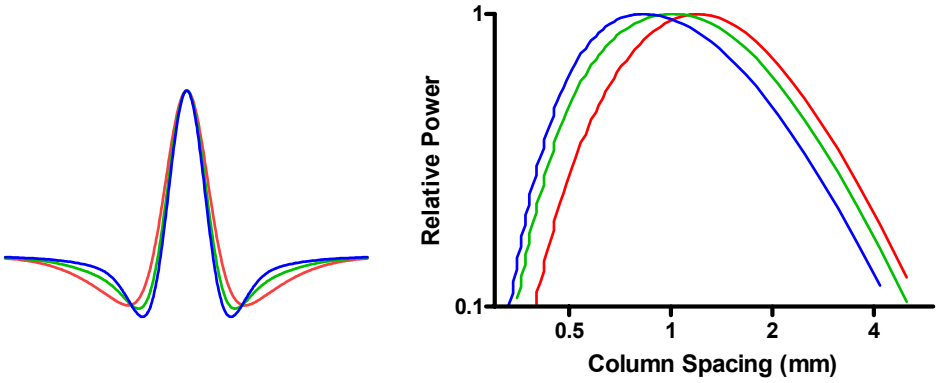


Fig. S 4.