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Reversible blockade of experience-dependent plasticity by calcineurin in mouse visual cortex

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Numerous protein kinases have been implicated in visual cortex plasticity, but the role of serine/threonine protein phosphatases has not yet been established. Calcineurin, the only known Ca^{2+}/calmodulin-activated protein phosphatase in the brain, has been identified as a molecular constraint on synaptic plasticity in the hippocampus and on memory. Using transgenic mice overexpressing calcineurin inducibly in forebrain neurons, we now provide evidence that calcineurin is also involved in ocular dominance plasticity. A transient increase in calcineurin activity is found to prevent the shift of responsiveness in the visual cortex following monocular deprivation, and this effect is reversible. These results imply that the balance between protein kinases and phosphatases is critical for visual cortex plasticity.

Brief monocular deprivation during the critical period of development dramatically alters neuronal responsiveness to subsequent stimulation of deprived and non-deprived eyes in the visual cortex. This experience-dependent phenomenon is called ocular dominance plasticity (ODP). Calcium influx through NMDA (*N*-methyl-D-aspartate) receptors is believed to be one of the initial steps of the mechanism of ODP[1,2], followed by the activation of protein kinases and phosphatases. The function of protein kinases in ODP has been studied extensively[3–5]. However the contribution of protein phosphatases, known to be involved in hippocampal synaptic plasticity and memory[6,7], has not been investigated.

Calcineurin is a serine/threonine protein phosphatase, highly sensitive to Ca^{2+} ($K_d = 0.1–1$ nM), and is the only phosphatase activated by Ca^{2+}/calmodulin[8]. It is selectively enriched in pyramidal cells of the CNS[9,10]. In primary visual cortex, its expression and laminar distribution are developmentally regulated and follow the inside-out pattern of cortical maturation[10]. Calcineurin can regulate a wide array of substrates involved in brain plasticity by direct dephosphorylation or through activation of the downstream protein phosphatase 1 (PP1). Calcineurin and PP1 dephosphorylate specific sites on NMDA and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, thereby contributing to the mechanisms of long-term potentiation (LTP), long-term depression (LTD) and depotentiation[7,11–16]. The pharmacological blockade of calcineurin impairs LTD[17] and enhances the induction of LTP[18] in visual cortex. Mechanistically, it may serve in part to antagonize the cAMP-dependent protein kinase A (PKA) by downregulating molecular substrates activated by PKA[6,19]. It may also control PKA activity itself by inhibiting specific isoforms of the cAMP-producing adenylyl cyclase (AC9, ref. 20). In neurons, calcineurin and PKA are active simultaneously, and their concerted action is facilitated by A kinase–anchoring proteins (AKAP)[21,22] through concomitant binding. Notably, previous results have demonstrated that type II PKA[23,24] and AKAP150 (Fischer *et al.*, *Soc. Neurosci. Abs.* 37.9, 2003) are necessary for ODP. In addition to PKA, calcineurin and PP1 controls other ODP-related substrates that include autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; ref. 4), a kinase with a similar pattern of mRNA and protein expression as calcineurin in cortical and hippocampal structures[9,25], and the cAMP–response element binding protein (CREB) transcription factor[26–28]. In the visual cortex, CRE-mediated transcription is increased after monocular deprivation[27], and blocking CREB activation prevents the loss of responses to the deprived eye[28]. Overall, these findings suggest the involvement of calcineurin in ODP.

To test this hypothesis, we took advantage of a line of transgenic mice expressing an active form of calcineurin inducibly and reversibly in the brain with the tetracycline-controlled transactivator (tTA) system[19] and examined ODP in these mutant mice. We observed that an excess of calcineurin activity in the visual cortex during the critical period impairs ODP in a reversible fashion. These findings indicate that calcineurin is critical for ODP and support the model that calcineurin negatively regulates various forms of brain plasticity.

RESULTS

Calcineurin activity in the visual cortex

To confirm that calcineurin activity is increased in primary visual cortex (V1) of the calcineurin-overexpressing (CNO) mice, we performed phosphatase assays on extracts from binocular V1 at the peak of the critical period (postnatal day (P) 28–29). Assays showed a 48 ± 7% increase in calcineurin activity in CNO mice compared with wild-type littermates (**Fig. 1a**). This increase in calcineurin activity was lower than that observed in the adult hippocampus[19] (112 ± 9%), most likely due to a different efficiency and time course of transgene

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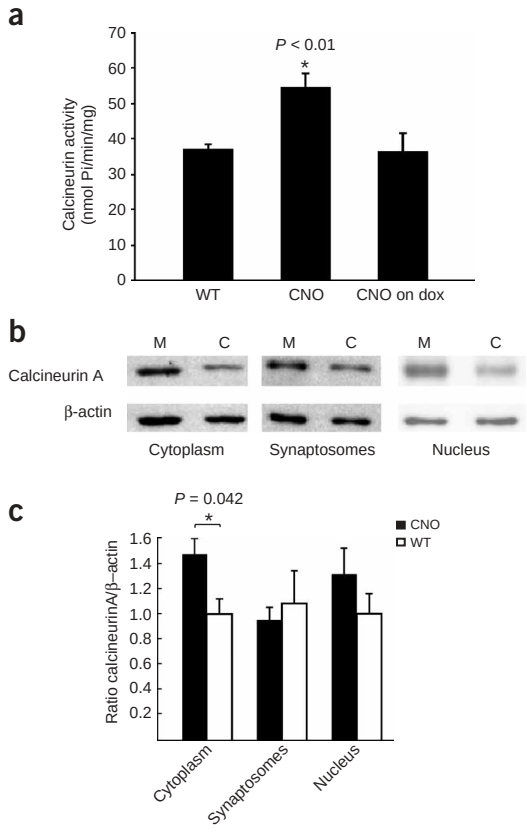

Figure 1 Calcineurin activity is increased in the visual cortex of CNO mice. (**a**) Enzyme activity in extracts from visual cortex of P28–29 CNO and wild-type (WT) mice. WT: 36.8 ± 1.8 nmol Pi min^{-1} mg^{-1}, $n = 4$; CNO mutants: 54.5 ± 4.0 nmol Pi min^{-1} mg^{-1}, $n = 3$, $P < 0.01$; CNO on doxycycline: 36.2 ± 5.4 nmol Pi min^{-1} mg^{-1}, $n = 4$, $P > 0.05$. Doxycycline was administered at least 7 d before tissue collection. (**b**) Representative examples of bands observed on immunoblots in three homogenate fractions from mouse cortex using an antibody against calcineurin A (M: CNO mutants, $n = 5$; C: WT, $n = 3$). (**c**) Relative calcineurin content normalized to β-actin (43 kDa). For all measures, data is expressed as mean ± s.e.m., if not stated otherwise.

expression in these structures. As in hippocampus, calcineurin overexpression in the visual cortex could be suppressed by doxycycline treatment, reflecting the full reversibility of the genetic manipulation (**Fig. 1a**). Analysis of the subcellular localization of calcineurin overexpression revealed a significant increase in calcineurin activity in cytoplasmic (46%) but not in synaptosomal or nuclear fractions in the mutant mice (**Fig. 1b,c**). This indicates that the calcineurin transgene concentrates in the cytoplasm in resting conditions.

Impaired ODP in CNO mutants

Four days of monocular deprivation during the critical period is sufficient to induce a robust and saturating ocular dominance shift to the open eye[29]. In wild-type littermates, neurons in the binocular zone of V1 were predominantly driven by the contralateral eye (**Fig. 2a**). After deprivation of the contralateral eye, the balance of inputs shifted to the open ipsilateral eye (**Fig. 2b**). Longer periods of deprivation did not induce any further ocular dominance shift (*t*-test, 4–5 d versus 6–7 d, $P = 0.58$). In non-deprived CNO mice, cortical neurons showed similar ocular dominance distribution as wild-type littermates (**Fig. 2c**). However, after monocular deprivation, no significant shift was observed in CNO mice (**Fig. 2d**), indicating impaired ODP. Ocular dominance distribution was still biased to the deprived contralateral eye even after 7–8 d of deprivation (two of seven mice), suggesting that the impairment in ODP was not because of a reduced sensitivity of the mutant mice to deprivation. Furthermore, the impairment was not because of a delay in the onset of the critical period, as 4- to 5-d monocular deprivation starting at P33–34 (after the typical critical period[29]) did not induce any apparent ocular dominance shift in CNO or wild-type mice (**Fig. 3**).

The impairment in plasticity can be rescued

To further determine if the impairment in plasticity was a direct consequence of the increased calcineurin activity, we suppressed calcineurin overexpression with doxycycline starting 7 d before monocular deprivation and examined whether this restored ODP in the mutant mice (**Fig. 4**). In wild-type controls, ocular dominance distribution was similar with or without doxycycline treatment in both non-deprived and deprived groups (**Fig. 4b,c**; compare to **Fig. 2a,b**). However, in mutants, doxycycline treatment before and during monocular deprivation (leading to transgene suppression) restored ODP and induced a robust ocular dominance shift (**Fig. 4e**), similar to that seen in wild-type littermates. The rescue was not due to a non-specific effect of doxycycline, as doxycycline itself had no effect on ODP in wild-type or CNO mice (**Fig. 4b–d**).

Weighted ocular dominance (WOD) scores for individual animals are shown in **Figure 4f**. Without monocular deprivation (open symbols), all mice had similar low WOD scores whether treated or not treated with doxycycline (wild-type, 0.29 ± 0.02; wild-type with doxycycline, 0.27 ± 0.01; CNO, 0.27 ± 0.02; CNO with doxycycline, 0.29 ± 0.01; *t*-test; $P = 0.38, 0.88, 0.85$, respectively), as in previous studies[23,24,29], indicating that calcineurin overexpression or doxycycline treatment had no effect on ocular dominance distribution in non-deprived animals. Following monocular deprivation (solid symbols), both wild-type groups showed a significant increase in WOD scores (with doxycycline = 0.55 ± 0.02, without doxycycline = 0.55 ± 0.02). CNO mice without doxycycline had only a small increase in WOD scores (0.33 ± 0.01; $P = 0.05$ relative to non-deprived wild-type),

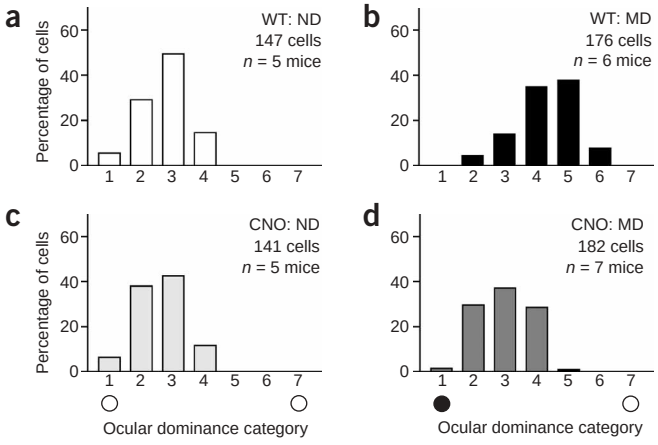

Figure 2 ODP is impaired by calcineurin overexpression. (**a**) Ocular dominance distribution in non-deprived (ND) wild-type (WT) mice. (**b**) After monocular deprivation (MD) initiated on P24, ocular dominance distribution shifts to the right in WT mice. (**c**) Without MD, CNO mice show an ocular dominance distribution similar to WT mice. (**d**) After MD, CNO mice show no ocular dominance shift.

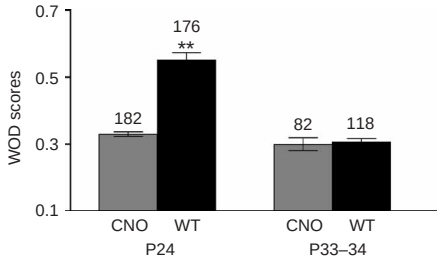

Figure 3 The impairment is not due to a delayed plasticity in CNO mutant mice. Columns show the effect of MD initiated during (P24, left) or after (P33–34, right) the critical period on WOD scores in CNO ($n = 7$ and 3 mice, respectively) and WT mice ($n = 6$ and 4 mice, respectively). The number of cells is labeled above the column. **, $P < 0.001$, t-test. Conventions as in **Figure 2**.

which was significantly lower than in deprived wild-type littermates (t-test, $P < 0.0001$). However, with both monocular deprivation and doxycycline treatment, WOD scores in CNO mice were similar to those in wild-type littermates (0.56 ± 0.01, t-test, $P = 0.62$) but significantly different from those in CNO mice without doxycycline (t-test, $P < 0.0001$), indicating a specific effect of calcineurin overexpression that was abolished by transgene suppression.

The impairment is not due to a developmental deficit

Calcineurin protein is not detectable until P4, although its mRNA is found as early as P1 in rat brain[30]. To exclude the possibility that an increase in calcineurin activity owing to transgene expression during early brain development (before the critical period) alters monocular deprivation–induced plasticity, we suppressed calcineurin overexpression at birth. Suppression was maintained either through development ('dox on/on' group), or calcineurin activity was elevated selectively during the critical period ('dox on/off' group)[31] (**Fig. 5**). In CNO mice with endogenous levels of calcineurin (because of permanent transgene suppression), ocular dominance distribution after deprivation shifted to the open eye, similarly to that in wild-type littermates (**Fig. 5a**). However, after restoration of transgene expression, we did not observe a shift in CNO mice after monocular deprivation (**Fig. 5b**, left). Both wild-type groups showed identical WOD scores (**Fig. 5c**, dox on/on, 0.56 ± 0.02; dox on/off, 0.52 ± 0.02). WOD values in the CNO groups were significantly different (**Fig. 5c**, dox on/on, 0.56 ± 0.03; dox on/off, 0.35 ± 0.01; t-test, $P < 0.001$), indicating that an overexpression of calcineurin during the critical period is sufficient to impair ODP (see also **Supplementary Fig. 1** online confirming that ODP is impaired by calcineurin even with an intact onset of the critical period).

Further, WOD scores in doxycycline on/off conditions were similar to those in non-treated conditions for both CNO and wild-type groups (compare **Fig. 5b** with **Fig. 2b**,**d**; $P = 0.37$ for wild-type, $P = 0.19$ for CNO), suggesting that the impairment in ocular dominance shift in CNO mice was not due to any developmental anomaly induced before the critical period. Consistently, the retinotopic map of V1 (**Fig. 6a**), the size of receptive fields (**Fig. 6b**), response strength and signal-to-noise ratio (**Fig. 6c**) were normal in CNO animals.

Plasticity can be rescued in later age

To examine whether calcineurin overexpression may alter the closing of the critical period and, if so, whether plasticity may be rescued later in life, we started doxycycline treatment at P38 and performed monocular deprivation 1 week later (P45). Four to five days of deprivation did not induce any shift in wild-type mice (**Fig. 7a**,**c**, WOD = 0.28 ± 0.03 compared to WOD = 0.26 (in ref. 29). However, in CNO mice, a low but significant shift was observed (**Fig. 7b**,**c**; WOD = 0.43 ± 0.06, $P = 0.003$, t-test), suggesting that calcineurin overexpression interfered with the closing of the critical period and thereby extended the critical period.

DISCUSSION

ODP is a form of sensory experience-dependent plasticity that has been extensively studied but whose molecular mechanisms remain poorly

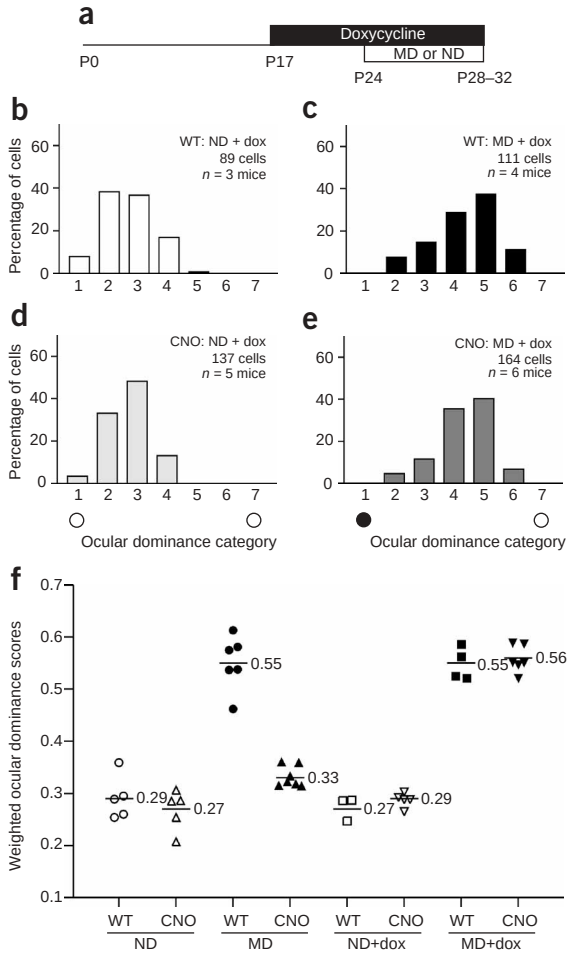

Figure 4 Plasticity is rescued by transgene suppression in CNO mice. (**a**) Suppression of calcineurin overexpression by doxycycline treatment starting on P17. (**b**) Doxycycline-treated WT littermates without deprivation (ND). (**c**) Doxycycline-treated WT littermates subjected to MD. (**d**) Doxycycline-treated CNO mice without deprivation. (**e**) After MD, doxycycline-treated CNO mice show a dramatic shift to the open eye. (**f**) WOD scores for individual animals. Without doxycycline treatment, non-deprived animals (open symbols) show low WOD scores (WT, open circles; $n = 5$ mice; CNO, open triangles; $n = 5$ mice). After MD (filled symbols), WT mice show increased WOD scores (filled circles, $n = 6$ mice), whereas CNO mice show impaired plasticity (filled triangles, $n = 7$ mice). After doxycycline treatment, WOD scores of non-deprived WT and CNO animals have no significant change (WT, open squares; $n = 3$ mice; CNO, open inverted triangles; $n = 5$ mice). After MD, doxycycline-treated CNO animals show high WOD scores (filled inverted triangles; $n = 6$ mice) similar to those in WT littermates (filled squares, $n = 4$ mice; $P < 0.001$). Each symbol represents the WOD score for a single mouse. Mean values are shown by horizontal bars with numbers. Conventions as in **Figure 2**.

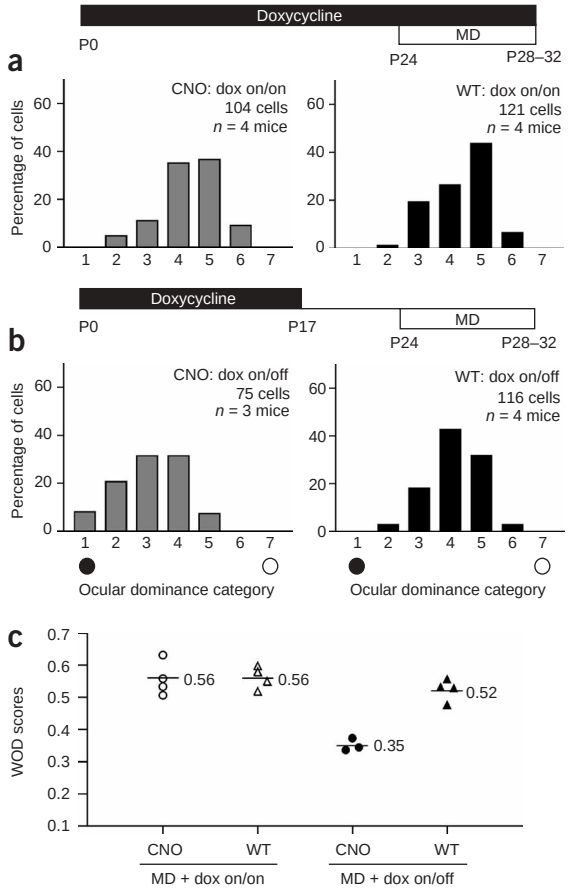

Figure 5 Calcineurin overexpression only during the critical period impairs ODP. (**a**) Doxycycline was administered to CNO and WT mice from P0 to the date of recording ('dox on/on'). MD induced a similar ocular dominance shift in CNO mutants (left) and WT littermates (right). (**b**) Doxycycline was removed 1 week before MD (P17, 'dox on/off'). No shift was observed in CNO mice (left), and plasticity was normal in WT mice (right). (**c**) WOD scores for individual animals. In dox on/on group, MD increases WOD scores in both CNO (filled circles) and WT (open triangles) mice. In contrast, CNO mice in the MD dox on/off group (filled circles) have significantly lower WOD scores than WT littermates (filled triangles, $P < 0.001$, *t*-test). Each symbol represents the WOD score for a single mouse. Mean values are shown by horizontal bars with numbers. Conventions as in **Figure 2**.

understood. This study provides evidence that the protein phosphatase calcineurin is involved in ODP by showing that an excess of calcineurin blocks the shift in ocular dominance normally induced by monocular deprivation. The impairment was induced by a transient increase in calcineurin activity during the critical period and could be rescued by restoring normal calcineurin activity, owing to the inducibility and reversibility of the genetic manipulation. These results are consistent with previous studies showing that downregulation of protein kinase activity impairs ODP[3–5] and emphasize the importance of the interplay between protein kinases and phosphatases in this form of plasticity.

As calcineurin is associated with PKA through anchoring proteins, it is likely to affect similar substrates during ODP. One of these substrates may be inhibitor-1 (I-1), a PP1 inhibitor activated by phosphorylation by PKA and blocked by dephosphorylation by calcineurin. Once calcineurin is stimulated by Ca^{2+} influx through NMDA receptors, it can activate PP1 via relief of I-1–mediated inhibition. Calcineurin can also stimulate the striatal-enriched protein tyrosine phosphatase (STEP), another protein phosphatase that is inactive in basal conditions[32]. A potential molecular cascade involved in ODP may thus engage calcineurin and PKA in a gate that controls PP1 activity and, in turn, the autophosphorylation of CaMKII (ref. 4,33), a critical player in ODP. Calcineurin and PKA may also gate the ERK pathway by modulating ERK activity through direct phosphorylation or dephosphorylation[34], or by interfering with its translocation through STEP[32]. An ultimate component of the cascade may implicate CREB and CREB-dependent gene expression in the nucleus, as ODP requires CREB[28] activation and protein synthesis[35]. Control of CREB-mediated transcription depends on CREB phosphorylation or dephosphorylation at Ser133 by protein kinases including PKA, CaMKIV, MAPK and possibly the protein phosphatase PP1, possibly activated by calcineurin[26,36]. Calcineurin also modulates intracellular Ca^{2+} by reducing NMDA receptor current decay time and regulating intracellular Ca^{2+} release[37], thereby controlling other Ca^{2+}-dependent enzymes. Thus, calcineurin may be involved in the control of signaling from the synapse to the nucleus during ODP. This is also consistent with the fact that in our mutants calcineurin is essentially overexpressed in cytoplasmic fractions and not at synapses and is therefore more likely to perturb intracellular signaling pathways than membrane receptors.

Recent work suggests that ODP induction and expression machinery may be different. Benzodiazepines restore the onset of the critical period, which is altered in GAD65$^{-/-}$ mice, but appear not to intervene in the expression of ODP[38]. Further, $GABA_A$ α1 knock-in mice show normal expression of ODP, although the transition to the precocious critical period by benzodiazepines is impaired[39] because benzodiazepines act through this subunit of GABA receptors. Thus, GABAergic inhibition seems to be uniquely responsible for the induction machinery. In our mutant mice, however, ODP is impaired by an

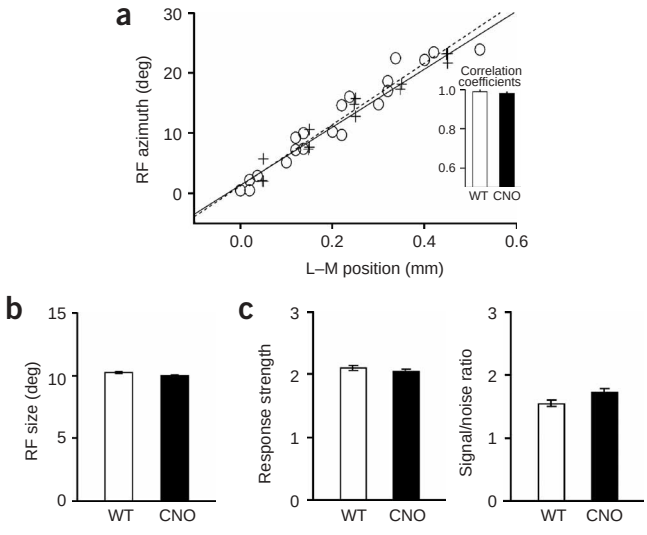

Figure 6 Response properties are normal in CNO mutant mice. (**a**) Retinotopic mapping in WT mice (open circles, $n = 15$ units in three mice) is similar to that in CNO animals (+; $n = 20$ units in four mice). Each symbol represents the average receptive field azimuth of a single penetration. Inset: correlation coefficients for three WT and four CNO regressions ($P = 0.5$, *t*-test). (**b**) Size of receptive field (RF; WT: $n = 118$ cells in four mice; CNO: $n = 128$ cells in five mice) is unchanged ($P = 0.2$, *t*-test). (**c**) Response strength (left) and ratio of signal and noise (right) are similar in WT and CNO animals (WT, $n = 118$ cells; CNO, $n = 128$ cells, $P > 0.1$ for both, *t*-test). Conventions as in **Figure 2**.

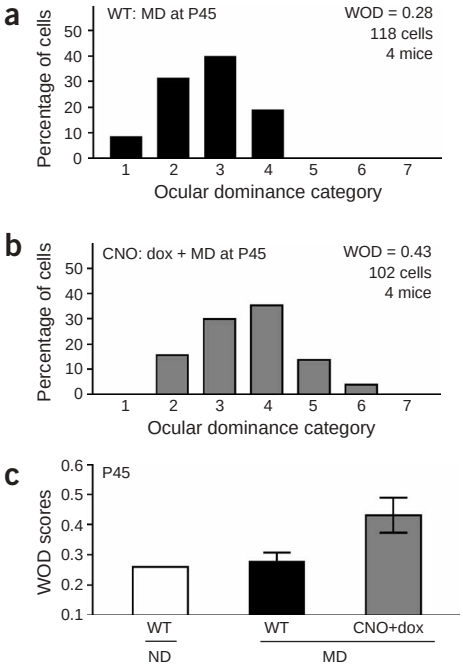

Figure 7 Restoring normal calcineurin action induces ODP in older mice. (**a**) Brief MD does not induce an ocular dominance shift in WT mice (P45). (**b**) Doxycycline treatment at P38 (7 d before MD) results in an ocular dominance shift in CNO mice ($P < 0.0001$, χ^2 test). (**c**) Average WOD score for non-deprived and deprived WT mice and deprived doxycycline-treated CNO mice at P45. Conventions as in **Figure 2**.

increase in calcineurin activity with an intact onset of the critical period (**Supplementary Fig. 1**, **Fig. 5**). Therefore, calcineurin may regulate ODP through mechanisms other than GABA inhibition during the critical period. Our results also show that calcineurin overexpression may disturb the closure of the critical period. The mechanisms of closure are not well understood but may involve the maturation of extracellular matrix[40] and intracortical myelination. Recent experiments reveal that ODP persists into adulthood in mice deficient for myelin-associated protein Nogo-A or its receptor (A.W. McGee, Y.Y., N.W.D. and S.S. Strittmatter, unpublished data). Thus, it could be of interest to examine whether the synthesis of these molecules is altered in CNO mice, as well as in other kinase-deficient mutant mice.

Short deprivation episodes during the critical period can induce structural changes in the brain. For instance, spine motility is significantly increased after monocular deprivation[41]. In the hippocampus, calcineurin is known to induce shrinkage of dendritic spines, possibly by dephosphorylating cofilin and perturbing cofilin-actin polymerization[42]. Together with PKA, it is also believed to control neurite extension and axonal regeneration[43,44]. Therefore, it is possible that calcineurin overexpression in our model impairs visual cortex plasticity by interfering with structural changes necessary for such plasticity. Further investigations would be necessary to examine this point.

ODP has been suggested to share mechanisms with LTD in the visual cortex[45]. However, previous work showed that calcineurin inhibition by FK506 impairs LTD in visual cortex[17], and calcineurin activity increases during LTD *in vivo*. Calcineurin overexpression in our mutant mice did not change LTD in layer II/III in visual cortex (**Supplementary Fig. 2**), consistent with its reported lack of effect on LTD in the hippocampus in the same mice[7]. This suggests that LTD and ODP may not be directly related or have a complex relationship as suggested by several other studies[23,46,47]. It may also be explained by the existence of several forms of LTD in different cortical layers (see review, ref. 48).

Finally, we did not observe any ODP in older wild-type animals (**Fig. 3**), similar to results from other single-unit studies[4,29]. This contrasts, however, with recent reports describing the existence of an adult form of visual plasticity measured by visually-evoked potentials[49,50]. This adult plasticity is strongly suppressed by nembutal[50] and may therefore not be observed in our experimental conditions.

METHODS

Generation and maintenance of Tet-CN279 transgenic mice. All procedures used in this study were approved by the Animal Care and Use Committee at Yale University and conform to the guidelines of the National Institutes of Health and The Society for Neuroscience. Tet-CN279 transgenic mice (TetO promoter-Δ*CaM-AI* transgene) were generated by microinjection of a linear DNA construct into fertilized one-cell eggs as previously described[19]. The founder mouse was backcrossed to C57BL6/J mice for 9–10 generations to generate heterozygous offspring and then crossed to heterozygous *CaMKIIα* promoter-tTA mice to generate Tet-CN279 mice. Genotyping was performed by PCR on tail DNA. Tet-CN279 double mutants (CNO) and wild-type littermates were used. The animals were maintained in the facility according to standard protocols. CNO and wild-type littermates were fed with normal food or with food supplemented with 2 mg kg^{-1} of doxycycline (Research Diets).

***In vivo* electrophysiology.** Electrophysiological recordings were performed under nembutal/chlorprothixene (50 mg kg^{-1}, i.p., Abott Laboratories; 10 mg kg^{-1}, i.m., Sigma) anesthesia using standard procedures[24]. Atropine (20 mg kg^{-1} s.c., Optopics) was injected to reduce secretions and parasympathetic effects of anesthetic agents, and dexamethasone (4 mg kg^{-1} s.c., American Reagent Laboratories) was administered to reduce cerebral edema. Mice were placed in a stereotaxic device, and a tracheal tube and intraperitoneal cannulae were inserted. A craniotomy was made over the right visual cortex, and agar was applied to enhance recording stability and prevent desiccation. Eyelids were removed from both eyes, and corneas were protected thereafter by frequent application of silicon oil. Body temperature was maintained at 37 °C by a homeostatically controlled heating pad. Heart rate and respiration were monitored continuously.

Four to six cells (>90 μm apart) through the full thickness of the cortex were evaluated in each of four to six penetrations spaced evenly (at least 200 μm apart) crossing the binocular region (azimuth <25°) of area 17 to avoid sampling bias. Cells were assigned to ocular dominance categories according to the seven-category scheme of Hubel and Wiesel. Ocular dominance histograms were constructed and WOD scores were calculated for each mouse with the formula: $\mathrm{WOD} = (1/6G_2 + 2/6G_3 + 3/6G_4 + 4/6G_5 + 5/6G_6 + G_7)/N$, where G_i is the number of cells in ocular dominance groups, and N is the total number of cells. Normal mice have an average WOD of about 0.28; that is, they are dominated by the contralateral eye. Response quality was assessed by rating the level of visually driven and spontaneous activity, each on a three-point scale (1 = low, 3 = high).

Monocular deprivation. Lid suture of the left eye was performed under 1–2% halothane anesthesia on postnatal day 24 (P24) or P33 for all mice receiving deprivation. Lid margins were trimmed and lids sutured together using 6-0 silk. Experiments were performed blind to genotype and drug treatment.

Phosphatase assay. Animals were killed by decapitation after anesthesia with halothane. Phosphatase assays were performed using an assay kit (Calbiochem). Pooled binocular visual cortices (L2–4 mm, P0–2mm) were homogenized and centrifuged. After desalting to remove free phosphates, supernatants were diluted in 50mM Tris (pH 7.5), 1 mM DTT, 100 μM EDTA, 100 μM EGTA, 0.2% NP-40 and were incubated at 30 °C for 30 min in reaction buffer. Okadaic acid (1 μM) was added to inhibit PP1 and PP2A activity. PKA regulatory subunit type II was used as substrate (DLDVPIPRFDRRV-pSer-VAAE). Calcineurin activity was expressed in nmol Pi released min^{-1} mg^{-1} protein. The protein concentration was measured using a Biorad protein assay. All measures were performed in triplicate.

Immunoblotting. Homogenates from cortex were subjected to sucrose gradient centrifugation, and cytoplasmic, nuclear and crude synaptosomal fractions were collected. Samples were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. After 1 h blocking (2% goat serum) at 22 °C, membranes were incubated with 1:6,000 anti-calcineurin (Chemicon, ab1695) and 1:3,000 anti–β actin (Sigma, A-5316) for 1 h, washed, then further incubated in 1:6,000 anti-rabbit (Upstate Biotechnology 12-348) and 1:3,000 anti-mouse horseradish peroxidase–conjugated secondary antibody (Upstate Biotechnology 12-349). Horseradish peroxidase was detected by adding 300 µl chemiluminescence reagent (Perkin Elmer Western Lightning) and exposing membranes to Kodak MR films. Quantification was conducted using the Image software. Immunoblots were prepared in duplicate or triplicate, and results were averaged.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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