Electrical synapses formed by connexin36 regulate inhibition- and experience-dependent plasticity

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The mammalian brain constantly adapts to new experiences of the environment, and inhibitory circuits play a crucial role in this experience-dependent plasticity. A characteristic feature of inhibitory neurons is the establishment of electrical synapses, but the function of electrical coupling in plasticity is unclear. Here we show that elimination of electrical synapses formed by connexin36 altered inhibitory efficacy and caused frequency facilitation of inhibition consistent with a decreased GABA release in the inhibitory network. The altered inhibitory efficacy was paralleled by a failure of theta-burst long-term potentiation induction and by impaired ocular dominance plasticity in the visual cortex. Together, these data suggest a unique mechanism for regulating plasticity in the visual cortex involving synchronization of inhibitory networks via electrical synapses.

gap junctions | development | cannabinoid receptor 1 | synchrony

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dynaptic interactions in the mammalian brain are sculpted by experience, particularly during “critical periods” in development. Accumulating evidence suggests that inhibitory neurons play a crucial role in reshaping neural networks in response to sensory perturbations (1). Inhibitory neurons in many areas of the brain, including the cerebral cortex, are coupled via gap junctions containing connexin36 (Cx36), which allow subthreshold fluctuations in membrane potential to spread between cells and promote synchrony of firing (2–5). Cx36-null mice (Cx36KO) display decreased coherence of rhythmic activity in populations of cortical interneurons in brain slices and decreased strength of evoked cortical inhibition in vivo (6–8). Similar effects of Cx36 signaling have been shown to electrically couple individual inhibitory neurons (6), and this coupling promotes synchrony of firing (2–5). However, the effect of coupling on the functional dynamics of inhibitory networks and their postsynaptic targets is unknown. We used patch-clamp recordings to determine the properties of the inhibitory network in Cx36KOs (6). In these mice, the coding region for Cx36 was replaced with a construct for β-gal and alkaline phosphatase. Because interneurons in Cx36 heterozygotes (HETs) form electrical and chemical connections similar to wild-type (WT) animals (18, 19), we compared putative changes in inhibitory synaptic transmission in KO cells to their HET counterparts, thereby controlling for possible effects of reporter gene expression.

We recorded inhibitory postsynaptic currents (IPSCs) from layer 4 (L4) neurons in mouse visual cortex (Fig. 1A). Because prolonged depolarization can affect inhibitory transmission (20), experiments were performed at −70 mV by using symmetric Cl− solutions (16). First, we investigated the properties of single inhibitory synapses and measured miniature IPSCs (mIPSCs; Fig. 1B). mIPSCs occurred at similar frequencies, amplitudes, and kinetics in both genotypes (Fig. 1B and C). Thus, unitary GABAergic transmission is not affected by the loss of Cx36. This notion is supported by quantitative PCR (qPCR) showing that GABA receptor subunit mRNA levels are similar in KOs and controls (Fig. S1).

Next we evaluated the effects of Cx36KO on the inhibitory network. We blocked glutamatergic transmission, electrically stimulated L4, and measured evoked IPSCs in postsynaptic neurons. Stimulation activated multiple inhibitory neurons and evoked a compound IPSC (eIPSC) in the recorded cell (Fig. 1D). The eIPSC represents a summation of IPSCs from the activation of multiple interneurons and increased with increasing stimulation intensity before saturating (Fig. 1 D and E). The maximal eIPSCs, which is a measure of the total GABAergic inputs converging onto neurons, were similar between control and KO.
cells (Fig. 1E). This result suggests that neurons in Cx36KO and control receive a comparable number of inhibitory inputs. The effects of gap junctional coupling on the network, however, might only be effective and revealed at submaximal stimulation intensities. To assess this possibility, we recorded IPSCs evoked with submaximal stimulation before and after application of the gap junction blocker carbenoxolone (CBX). CBX reduced eIPSC amplitudes in both genotypes but to a lesser degree in Cx36KO (Fig. S2). Although CBX has nonspecific effects (21) and potentially also blocks non-Cx36-containing gap junctions, the larger decrease in control suggests that a fraction (∼25%) of the CBX-induced reduction in eIPSC amplitude is specific to gap junctions containing Cx36. Because coupling between inhibitory neurons enhances their synchrony (2–5), the eIPSC fraction mediated by Cx36 could be due to postsynaptic summation of synchronous IPSCs. This result is consistent with in vivo results showing that Cx36 removal reduces the amplitude of inhibition (8).

Because the removal of Cx36 also results in longer-lasting inhibition in vivo (8), and because Cx36 enhances the synchrony between inhibitory neurons (2–5), we next investigated the dynamic properties of inhibition. Inhibitory transmission is attenuated during repetitive activation, and this attenuation is thought to be due to vesicle depletion (16, 22, 23). Because Cx36 enables synchronous firing of inhibitory neurons, the lack of Cx36 in KOs might affect adaptation during repetitive stimulation. Therefore, we studied the inhibitory response of L4 cells to trains of stimulation pulses delivered at different frequencies (50, 30, 10, and 1 Hz; Fig. 1F). Stimulus intensities that produced similar eIPSC amplitudes during the first pulse between the two genotypes were chosen for the comparison (Fig. 1G). During the stimulus trains, eIPSC amplitude rapidly decreased until it reached a steady level, the amplitude of which was inversely related to the stimulation frequencies (Fig. 1E and G). The amount of reduction at the steady state for all frequencies tested and the total charge transferred at 50 Hz were similar in both genotypes, suggesting that...
loss of Cx36 does not cause major deficits in the synthesis of GABA and its source of readily releasable vesicles (Fig. 1 G–I). Although the steady-state levels were similar, we observed changes in the temporal properties of inhibitory transmission during stimulus trains. At a repetition rate of 1 Hz, amplitudes were similar between genotypes (Fig. 1J), but at higher stimulation frequencies, amplitudes were larger in Cx36KOs than controls for the first couple of stimulus pulses (Fig. 1J). In particular, in Cx36KO, eIPSCs at the second pulse of the 10- and 30-Hz pulse trains were larger than at the first pulse, thus displaying facilitation that was not observed in control (Fig. 1J). In addition, the normalized amplitudes at the subsequent 3–6 pulses for 10- to 50-Hz stimuli were larger in Cx36KO than in control (Fig. 1K). Thus, during high-frequency discharge, inhibition is more effective in Cx36KO within a time window of 30–100 ms (Fig. 1K). The facilitatory response observed in Cx36KO is reminiscent of reduced synaptic release, consistent with a Cx36-dependent component of the eIPSC (Fig. S2). Together, these in vitro results suggest that the removal of Cx36 results in initially weaker but longer-lasting inhibition, consistent with in vivo observations (8).

**Cx36 Affects Synaptic Plasticity.** Our in vitro results suggest that temporal dynamics of inhibition is altered in the Cx36KO. Altered inhibition can affect the function of the whole cortical network, and it has been shown to interfere with long-term synaptic plasticity (16). Thus, we investigated whether removal of Cx36 compromises LTD and LTP in layer 2/3 (L2/3; ref. 24). LTP was induced by TBS of L4, whereas LTD was induced by stimulating at a low frequency (1 Hz). We compared baseline amplitudes of local field potentials (LFPs) to postinduction (0–60 min) LFP amplitudes. After low-frequency stimulation, slices from all genotypes showed comparable reduction in LFP amplitudes (Fig. 2A). These results suggest that Cx36 removal does not affect LTD.

TBS led to increased LFP amplitudes in WT slices (112.3 ± 8.3%; P < 0.01; n = 8 slices; n = 3 animals; Fig. 2B). In contrast, Cx36KO slices (n = 10 slices; n = 6 mice) did not show LFP amplitude increases (P < 0.05), but instead showed a trend to decrease (94.9 ± 8.5%; P < 0.09; Fig. 2B). The mean LFP amplitude after LTD in both WT and HET controls was significantly larger than in Cx36KO (P < 0.0001 and P < 0.04, respectively; Fig. 2D), but LTD increases in HETs showed more scatter and slightly lower mean increases than in WT (Fig. 2B). These data suggest that removal of Cx36 prevents synaptic strengthening with high-frequency stimuli. In contrast, the ability of synapses to undergo synaptic weakening with low-frequency stimuli does not depend on Cx36.

The deficits in TBS-LTP could be due to intrinsic deficits in synaptic plasticity. We thus investigated whether pairing LTP was present in Cx36KOs. L2/3 neurons were recorded via whole-cell patch clamp, and pairing LTP was induced by pairing 0 mV depolarization with low-frequency stimulations (1 Hz, 200 pulses). Both WT and Cx36KOs exhibited comparable pairing LTP (P = 0.635; Fig. 2C). Thus, together our results suggest that, although excitatory synapses retain the capability of expressing LTD, the induction of LTD with high-frequency stimuli is selectively impaired.

The deficits in TBS-LTP could potentially be due to altered glutamatergic signaling. To explore this possibility, we obtained recordings from L2/3 neurons under conditions that evoke isolated AMPA or NMDA currents (Fig. 3A). Neither the ratio of AMPA-to-NMDA currents (Fig. 3A) nor NMDA current decay times, which depend on the proportion of NR2A and NR2B subunits (25, 26), showed any significant difference between genotypes (Fig. 3B). Consistent with these observations, we found similar levels of NR2A and NR2B mRNAs in Cx36KOs and controls (Fig. 3C). Therefore, the absence of Cx36 in neuronal networks does not grossly influence excitatory transmission or intrinsic plasticity mechanisms. However, we found that the responses during the TBS suppressed more in the KOs compared with HETs and WTs, which was quantified by comparing the

![Fig. 2.](https://www.pnas.org/cgi/doi/10.1073/pnas.1100166108 Postma et al.)

A Upper) Plotted are field potential (LFP) amplitudes before and after application of 1-Hz stimulus. (Lower) Time course of LTD and relative LFP amplitudes after 60 min. Filled circles indicate individual slices. The reduction in LFP amplitudes was similar (KO: 94.3 ± 6.1%, 11 slices, 6 animals; HET: 92.2 ± 8.0%, 11 slices, 6 animals; WT: 92.9 ± 7.8 12 slices, 2 animals; all P < 0.05, *). (B Upper) Plotted are LFP amplitudes before and after application of TBS stimulus. (Lower) Time course of LTD and relative LFP amplitudes (rel. to baseline) after 60 min. *P < 0.05. (C) Paring LTP. Traces show whole cell EPSPs in L2/3 neurons before and after pairing in WT and KO. Main graph shows input resistance and membrane potential. Bar graph shows %EPSP slope after 30 min in WT (143 ± 7.2%; 5 cells, 4 animals; P < 0.004) and KOs (138 ± 6.8%; 6 cells, 5 animals; P < 0.003). Filled circles indicate individual cells.
normalized LFP area during the TBS (Fig. 3D). This result is consistent with an interpretation that there is enhanced inhibition during TBS in the KOs, because the degree of frequency depression in L2/3 of visual cortex depends on the strength of inhibition (27). This result is also consistent with the frequency facilitation of eIPSCs seen in Cx36KO (Fig. 1J and K).

**Cx36 Regulates OD Plasticity.** Our in vitro results reveal specific deficits in temporal profile of inhibitory efficacy and synaptic plasticity in Cx36KO. Both inhibitory efficacy and synaptic plasticity are important for normal developmental refinement and plasticity of cortical organization (1, 17). Thus, we investigated whether OD plasticity of visual cortex requires the presence of Cx36 (Fig. 4A). We chronically recorded visually evoked potentials (VEPs) in the binocular zone (BZ) of the primary visual cortex to measure the input strength of both ipsilateral and contralateral eyes before and after 5–6 d of MD. After 2 d of habituation, baseline VEPs were obtained in response to stimulation of each eye (Fig. 4B). To circumvent the effects of Cx36KO on rod photoreceptor signalling (19), we recorded VEPs under photopic illumination, which activates cone photoreceptors (>50 cd/m²). Baseline VEP waveforms (Fig. 4B) and amplitudes (Fig. 4C) at P25 were similar in Cx36KOs and controls. Thus, the initial development of OD does not require Cx36.

Next, we induced OD shifts by monocularly depriving animals for 5–6 d through the contralateral eyes starting at ~P25 (Fig. 4D, post). OD plasticity varies between mouse strains (28). Because Cx36KOs are kept on a mixed background (Methods), we used both WT and HET littermates as controls. In control mice, we observed the expected reduction in VEP amplitude to DE stimulation and increase of VEP amplitude to NDE stimulation (Fig. 4D and E). We evaluated the OD shift by calculating the difference in the DE/NDE VEP ratios (Fig. 4F). A reduction of the ratio after MD indicated a shift in OD toward the NDE. In control animals, we observed a reduction in the ratio, indicating the expected OD shift toward the NDE (13). In contrast, Cx36KOs displayed reduced VEP amplitudes after stimulation of either NDE or DE (Fig. 4D and E), which manifested as no change in OD ratio after MD (Fig. 4F). The abnormal NDE weakening (Fig. 4F) therefore undermined OD plasticity in Cx36KOs (Fig. 4F).

VEP recordings detect physiological changes in the relative strength of both eyes caused by MD, they do not reveal changes in the spatial extent of the functional representation of both eyes that typically accompany OD plasticity, i.e., the expansion or contraction of regions responding to the NDE or OD. To explore this issue, we measured the expression of Arc, an immediate early gene that is rapidly expressed in visual cortical neurons after brief visual stimulation (11, 29–31) with in situ hybridization. The visually induced expression pattern of Arc is a sensitive indicator of OD plasticity in both mice and cats (11, 29–31). As expected, after monocular enucleation (ME) in control, the functional representation of the NDE increased in control (Fig. S3). In contrast, no discernible expansion of the functional NDE representation after ME was detected in Cx36KO, and the reduced Arc expression intensity suggests a weakening of the NDE-driven activity (Fig. S3). Together with the VEP data, these results show that Cx36 is required for normal OD plasticity.

The abnormal OD plasticity in Cx36KO might be due to the enhanced frequency facilitation of IPSCs, which is a consequence of a reduction in release. Hence, enhancing GABA release might rescue OD plasticity. CB1 endocannabinoid receptors (CB1Rs) are expressed in electrically coupled interneurons (32) and regulate IPSC amplitudes by affecting GABA release. CB1 activation decreases IPSC amplitudes by lowering GABA release, whereas blocking CB1 has the opposite effect (33–35). Moreover, increasing GABA release by blocking CB1 decreases frequency facilitation of IPSCs (36). Thus, we blocked CB1R with the selective antagonist AM251 and reassessed OD plasticity. First, we investigated whether AM251 injection by itself had any effect. Injections of AM251 in unmanipulated animals did not alter VEP amplitudes in either genotype (Fig. S4). Furthermore, MD in control animals concurrent with AM251 injections produced normal OD shifts (Fig. 4E and F and Fig. S4), consistent with previous results (37). In contrast, AM251 treatment on Cx36KOs restored normal OD shifts after MD (Fig. 4E and F and Fig. S4). The restoration of OD plasticity in Cx36KOs by AM251 was due to a rescue of NDE strengthening, as evidenced by increased NDE VEP amplitudes after MD (Fig. 4E and F). Together, these data support the idea that the altered OD plasticity in Cx36KOs is the result of abnormal inhibition.

**Discussion**

Our studies show that Cx36 signaling in inhibitory neurons is required for normal LTP and OD plasticity. A major difference between control and Cx36KO on a synaptic level is that high-frequency stimuli, such as TBS, that normally evoke LTP fail to do so in Cx36KO. The synaptic effect of Cx36KO is reflected in vivo by a change in response to MD. After MD, NDE inputs to the visual cortex weaken rather than strengthen as they do in control. The physiological data are consistent with the Arc induction results. Arc induction showed that the NDE increase is almost absent in Cx36KO and that Arc intensity of the remaining region driven by the NDE is reduced. This result suggests a net decrease of NDE-driven activity in Cx36KOs. Thus, in Cx36KO, MD leads to a weakening of both eyes and therefore does not lead to a net shift of OD toward the NDE.

Our results suggest that the effects of Cx36 deletion on LTP and OD plasticity are not due to gross deficiencies in glutamatergic transmission or mechanisms of synaptic plasticity. However, we found a specific impairment of inhibition in the absence of Cx36. Our data show facilitatory responses to high-frequency stimulation. Thus, inhibition is longer lasting in the absence of Cx36. Because our stimulation protocol was adjusted to normalize the IPSC amplitude of the first pulse of a stimulus.
train, we did not observe large differences in IPSC amplitude between cells. However, our pharmacological manipulations showed that (~25%) of the IPSCs is due to coupling via Cx36, given the lower efficacy of CBX in the Cx36KO. This result is consistent with in vivo results, which showed that removal of Cx36 leads to reduced inhibitory amplitudes and prolonged duration of inhibition (8). At a single synapse, the postsynaptic amplitude depends on the probability of synaptic release ($p_s$), the evoked amplitude of the transmitter contained in a synaptic vesicle ($A_0$), and the number of vesicles released ($n$): $A_n = p_s^n a_0$. In a network with independently acting neurons, the compound amplitude ($A_n$) depends on the single neuron firing probability ($p_s$), the evoked amplitude $A_0$, and the number of neurons ($m$): $A_n = p_s^n a_0^m = p_s^m p_s^n a_0$. Thus, the release probability of the network ($p_m$) depends on both $p_s$ and $p_m$: $p_m = p_s p_m$. Our data are consistent with decreased $p_r$. Desynchronization reduces $p_s$ and thereby $p_m$. Thus, a facilitating network response in Cx36KO is consistent with reduced activation of the inhibitory network for low-frequency inputs and an increased facilitation for a brief time window (~30–100 ms) during high-frequency stimulation. AM251 increases synaptic release probability ($p_s$), which can then increase $p_r$. Consistent with this scenario, our results show that AM251 injections rescue OD plasticity in Cx36KO.

How, then, do these deficits in inhibition in Cx36KO relate to the observed changes in LTP and OD plasticity? Altered temporal profile of inhibitory efficacy can alter timing relationships between thalamic and cortical activity consistent with reduced gamma activity in Cx36KO (8, 38, 39). Such altered timing relationships can alter synaptic plasticity. For example, in studies where thalamic inputs to visual cortex were experimentally decorrelated from cortical neurons by cortical silencing, NDE weakening was also observed (~40–42). Altered inhibitory efficacy can produce LTP deficits through multiple mechanisms. Most likely, enhanced inhibition during high-frequency stimulation may attenuate NMDA receptor activation and, consequently, LTP (27). Consistent with this scenario, we showed that Cx36KOs display increased inhibition (within a window of 30–100 ms) and stronger suppression of excitatory responses during repetitive high-frequency stimulation.

In Cx36KO, the coding region for Cx36 was replaced with β-gal and alkaline phosphatase. Thus, as a sensitive control for possible effects of reporter gene expression, we compared KO cells to their HET counterparts (18, 19). Electrical coupling in thalamic reticular nucleus neurons from Cx36 HET is qualitatively similar to WT (18), but effects of gene dosage on connexin levels have been reported for other connexins (43). Although effects of MD on OD plasticity were largely similar in WT and HET, we observed slight differences in LTP between both groups. Thus, the differences in LTP we observed in HET could be related to gene dosage effects and a higher sensitivity of LTP to slight perturbations in coupling. Regardless, the difference between WT and HET responses after LTP did not reach statistical significance, whereas the difference between control and Cx36KO did.

The notion that an alteration in the overall levels of inhibition accounts for the impaired OD plasticity in the Cx36KO is well supported. Our results are consistent with the view that a certain level of inhibition is required for OD plasticity to occur (1). For example, knockout of the 65-kD isoform of the GABA synthetic enzyme glutamic acid decarboxylase (GAD65) impairs OD plasticity in Cx36KO mice.
ticity and LTD and causes deregulation of NMDA receptor levels (16, 31, 44). We show that removal of Cx36 impairs OD plasticity and LTP. Thus, our results extend the previous model by showing that inhibition can affect either LTP or LTD and that in both cases OD plasticity is impaired. Our results also show that the strength and duration of inhibition can be regulated by at least two complementary pathways: synchrony of inhibitory neurons via Cx36 and GABA production/release via GAD65.

Modulation of the strength and dynamic properties of inhibition by synchronizing the output of coupled neurons has distinct advantages over other known mechanisms for regulation of synaptic strength. Several neurons synchronizing their output on a single target neuron constitutes a form of gain control, which can be dynamically modulated in vivo by activity-dependent mechanisms on a second-to-minute time scale (45). This finding complements other mechanisms, such as altered levels of receptor expression, that operate on timescales of minutes to days. Regardless of whether or not such temporal factors play a role in inhibition, our results demonstrate that electrical coupling between inhibitory interneurons has profound effects on the temporal dynamics of inhibition and is critical for normal OD plasticity.

Methods

See SI Methods for detailed methods. Experiments were conducted in accordance with the Animal Care and Use Committee of the University of Maryland and Harvard Medical School. Cx36KO mice were generated as described (6). Slice electrophysiology was performed as described (24, 31). To record EPSCs, pipettes were filled with a cesium-based solution. AMPA and NMDA EPSCs were isolated by holding neurons at –70 and +40 mV, respectively, and blocking GABAergic transmission. IPSCs were isolated by blocking glutamate receptors. To measure IPSCs at –70 mV, respectively, and blocking GABAergic transmission. IPSCs were isolated by using stimuli oriented perpendicular to the baseline stimuli. OD ratio finding is corrected by blocking glutamate receptors. To measure IPSCs at –70 mV, respectively, and blocking GABAergic transmission. IPSCs were isolated by using stimuli oriented perpendicular to the baseline stimuli.

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Supporting Information

Postma et al. 10.1073/pnas.1100166108

SI Methods

The methods for slice physiology, RT-PCR, in situ hybridization, and in vivo recordings were similar to those in our previous studies (1–6). The experiments were conducted in accordance with the Animal Care and Use Committees of the University of Maryland and Harvard Medical School.

Cx36KO Mice. The Cx36-knockout mice (Cx36KO) were generated as described (7). The Cx36 coding sequence was replaced by a bicistronic cassette containing two reporter genes: β-galactosidase and placental alkaline phosphatase. Cx36KO mice were kept in a mixed C57B6–129SvEv background.

In Vitro Electrophysiology. For whole-cell patch recordings, animals were euthanized under deep isoflurane anesthesia, and a block of brain containing visual cortex was removed rapidly. Slices (350–450 μm) were cut on a vibrating microtome in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3 KCl, 1.25 KH2PO4, 20 NaHCO3, 10 glucose, 1.3 MgSO4, and 2.5 CaCl2 (pH 7.35–7.4, equilibrated with 95% O2–5% CO2) and were then incubated for at least 1 h in ACSF at 30 °C. For recording, slices were held in a chamber on a fixed-stage microscope (Nikon FN1) and superfused (2–4 μL/min) with ACSF at ~25 °C. Recordings were performed with a patch clamp amplifier (Multiclamp 700B) in voltage clamp mode using 4–8 Mohm pipettes. To record EPSCs, pipettes were filled with (in mM): 115 CsMeSO4, 5 NaF, 10 GTP, 10 Hepes, 3.5 Mg-ATP, and 3 QX-314. AMPA and NMDA currents were isolated by holding neurons at −70 and +40 mV, respectively, followed by blocking GABAergic transmission with picrotoxin and reducing excitability in the slice by using a modified ACSF containing 5.0 mL. AMPA/NMDA ratios were determined by first calculating the respective conductances (gAMPA and gNMDA). gAMPA was calculated from the peak AMPA EPSC at −70 mV, and gNMDA was calculated from the EPSC at 40 mV at 100 ms after stimulus onset.

IPSCs were isolated by blocking glutamate receptors with 100 μM CNQX or NBQX, and 50 μM APV. To allow the measurement of IPSCs at −70 mV, the reversal potential of Cl− was shifted to 0 mV by using an electrode solution that contained (in mM): 40 CsCl, 8 KCl, 10 EGTA, 10 Hepes, and 1 QX-314, pH 7.4 (275–285 μMn) (8). Signals were digitized by a Digidata AD board (Molecular Devices) under pClamp (Molecular Devices; Version 9). NMDA currents were analyzed with MATLAB using custom routines. Electrical stimulation was performed by using a bipolar stimulating electrode (Microprobe; ~200-μm tip spacing, bifilar, 0.2 ms) coupled to a stimulus isolator (Cygnus). The electrode was placed into L4 ~500–800 μm horizontally from the recorded cell for L4 recordings and radially below the recorded cell for L2/3 recordings. Stimuli were applied at varying stimulus levels for each cell to determine threshold and saturation level. For comparison L/ O curves were normalized to the maximum evoked IPSC. In each recording session, we recorded cells from control and KO animals from the same litter. Analysis was done blind to genotype.

For pairing-LTP experiments, L2/3 neurons were recorded in current clamp, and evoked excitatory post synaptic potentials (eEPSPs) were monitored by baseline stimulation at 0.1 Hz delivered through a stimulating electrode placed in L4. Pairing-LTP was induced by switching to voltage-clamp, and holding the neurons to 0 mV and stimulating at 1 Hz (200 pulses).

For field potential recordings, animals were euthanized by decapitation under deep isoflurane anesthesia, and the brains were transferred to the ice-cold dissection buffer (in mM: 212.7 sucrose, 2.6 KCl, 1.23 NaH2PO4, 26 NaHCO3, 10 dextrose, 3 MgCl2, and 1 CaCl2) saturated with a mixture of 5% CO2 and 95% O2 (pH ~7.4). A block of primary visual cortex was removed and sectioned into 400-μm-thick slices by using a vibratome. Visual cortex slices were recovered for 1 h at room temperature in ACSF (in mM): 124 NaCl, 5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 10 dextrose, 1.5 MgCl2, and 2.5 CaCl2 (saturated with a mixture of 5% CO2 and 95% O2) and transferred to a submersion-type recording chamber perfused with ACSF (29.5–30.5 °C; 2 mL/min). All field potentials were recorded as described (9).

Synaptic responses were evoked through a double-barreled glass electrode filled with ACSF placed in L4, and recorded through a glass electrode filled with ACSF placed in L2/3. Baseline stimulation was set at the intensity that yielded 1/2–2/3 of the max FP amplitude and delivered at 0.033 Hz. There was no significant difference in the basal FP amplitude across groups [WT: 0.56 ± 0.042 mV, n = 20; HET: 0.64 ± 0.081 mV, n = 25; KO: 0.53 ± 0.038 mV, n = 22; ANOVA: F(2, 64) = 0.981, P = 0.3806]. LTP was induced by delivering three trains (intertrain interval of 10 s) of theta-burst stimulation (TBS; four pulses of 100-Hz bursts repeated 10 times at 5 Hz), and LTD was induced by using a train of 1-Hz stimulation (900 pulses; ref. 10). Only the slices showing a stable baseline (~10% drift over a 20-min baseline period) were used for analysis. Field potential amplitude was normalized to the average value of the 20-min baseline and expressed as percentage of baseline ± SE of mean (SEM). One-way ANOVA and t test were used, respectively, for statistical comparison of LTP and LTD magnitude between the groups, and P < 0.05 was considered statistically significantly different. Experiments and analysis were done blind to genotype.

In Vivo Electrophysiology. VEP recordings were performed in awake, head-restrained mice by using chronically implanted microelectrodes (11). Recording electrodes were implanted into L4 of the right hemisphere between P22 and P25. Animals were anesthetized and placed in a stereotactic frame. A midline incision was made in the scalp, after which skin and connective tissue were retracted. A small burr hole was made in the skull overlying binocular visual cortex (3 mm lateral of lambda) through which a tungsten microelectrode (FHC) was lowered 450 μm below the cortical surface, corresponding to L4. A plug of cranial cement held the electrode and head mount in place. The skin was treated with topical antibiotics and sutured closed. Animals were maintained at ~37.5 °C throughout the procedure, and recovery, general condition, and reflex signs were monitored. For recording, animals were secured to a stereotactic frame using the head mount. Animals were gradually acclimated to the restraint system before testing (~2 d). After habituation, baseline VEPs were obtained in response to stimulation of each eye separately. Recording sessions from 1 to 6 h, once per day, were carried out over periods ranging from one to several days. Signals were amplified (WPI DAM80) and digitized with a 16-bit AD board (National Instruments) by using custom software. All subjects were eventually killed for histological or biochemical analysis. VEP recordings were performed by using custom software in MATLAB using Psychosys Toolbox (psychtoolbox.org). VEPs were elicited by using full-field, sine-wave gratings (measured at 0.01–1 cycle per degree; 100% contrast) with fixed temporal frequency (1 Hz). VEPs were analyzed offline by using MATLAB. Maximal screen luminance was 300 cd/m2 with a contrast ratio of 2,000:1. Luminance changes were captured in
a small part of the screen with a photodiode (Thorlabs) to ensure the equal illumination between animals. For MD experiments, eyes were reopened and tested by using stimuli oriented perpendicular to the baseline stimuli, which eliminates the contaminating influence of stimulus-selective response potentiation on our measurements of long-term plasticity (11, 12). We computed the OD ratio as contra/ipsi VEP amplitude ratio and VEP change as the difference in VEP amplitude before and after deprivation. VEP amplitudes and amount of OD shift were compared between genotypes at 0.05 cycles per degree (11, 12). One-way ANOVA and paired t test were used, respectively, for statistical comparisons between the genotypes, and P < 0.05 was considered statistically significantly different. Experiments and analysis were done blind to genotype.

**OD Plasticity Experiments Using Arc.** Animals were deeply anesthetized with 4% isoflurane (Halocarbon), and monocular enucleation (ME) was performed at P25 under aseptic conditions as described (4, 5, 13, 14). ME at the ages and for the periods of time used here (~10 d) does not change the spatial pattern of LON activation but results in robust OD shifts in visual cortex (4, 5, 13, 14). Arc expression measures provide a sensitive measurement of OD changes in all layers of visual cortex in mice and cat after MD or ME (4, 5, 13, 14). ME is well suited for Arc induction experiments, as it yields a robust intraocular activity imbalance in the induction phase compared with MD where light can penetrate the closed eyelid and increase activity in connected circuits. Thus, ME enhances the signal-to-noise ratio compared with MD. Sections in which Arc signal was weak were excluded from the analysis. Experiments and analysis were done blind to genotype.

**Real-Time qPCR Analyses.** Real-time quantitative PCR (qPCR) was performed as described (3, 5). Mice were euthanized, and brains were removed. V1 and V2 were microdissected from coronal 2-mm-thick brain slices cut on an acrylic matrix (Ted Pella) and frozen immediately on dry ice. Total RNA was isolated by using TRIzol (Gibco BRL). cDNA was synthesized by using the iScript cDNA Synthesis Kit (BioRad Laboratories). A reaction mix contained 1× iQ SYBR Green Supermix (Bio-Rad Laboratories), 100 nM each oligonucleotide primers and 10 ng of cDNA in a 25-μL total volume. The relative amount of mRNA was normalized to the level of internal control message, hypoxanthine phosphoribosyltransferase (HPRT).

HPRT (nm_013556): Fwd: TGCTCGAGATGTCACTAGAAGG, Rev: TATGTCCCCGTGTGACTGT.

GABAa1 (m86566): Fwd: CCGTTCAGTTGGTTGAGCA, Rev: CTCGTTAGGCAAGAAGGACG.

GABAa2 (m86576): Fwd: TCACTCGAAGCGCAATGGT, Rev: AACGGAGCTCAAGACGTTAAGG.

GABaa3 (nm_800867): Fwd: GCTCCAGTGGCTCTTGC, Rev: TGATAGCTGATTCCGGGTTC.

KCC2 (nm_020333): Fwd: CACAGCATTTCCATGAGTG, Rev: CTCTGTTGAGCCAGAAGGAGAC.

Nr2b (nm_008170): Fwd: TGCTACAAACCCACAGGAA, Rev: CTCTTCAGAAGTAAAGCTG.

Real-time PCR data analysis was performed according to the comparative threshold cycle (Ct) method. Differences in threshold crossing cycle between mRNA of target gene and HPRT (=dgene) were calculated for each condition. Levels of mRNA expression were computed as 2−P(dgene). mRNA levels (or ratios) in Cx36KO were normalized to average level (or ratio) in control.

**In Situ Hybridizations.** Animals were killed with an overdose of sodium pentobarbital (200 mg/kg IP, to effect), and brains were removed and frozen in cryoprotective medium (M1; Shandon). Horizontal sections were cut (12-15 μm) on a cryostat. In situ hybridizations were performed as described (2–5). Template sequences (~700 bp long) were generated by RT-PCR from a mixture of P0, P7, P14, and P30 total RNA by using primers that were obtained from the GenBank sequences for Cx36 and Arc. 5′-labeled riboprobes were generated by in vitro transcription. After hybridization, sections were processed, dipped with autoradiographic emulsion (Kodak NTB-2), and exposed for 3–6 wk. Darkfield images were acquired with a CCD camera (Spot). The borders between layers were chosen according to adjacent cresyl violet-stained sections. Sense probes yielded only background levels of signal.

**Densitometric Scans of Arc Induction in Specific Cortical Layers.** Quantitative analysis of Arc expression was performed in MATLAB (MathWorks) by line scans in L4 as described (4, 5). Five to 10 sections from each condition were scanned. The analyses were performed blind to genotype and manipulation; slides from different animals and manipulations were interleaved with each other and only reassembled once they were decoded. For each section, a line along the center of the chosen layer was generated by selecting 20–100 points and then performing a cubic spline interpolation between these points. At every pixel along this line, a perpendicular line through the layer (30–60 pixels long; 1 pixel = 1.75 μm) was computed and the average signal intensity of pixels along this line was measured. The resulting intensity line scan was low pass filtered (7 pt triangular), generating a curve of Arc signal intensity versus distance along the layer of interest. Arc signal rose from a minimum in both V1 and V2 to a maximum within the binocular zone (BZ). The width of the BZ was measured as the region around the intensity maximum in which signal intensity is greater than 2 SDs of the Arc background signal intensity (determined as average intensity of 30 pixels in the region of minimum Arc induction outside the BZ; this method would if anything underestimate BZ width). The area of V1 and V2 in which Arc induction is at a minimum is defined as the monocular zone. To quantify the strength of Arc induction, the average signal intensity above background within the BZ was computed; the normalized intensity = total signal/width of BZ, where normalized intensity is interpreted as a measure of strength of eye input.

**Statistics.** ANOVA, t test, or Wilcoxon was used for statistical comparisons between the genotypes, and P < 0.05 was considered statistically significantly different.

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**Fig. S1.** Quantitative RT-PCR for GABA$_A$ receptor subunits and KCC2 in visual cortex. Normalized mRNA levels were similar between genotypes ($n = 9$, control; $n = 5$, Cx36KO; $\alpha_1$: 1.0 ± 0.02 vs. 0.99 ± 0.02, $P > 0.1$; $\alpha_2$: 1 ± 0.017 vs. 0.99 ± 0.014, $P > 0.1$; $\alpha_3$: 1 ± 0.02 vs. 1.0 ± 0.01, $P > 0.1$; $\gamma_2$: 1.0 ± 0.015 vs. 1.01 ± 0.012, $P > 0.1$; KCC2: 1.0 ± 0.023 vs. 1.016 ± 0.014, $P > 0.1$).

**Fig. S2.** Cx36 increases the amplitude of cortical IPSCs. (A) eIPSC before and after application of 200 $\mu$M CBX. Left traces show absolute amplitude, and right traces are scaled to match the peak amplitude. (B) Box plots (line indicates median) show the effect of CBX on peak amplitude in ctrl and Cx36KO. Outliers are indicated in green. CBX application reduced the amplitude in both ctrl and Cx36KO but the decrease was larger in ctrl ($−73 ± 7.9\%$ and $−48 ± 17.9\%; n = 6$ and $10; P < 0.005$). Application of CBX reduced the amplitude of the eIPSCs both in control and KO animals, suggesting effects of CBX on inhibitory synaptic transmission independent of Cx36. However, the larger magnitude in ctrl suggests that Cx36 containing gap junctions contribute to a larger amplitude of evoked IPSCs.
Fig. S3. Impaired expansion of the NDE in Cx36KO mice. The representation of the ipsilateral eye in L4 of the BZ of control and Cx36KO mice was compared at P25 by measuring visually evoked Arc mRNA induction. (A and B) Dark-field images of in situ hybridizations for Arc showing representation of the ipsilateral eye (white silver grains indicate Arc mRNA). (A) Unmanipulated animals at P25. Arc induction is confined to the binocular zone (BZ) of the visual cortex (arrowheads) in both Cx36KO (n = 10) and control (n = 11). (B) Six days after monocular enucleation (ME) the ipsilateral representation expands in the control (n = 9) but not in the Cx36KO (n = 16). In a fraction of sections from Cx36KO after ME no or only very weak ipsilateral patches were detected (bottom). (C) Average Arc expression profiles for the four animal populations (animal averages aligned to lateral border of ipsilateral patch). Note that the NDE expansion and peak Arc expression levels in the Cx36KO are reduced. (D) Plot shows the cumulative distribution of the average width of the ipsilateral representation for all animals. Width is similar in control (black) and Cx36KO (red) (solid lines) (1,070 ± 100.2 μm vs. 1,129 ± 68 μm; P > 0.1) and also similar to previous measurements in other genotypes (1, 2). This result suggests that initial segregation and refinement of the BZ (2) does not depend on Cx36. Mean width increases after ME (dashed lines) in control (2,217.6 ± 331 μm; P < 0.001) and Cx36KO (1,389 ± 408 μm; P < 0.05), but increases were much larger in control (P < 0.01). (E) Plotted is the distribution of signal intensities for each animal. The integrated signal intensity in L4 was similar in control (black) and Cx36KO (red) (solid lines) (9,758 ± 2,077 vs. 9,367 ± 2,750; P > 0.1), suggesting that Arc induction is not impaired by removal of Cx36. After ME integrated signal intensity in control increased (black dashed line, 17,813 ± 7,790; P < 0.05; Wilcoxon). In contrast, integrated signal intensity in Cx36KO decreased after ME (red dashed line, 6,748 ± 2,601; P < 0.05; Wilcoxon), suggesting lower NDE driven activity in visual cortex.

Fig. S4. AM251 rescues NDE strengthening in Cx36KO mice. (A) Increasing GABA release by CB1 receptor blockade with AM251 does not affect VEP amplitudes or OD in heterozygous control (n = 7) or Cx36KO (n = 10) animals (P > 0.05). Shown are VEP amplitudes to stimulation of the contralateral (Left) and ipsilateral (Right) eye and the OD shift (Lower) after 6 d of daily AM251 injection. Plotted are means ± SD. Blue lines indicate 0.05 cycles per degree. (B) AM251 injections during monocular deprivation (MD) do not affect OD plasticity in heterozygous control (n = 8) but restore OD plasticity in Cx36KO (n = 10). Graphs show VEP amplitudes to stimulation of the contralateral and ipsilateral eye after 5 d of MD. Blue lines indicate 0.05 cycles per degree. (C and D) Mean difference in VEP at 0.05 cycles per degree after 6 d of AM251 (C) or after 6 d of AM251+ MD (D). (C) After 6 d of AM251, no change is observed in ipsi or contra eye responses (P > 0.05). (D) After 6 d of AM251+ MD inputs from the NDE strengthened both in heterozygous control and Cx36KO (*P < 0.05). Six out of 8 control and 8 out of 10 Cx36KO animals did show significant DE weakening at 0.05 cycles per degree (P < 0.02 and P < 0.05). No differences between genotypes were observed (P > 0.1).