Brief Communications

# Brief Dark Exposure Reduces Tonic Inhibition in Visual Cortex

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**Tonic inhibition mediated by extrasynaptic $GABA_A$ receptors (GABARs) sensing ambient levels of GABA can profoundly alter the membrane input resistance to affect cellular excitability. Therefore, regulation of tonic inhibition is an attractive mechanism to control the levels of cortical firing. In cortical pyramidal cells, tonic inhibition is regulated by age and several neurotransmitters and is affected by stroke and epilepsy. However, the possible role of sensory experience has not been examined. Here, we report that a brief 2-day exposure to dark reduces by 1/3 the inhibitory tonic conductance recorded in layer II/III pyramidal cells of the mouse juvenile (postnatal day 12–27) visual cortex. In these cells, tonic inhibition is carried primarily by GABARs containing the δ subunit. Consistently, the dark exposure reduction in conductance was associated with a reduction in δ subunit levels, which were not affected in control frontal cortex. We propose that a deprivation-induced reduction in tonic inhibition might serve a homeostatic function by increasing the firing levels of cells in deprived cortical circuits.**

*Key words:* extrasynaptic receptors; δ-$GABA_A$ receptor; layer 2/3; pyramidal cells

**Significance Statement**

**Previous *in vivo* studies reported rapid increases in spontaneous activity after visual deprivation. These adaptive responses to deprivation are believed to reflect a reduction in the recruitment of inhibitory circuits. Notably, the possible role of tonic GABAergic inhibition, which strongly limits cellular and network excitability, has not been examined. We report that a brief 2-day exposure to dark reduces both the conductance of tonic inhibition in layer 2/3 pyramidal cells and the expression of receptors containing the δ-$GABA_A$ receptor subunit, the principal carrier of tonic inhibition in these cells. These results suggest that the early phases of homeostatic adaptations to sensory deprivation might result from modulation of GABAergic function at multiple levels.**

## Introduction

In multiple systems, particularly in cortex, GABAergic neurotransmission plays a crucial role in controlling the temporal and spatial spread of neural activity (Isaacson and Scanziani, 2011). This function is primarily subserved by ionotropic $GABA_A$ receptors (GABARs), which can be differentiated into two complementary functional types: (1) GABARs that accumulate at synaptic sites and mediate phasic inhibition (also termed fast inhibition), which is rapid enough to influence the temporal firing pattern of cortical cells; and (2) GABARs that distribute perisynaptically and extrasynaptically that have a high affinity for GABA, which allows them to sense changes in ambient GABA levels and to mediate the slower process of tonic inhibition (Farrant and Nusser, 2005). Activation of these extrasynaptic GABARs increases the membrane conductance constraining cellular excitability (Lee and Maguire, 2014), dendritic integration (Groen et al., 2014), and the induction of synaptic plasticity (Smith, 2013; Groen et al., 2014).

Because of their role in cortical excitability, GABAergic circuits are well positioned to mediate adaptive homeostatic responses to changes in excitatory drive. In visual cortex, for example, reducing excitatory drive by dark rearing can arrest the normal postnatal maturation of GABAergic circuits, which includes an increase in synapse number (Morales et al., 2002; Chattopadhyaya et al., 2004) and changes in their release properties (Jiang et al., 2010). Similar to fast GABAergic transmission, the magnitude of tonic inhibition also exhibits a postnatal increase

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(Jang et al., 2010) that is strongly modulated by a variety of G-protein receptors and is severely altered in several pathological conditions, including epilepsy (Roberts et al., 2005; Ferando and Mody, 2012; Grabenstatter et al., 2012) stroke (Hines et al., 2012), and injury (Imbrosci et al., 2013). Notably, however, the possible role of sensory experience in tonic inhibition remains largely unknown.

An additional motivation to address whether sensory experience regulates tonic inhibition is the observation that brief visual deprivation triggers in visual cortex an increase in spontaneous activity and network excitability that compensates for the loss of sensory drive (Maffei and Turrigiano, 2008; Kuhlman et al., 2013). These rapid deprivation-induced increases in cortical excitability would be consistent with a concomitant reduction in tonic inhibition. Moreover, those excitability changes do not associate with reductions in phasic inhibition. Indeed, some forms of visual deprivation induce an increase, rather than a decrease, in the postsynaptic strength of fast GABAergic transmission (Morales et al., 2002; Maffei et al., 2006). Prompted by these considerations, we examined the effects of a brief dark exposure (2 days) on tonic inhibition measured in layer II/III pyramidal cells of the mouse visual cortex and on the levels of δ subunits that assemble into GABARs, which mediate tonic inhibition in these cells. We found that the brief deprivation epoch reduced both the conductance of tonic inhibition and the levels of δ subunit protein. These findings suggest that homeostatic regulation of tonic inhibition via a change in δ-containing GABARs (δ-GABARs) may explain the contribution of adaptive cortical responses to changes in input drive.

## Materials and Methods

*Visual cortical slices.* Twenty-one- to 27-d-old C57BL/6 mice of either sex reared in normal light/dark 12 h cycles or in the dark for 2 d with care provided under infrared illumination were used in these studies. All procedures were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. Visual cortical slices (300 μm) were cut as described previously (Huang et al., 2012, 2013; Gu et al., 2013) in ice-cold dissection buffer containing the following (in mM): 212.7 sucrose, 5 KCl, 1.25 $NaH_2PO_4$, 10 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose, bubbled with 95% $O_2$/5% $CO_2$, pH 7.4. Slices were transferred to normal artificial CSF (ACSF) for at least 1 h before recording. Normal ACSF was similar to the dissection buffer except that sucrose was replaced by 124 mM NaCl, $MgCl_2$ was lowered to 1 mM, and $CaCl_2$ was raised to 2 mM.

*Visualized whole-cell voltage-clamp recordings.* Visualized whole-cell voltage-clamp recordings were made from layer II/III regular spiking pyramidal cells with glass pipettes (4–6 M) filled with intracellular solution containing the following (in mM): 120 CsCl, 8 NaCl, 10 HEPES, 2 EGTA, 5 QX-314, 0.5 $Na_2GTP$, 4 MgATP, and 10 $Na_2$-phosphocreatine, pH adjusted to 7.25 with KOH, 280–290 mOsm. Membrane currents were recorded at −60 mV in the presence of 20 μM CNQX and 100 μM APV. Only cells with series resistance <20 MΩ (8–18 M) and input resistance >100 MΩ were studied. Membrane properties ($R_{input}$ and capacitance) and series resistance were monitored with 100 ms negative voltage commands (−4 mV) delivered every 1 min. Cells were excluded if series resistance changed >15% over the experiment. Data were filtered at 2 kHz and digitized at 10 kHz using Igor Pro software (WaveMetrics). All drugs except compound 6 (a gift from Dr. M. Gallagher, Johns Hopkins University, Baltimore, MD) were purchased from Sigma-Aldrich or R&D Systems. L655708 and Compound 6 were dissolved in DMSO then diluted 1:1000 in ACSF. THIP and bicuculline (BMI) were dissolved in water.

*Measurement of spontaneous IPSCs (sIPSCs).* The rate and amplitude of the sIPSCs were computed using the Mini Analysis Program (Synaptosoft) as described previously (Morales et al., 2002). For event discrimination, we used a threshold of 3× the RMS noise. At least 100 events were used in these calculations.

*Measurement of tonic inhibition.* Tonic currents were determined as the shift of baseline holding currents after the addition of the GABAR antagonist BMI (20 μM) to the ACSF containing 5 μM GABA. First, all-points histograms (bin width 2.5 pA) were generated for the control and bicuculline epochs (30 s). Then, the histograms were fitted with a Gaussian model and nly the left side of the distribution was used to avoid contamination with spontaneous events (Bright and Smart, 2013). The shift in the peaks of the Gaussian fits was used as the value for the tonic current (see Fig. 1*A*). To confirm that the left-side Gaussian fit approach was not affected by sIPSCs, in a random subset of 20 cells (with an sIPSC frequency of 7.4 ± 0.6 Hz), we obtained the holding currents by averaging 5 ms windows every 100 ms and removing manually the selections that happen to fall on the IPSC decay. No significant difference was found between the tonic currents calculated using the Gaussian fit and the sampling methods (Gaussian: 41.19 ± 3.67 pA; sampling: 41.16 ± 3.68; paired *t* test: $p = 0.858$). In these cells, we also examined the correlation between sIPSC frequency and tonic inhibition and found it negligible ($r^2 = 0.054$), further indicating that the sIPSCs did not compromise the measurement of tonic inhibition. To account for cell-to-cell size variability, the tonic currents were normalized to membrane capacitance ($C_m$), which was calculated from the integral of the transients of the test pulses. The $C_m$ in the cells from normal reared (NR) and dark-exposed mice (DE) was not different (NR = 93.6 ± 0.5 pF, $n = 21$ cells; DE = 105.1 ± 0.3 pF, $n = 15$; *t* test: $p = 0.25$). A Pearson analysis indicated that, in both cases (NR and DE), the magnitude of the tonic currents was independent of the $C_m$ values (NR: $r^2 = 0.039$, $p = 0.450$, 21 pairs; DE: $r^2 = 0.027$, $p = 0.558$, 15 pairs). Therefore, we can rule out that the small, nonsignificant increase of $C_m$ after DE compromised the computation of normalized tonic currents. A similar general procedure was used to measure the shifts of baseline holding currents in the application of THIP, L655708, and Compound 6. This analysis was done using a custom-made function in MATLAB R2014a (The MathWorks).

*Immunoblot analysis.* Western blot was performed using traditional methods in the Russek laboratory (Hu et al., 2008). Protein (30 μg) extracted from frontal and visual cortices was loaded onto 10% Tris-glycine gels and run for 1.5–2 h at 115 V. Blots were transferred to a nitrocellulose membrane and blocked in 5% milk/TBS with Tween 20 (TBS-T). Membranes were incubated in primary antibodies for GABARα1 (Millipore, catalog #06–868, 1:1000 in 5% milk/TBS-T), GABARδ (Santa Cruz Biotechnology, catalog #sc31438, 1:200 in 5% milk/TBS-T), and GABARγ2 (Novus Biologicals, catalog #NB 300–190, 1:500 in 5% milk/TBS-T) overnight at 4°C. Membranes were washed and incubated in either goat anti-rabbit (for GABARα1 and GABARγ2) or donkey anti-goat HRP for GABARδ. All secondary antibodies were from Santa Cruz Biotechnology (1:2000 in 5% milk/TBS-T for 1 h). Protein bands were detected using chemiluminescence with ECL Western Blotting Detection Reagent (GE Healthcare). Protein was analyzed based on band size (GABARα1 and GABARδ, ~50–51 kDa and GABARγ2 ~46 kDa). For β-actin detection, membranes were incubated in anti-β-actin (Sigma-Aldrich, catalog #A5441, 1:5000) in TBS-T for 45 min, washed, and incubated in horse anti-mouse HRP secondary (Vector Laboratories, 1:2000) in TBS-T for 45 min. All protein bands were normalized to β-actin in the same samples and expressed as percentage change over control animals. Densitometry was performed using ImageQuant software (GE Healthcare).

*Statistical analysis.* All data are presented as mean ± SEM. Significance was considered to be $p < 0.05$, as determined by unpaired two-tailed *t* tests on the dataset that passed the D'Agostino and Pearson omnibus normality test.

## Results

In these studies, we tested the effects of a brief period of dark exposure (2 d) on the magnitude of tonic inhibition in layer II/III pyramidal cells in the visual cortex of P21–P27 BL6 mice. Tonic inhibition is often quantified as the holding current that is suppressed by applied $GABA_A$ antagonists. The magnitude of these currents, however, depends, not only on the tonic conductance, but also on the concentration of external GABA, which in turn is determined

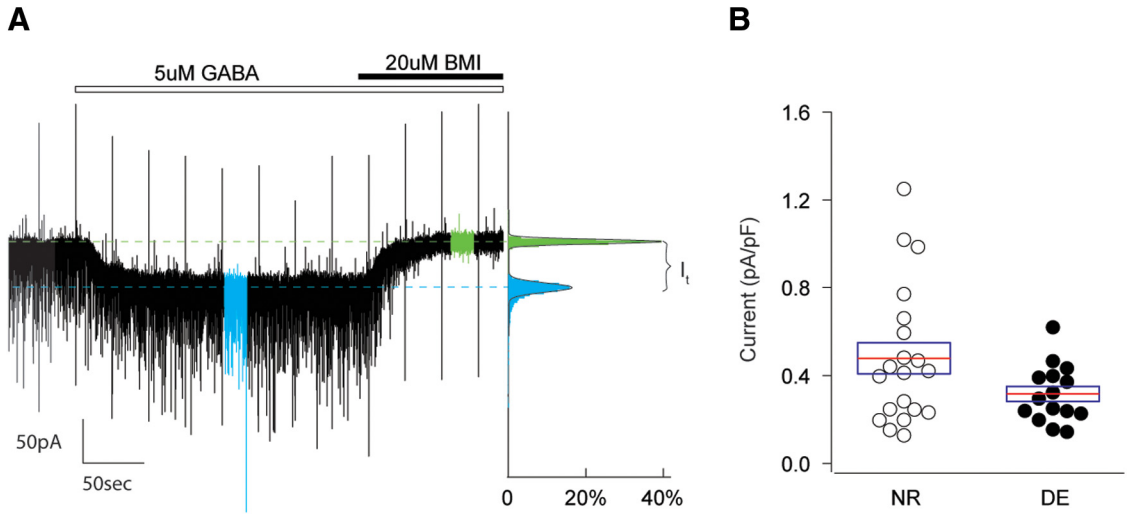


**Figure 1.** Brief dark exposure (2 d) reduces tonic inhibition in layer II/III pyramidal cells of the visual cortex. ***A***, Example of tonic synaptic response ($I_t$) evoked by bath-applied GABA (5 $\mu$M) and eliminated by the antagonist BMI (20 $\mu$M). Tonic inhibitory currents ($I_t$) were computed by taking the difference in the median holding current (dotted lines) recorded during GABA (blue) and during BMI (green) and normalizing them to cell capacitance. All-points histograms of the highlighted windows of the current are shown to the right, along with Gaussian fits. ***B***, Dark exposure (2 d) reduces the magnitude of tonic inhibition. Averages are represented by the horizontal red lines; boxes indicate mean ± SEM.

by the balance of extrusion and supply processes. Because these last two processes are difficult to assess and/or control in slices, we focused on the changes in conductance.

Tonic inhibition was quantified as the postsynaptic current induced by bath application of 5 $\mu$M GABA, a concentration at which GABA maximally activates the extrasynaptic receptors responsible for tonic inhibition, but without affecting synaptic receptors involved in fast GABAergic transmission (Jang et al., 2010). Therefore, the difference in the holding current recorded first in the presence of GABA and then after adding the $GABA_A$ blocker BMI (20 $\mu$M) provided a direct estimate of the total tonic conductance available in a given cell (Fig. 1*A*). Moreover, we confirmed in a subset of 9 cells that, at this age (<5 weeks), ambient GABA is insufficient to significantly activate tonic current (Jang et al., 2010): the difference in holding current between before adding GABA and after BMI, as shown in Figure 1*A*, was minimal (3.1 ± 1.6 pA) compared with the current determined in 5 $\mu$M GABA (51.2 ± 5.2 pA). To compare results from cells of different sizes, we normalized the tonic current values by the cell capacitance, a direct measure of the cell surface (see Materials and Methods). The results, shown in Figure 1*B*, indicate a significant reduction in the normalized magnitude of tonic inhibition measured in layer II/III pyramidal cells of the 2 d DE mice group compared with the NR group (NR: 0.47 ± 0.07 pA/pF, $n = 5$ mice, 20 cells; DE: 0.31 ± 0.03 pA/pF, $n = 4,15$; $p = 0.047$). The significant decrease in tonic inhibition is in marked contrast to the recently reported absence of changes in the amplitude and frequency of miniature IPSCs after 2 d of dark (Gao et al., 2014). Therefore, we confirmed, in a subset of cells not exposed to the drugs, that the sIPSCs (a group that includes miniature IPSCs) were not affected by the brief dark exposure in either their amplitude (NR: 38.15 ± 2.3 pA, $n = 10$; DE: 38.13 ± 2.14 pA, $n = 7$; $p = 0.989$) or their frequency (NR: 6.03 ± 0.61 Hz; DE: 6.38 ± 0.70; $p = 0.706$). Altogether, the results in layer II/III pyramidal cells indicate that a brief event of visual deprivation in the form of 2 d dark exposure decreases tonic currents without affecting fast GABAergic transmission.

Tonic inhibition is mediated by extrasynaptic GABARs containing primarily either the δ or the α5 subunit (Lee and Maguire, 2014). In neocortex, α5-containing GABARs are the principal components of tonic inhibition in layer V pyramidal cells and δ-containing receptors seemingly predominate in layer II/III (Drasbek and Jensen, 2006; Jang et al., 2013). However, both types of receptors have been reported functional in layer V in some cortical regions (Yamada et al., 2007). Therefore, we set out to use pharmacology to identify the role of both subunits in layer II/III pyramidal cells in the visual cortex. First, we tested the involvement of the δ subunit by applying the specific agonist THIP because specific antagonists that block δ-containing receptors are not yet available. As shown in Figure 2, the application of THIP caused a dose-dependent increase in the holding current that was fully eliminated by a subsequent addition of BMI (Fig. 2*B*; 1 $\mu$M: 0.34 ± 0.02 pA/pF, $n = 8$; 5 $\mu$M: 1.64 ± 0.09 pA/pF, $n = 6$; 10 $\mu$M: 1.75 ± 0.25 pA/pF, $n = 4$; 20 $\mu$M: 1.89 ± 0.24 pA/pF, $n = 6$), indicating that δ-GABARs can support tonic inhibition in layer II/III pyramidal cells. In contrast, ligands specific for the receptors containing the α5 subunit were ineffective. Bath application of reverse agonist L655708 (10 $\mu$M) did not affect the holding current recorded that was evoked by GABA (5 $\mu$M) (ΔI: 0.06 ± 0.07 pA/pF, $n = 4$), yet a subsequent application of BMI did suppress the holding current (ΔI: 0.31 ± 0.04 pA/pF, $n = 4$). Similarly, the recently developed α5-specific agonist Compound 6 (van Niel et al., 2005; Koh et al., 2013) applied at 5 $\mu$M had no effect on the holding current (Δ*I*: −0.005 ± 0.03 pA/pF, $n = 6$). These data confirm the idea that δ-containing GABA receptors, but not those with α5 subunits, contribute to the tonic inhibition in layer II/III pyramidal cells of visual cortex.

The expression levels of the δ subunit can be modified by conditions that alter neural activity, such as epilepsy (Roberts et al., 2005; Ferando and Mody, 2012; Whissell et al., 2015). Therefore, we investigated whether brief visual deprivation reduces, not only tonic inhibition, but also the expression levels of the δ subunit. To that end, we used immunocytochemistry to quantify the protein levels of the δ subunit in 10 DE mice (P21–P22) and 9 age-matched NR control littermates. In these studies, we also determined the levels of α1 subunits, which primarily reflect fast synaptic GABAergic neurotransmission (Farrant and Nusser, 2005) due to their assembly with the γ2 subunit, and replaces the

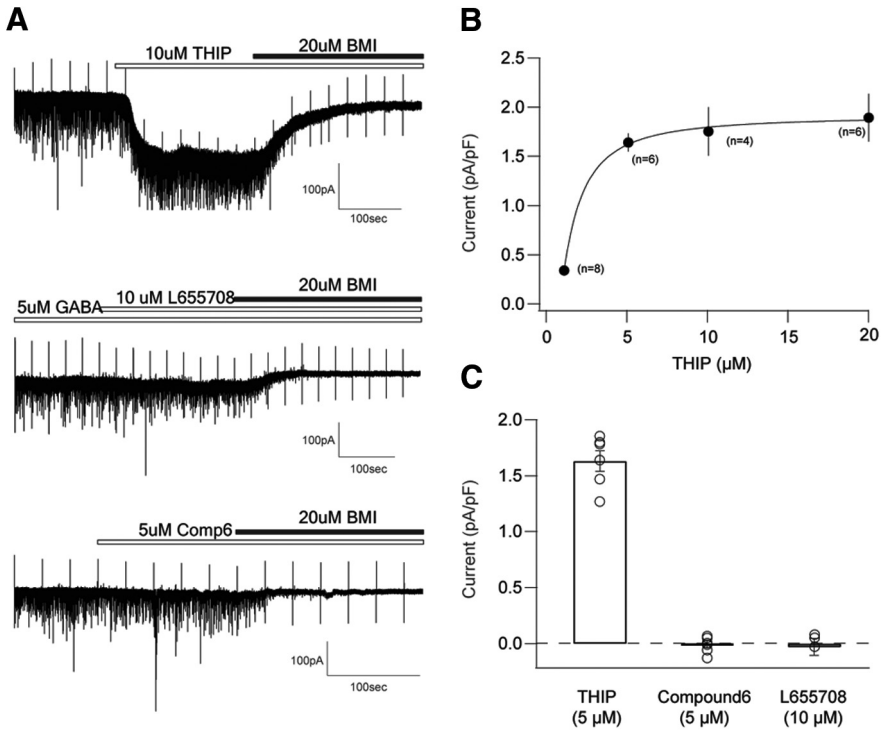


**Figure 2.** Tonic inhibition in layer II/III pyramidal cell is carried by δ-containing GABARs, but not by α5-containing receptors. ***A***, Representative experiment showing the effects of the δ-agonist TIHP (top trace), along with the lack of effect of both, the α5 inverse agonist L655708 (middle trace), and the α5 agonist Compound 6 (bottom trace). ***B***, Dose–response relationship for THIP-induced tonic current. ***C***, Summary of the effects of the specific drugs. Plotted is the amplitude induced by the agonists (δ:THIP; α5: Compound 6) and the current suppressed by the α5 inverse agonist L655708.

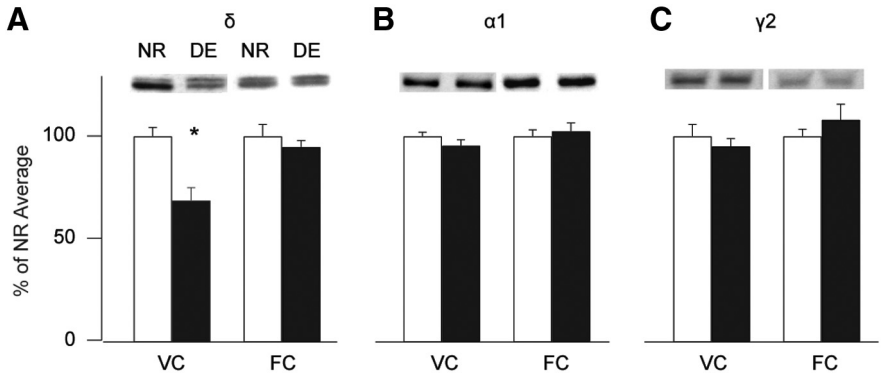


**Figure 3.** Brief dark exposure (2 d starting at P21–P22) selectively decreases δ subunit levels, but not γ2 or α1 subunit levels, in visual cortex. Shown are the results of Western blot analysis of δ (***A***), α1 (***B***), and γ2 (***C***) subunit expression levels as sampled in the visual cortex (VC) and frontal cortex (FC) of NR and DE mice. The bars indicate the average normalized intensity values for the three subunits in NR (open bars, $n = 9$ mice) and DE (filed bars, $n = 10$ mice) mice. *$p < 0.05$. Example blots are shown on top.

δ subunit in extrasynaptic receptors (Vithlani et al., 2011). In addition, we also measured those subunit levels in the frontal cortex, which typically is not affected by visual deprivation (Goel et al., 2006). The results, shown in Figure 3, indicate a clear down-regulation in the expression of δ subunit protein levels in the visual cortex (Fig. 3*A*; DE: 68.8 ± 6.2%, $n = 10$ mice; NR: 100.0 ± 4.2%, $n = 9$ mice), but not in the frontal cortex (DE: 94.8 ± 3.0%; NR: 100.0 ± 5.7%). A one-way ANOVA test ($F_{(3,34)} = 9.39$, $p = 0.0001$) followed by Sidaks' multiple-comparisons test confirmed the significance of these differences. Conversely, and consistent with previous studies reporting no changes in fast GABAergic neurotransmission after brief deprivation (Gao et al., 2014), the 2-day dark exposure did not affect the expression levels of the α1 subunit in either the visual cortex (Fig. 3*B*; DE: 102.4 ± 4.0%; NR: 100.0 ± 3.1%) or the frontal cortex (DE: 95.4 ± 2.8%; NR: 100.0 ± 1.9%; one-way ANOVA test: $F_{(3,34)} = 0.947$, $p = 0.429$). Similarly, the levels of the γ2 subunit were not affected in either the visual cortex (Fig. 3*C*; DE: 108.0 ± 7.5%; NR: 100.0 ± 3.4%) or the frontal cortex (DE: 95.24 ± 3.7%; NR: 100.0 ± 5.7%; one-way ANOVA test: $F_{(3,34)} = 1.014$, $p = 0.399$). Altogether, the results support the idea that a brief exposure to dark specifically reduces the δ subunit content of GABARs and only in the visual cortex.

## Discussion

Here, we show that two days of dark exposure substantially reduces the conductance of tonic inhibition in layer II/III pyramidal cells and also the expression of the δ subunit of GABARs. We also confirmed that δ-GABARs, and not those containing the α5 subunit, are the principal carriers of tonic inhibition in layer II/III pyramidal cells in visual cortex. Based on these findings, we propose that the number of extrasynaptic δ-GABARs is dynamically regulated by sensory experience and can contribute to rapid, experience-driven adaptive changes in cortical excitability.

Changes in tonic inhibition induced by a variety of stimuli and conditions, including estrus cycle, stress, and epilepsy (Whissell et al., 2015) are well established in multiple brain circuits. In layer II/III cortical pyramidal cells, previous studies reported that basal tonic inhibition increases after stroke or local injury (Hines et al., 2012; Imbrosci et al., 2013). This increase was associated with an increase in ambient GABA levels caused by a reduced uptake (Hines et al., 2012). In contrast, we report that in these cells sensory deprivation reduces the conductance of tonic inhibition, which was associated with reduced expression levels of the δ subunit. Similarly, in the principal cell of layer IV barrel cortex, tonic conductance carried by δ-GABARs is enhanced after prolonged associative training (Urban-Ciecko et al., 2010). Therefore, many seemingly different mechanisms can regulate tonic inhibition in the cortex. It seems plausible that these distinct mechanisms subserve different functions. The changes in the levels of ambient GABA may reflect a global neuroprotective mechanism, whereas the regulation of total conductance, through changes in channel number, might reflect local compensatory responses to changes in neural activity.

Experience-dependent changes in synaptic gain are well documented for glutamatergic excitatory neurotransmission in several sensory cortices. In pyramidal cells of the visual cortex, brief exposure to dark promotes the incorporation of AMPA receptors into the synapses (Goel et al., 2006). This deprivation-induced increase in excitatory strength is widely regarded as a homeostatic

adaptive mechanism crucial to maintain cortical excitability that will compensate for the loss of input activity. In contrast, although GABAergic synapses are modifiable by neural activity, rapid adaptive changes in strength after deprivation have not been reported for fast GABAergic neurotransmission. Dark exposure can arrest the postnatal increase in GABAergic synapses and the maturation of their release properties (Morales et al., 2002; Chattopadhyaya et al., 2004; Jiang et al., 2010); however, this process is very slow and the effects of dark exposure require at least a week to be reliably detected. Postsynaptic changes can be evoked in layer II/III, but only as a transient increase in $GABA_A$ channels at the synapse termed rebound potentiation, which occurs during light exposure after a prolonged deprivation in the form of 1 week of dark-rearing (Gao et al., 2014). Therefore, to the best of our knowledge, the changes in the expression and function of extrasynaptic δ-GABARs that we report here is the only GABAergic mechanism capable of mediating a rapid homeostatic response to visual deprivation. Such rapid homeostatic responses do occur in the visual cortex, in which spontaneous spiking increases within 1–2 d after visual deprivation and compensates for the initial fall in overall firing due to reduced drive (Hengen et al., 2013; Keck et al., 2013). This increase in spontaneous activity precedes, and possibly gates, the subsequent remodeling of excitatory connections between the pyramidal cells in many deprivation paradigms. The likely role of reduced tonic inhibition in pyramidal cells in the rapid increase in cortical excitability and its consequences for cortical plasticity remain to be determined.

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