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Developmental maturation of excitation and inhibition balance in principal neurons across four layers of somatosensory cortex

Zhi Zhang, Yuan-Yuan Jiao, and Qian-Quan Sun*

Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071

Abstract

In adult cortices, the ratio of excitatory and inhibitory conductances (E/I ratio) is presumably balanced across a wide range of stimulus conditions. However, it is unknown how the E/I ratio is postnatally regulated, when the strength of synapses are rapidly changing. Yet, understanding of such a process is critically important, because there are numerous neuropsychological disorders, such as autism, epilepsy and schizophrenia, are associated with disturbed E/I balances. Here we directly measured the E/I ratio underlying locally induced synaptic conductances in principal neurons from postnatal day 8 through 60. We found that 1) within each developmental period, the E/I ratio across 4 major cortical layers was maintained at a similar value under wide range of stimulation intensities; and 2) there was a rapid developmental decrease in the E/I ratio, which occurred within a sensitive period between P8 to P18 with exception of layer II/III. By comparing the excitatory and inhibitory conductances, as well as key synaptic protein expressions, we found a net increase in the number and strength of inhibitory, but not excitatory synapses, is responsible for the developmental decrease in the E/I ratio in the barrel cortex. The inhibitory markers were intrinsically co-regulated, gave rise to a sharp increase in the inhibitory conductance from P8 to P18. These results suggest that the tightly regulated E/I ratios in adults cortex is a result of drastic changes in relative weight of inhibitory but not excitatory synapses during critical period, and the local inhibitory structural changes are the underpinning of altered E/I ratio across postnatal development.

Keywords

E/I balance; synaptic conductance; postnatal development; neocortex; critical period

Introduction

Both theoretical modeling and direct electrophysiological recordings reveal that the neocortical network activity *in vivo* is generated through a dynamic balance of excitation and inhibition (Borg-Graham et al., 1998; Salinas and Sejnowski, 2000; Wang, 2001; Haider et al., 2006). The excitation–inhibition ratio (E/I ratio) of synaptic inputs in adult cortical neuron is dynamically regulated to avoid runaway excitation or quiescence in response to alterations in input strength (Wehr and Zador, 2003; Wilent and Contreras, 2004; Wehr and Zador, 2005; Pouille et al., 2009). Balance between feedback or feedforward inhibition and recurrent excitation can give rise to balanced network amplification (Carvalho and

*neuron@uwyo.edu, telephone 307 766 5602, fax 307 766 5526.

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Buonomano, 2009; Murphy and Miller, 2009) or stability (Marino et al., 2005). In sensory cortices, such a balance serves to increase temporal precision and reduce the randomness of cortical operation (Monier et al., 2003; Wehr and Zador, 2003; Cruikshank et al., 2007; Pouille et al., 2009), but see Sun et al., (Sun et al., 2010)

The establishment of E/I balance in adult cortical neurons may result from developmental and experience-dependent co-regulation of developing glutamatergic and GABAergic synapses (Desai et al., 2002; Turrigiano and Nelson, 2004; Liu et al., 2007). Earlier in development, there are dramatic changes in the number, distribution and subtype of GABA_A receptors (Luhmann and Prince, 1991; Chattopadhyaya et al., 2004; Jiang et al., 2010) and glutamate receptors (Mierau et al., 2004; Bannister et al., 2005; Brill and Huguenard, 2008; Oswald and Reyes, 2008), which produce profound alterations in synaptic transmission. However, the experimental E/I ratio data in principal neurons from different cortical layers during major postnatal critical periods are absent. Therefore, little is known how the E/I ratio is dynamically adjusted through the postnatal critical periods, when the strength of excitatory and inhibitory synapses are rapidly changing. Yet, understanding of such a process is critically important, because abnormal regulation of the E/I balance has been involved in a number of nervous system disorders [reviewed by (Eichler and Meier, 2008)], such as epilepsy (Gale, 1992; Bradford, 1995; Thompson et al., 1996; Olsen and Avoli, 1997; Cobos et al., 2005), schizophrenia (Lewis et al., 2003; Kehrer et al., 2008), autism (Jamain et al., 2003; Laumonnier et al., 2004) and acoustic trauma (Scholl and Wehr, 2008). Using thalamocortical (TC) brain slices, we directly measured the E/I ratio in principal neurons from four major layers (II/III, IV, V) of barrel cortex at four critical postnatal ages (P8, P18, P30 and P60). Our results indicate that there is a significant developmental decrease in the E/I ratio, which occurred rapidly within a critical period between P8 and P18. Furthermore, we found that the decrease in E/I ratio is due to a significant increase of inhibitory, but not excitatory conductances. In addition, we found that the balance of E/I synapses remained highly constant over a range of stimulus conditions across all major layers of barrel cortex at given developmental stages.

Materials and methods

Mice and slice preparation

The use of animals was based on protocols approved by the Institutional Animal Use Committee of the University of Wyoming. We used GAD67-GFP mice in order to distinguish GABAergic cells from non-GABAergic cells (Tamamaki et al., 2003; Jiao et al., 2006). Mice were divided into the following four age groups: P8, P18, P30 and P60; 8–10 mice for each age group. Mice were deeply anesthetized with nembutal (40 mg/kg) and decapitated. Brains were quickly removed and transferred into a cold (~4°C), oxygenated cutting solution containing the following (in mM): 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 11 glucose, and 234 sucrose. TC slices were prepared according to the methods described by Agmon and Connors (Agmon and Connors, 1991; Agmon et al., 1995). The reason why we use TC slices is because this preparation maximally preserves thalamic (Agmon and Connors, 1991; Agmon et al., 1995) as well as intracortical connections between layer IV and layer II/III (Stern et al., 2001; Bender et al., 2003; Bureau et al., 2004; Micheva and Beaulieu, 1996). The slices were cut using a vibratome (TPI, St. Louis, MO) and incubated in a holding chamber at 35°C for 1 hour and subsequently at room temperature before being transferred to a recording chamber. For P8, P18 and P30 mice, the brain slices were 200 μm. For P60 mice, the brain slices were 300 μm to compensate the developmental increase in brain volume (Shao and Dudek, 2009). The slices were fixed to a modified microscope stage, and allowed to be equilibrated for at least 30 min before recording. The slices were minimally submerged and continuously superfused with oxygenated (95% O₂-5% CO₂) artificial CSF (aCSF) containing the following (in mM): 126

NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 glucose, pH 7.4, at the rate of 4.0 ml/min.

Whole-cell Patch Recording

Recordings were obtained at $35 \pm 1^\circ\text{C}$ from excitatory neurons (i.e. GFP- cells) in layer II/III, IV and V barrel cortex. Since the local circuitry of cells on the surface may be damaged during cutting, the recordings were not made from surface cells. Capillary glass pipette recording electrodes (1.5–2 μm tip diameters, 3–6 M Ω) were filled with solution containing (in mM): 120 cesium gluconate, 10 phosphocreatine-Tris, 3 MgCl₂, 0.07 CaCl₂, 4 EGTA, 10 HEPES, 4 Na₂-ATP, and 1 Na-GTP, (pH 7.4 adjusted with Cs-OH, 280 mOsm). Neurobiotin (0.5%; Vector Labs, Burlingame, CA) was regularly added to patch pipette solution for morphological confirmation of excitatory neurons. A sharpened bipolar tungsten electrode (length 76 mm, shaft diameter 0.356 mm, tip diameter 1 μm , normal impedance 1.0 ± 0.2 M Ω ; WPI, Sarasota, FL), placed in close proximity to the recorded neurons, was used to deliver synaptic stimulation at low frequency (0.1 Hz). Three different stimulation intensities were used: the minimum, 5% over the minimum, and 15% over the minimum. The minimum was defined as a large proportion of failures (Dobrunz and Stevens, 1997). For example, during 5 consecutive recordings, the stimulation could only induce a postsynaptic response in only one or two of the recordings. The stimulation intensity to induce inhibitory minimum response was used as minimum stimulation intensity. Recordings were made at seven different holding potentials: +30 to –90 mV. Synaptic currents were recorded with a multiclamp 700B amplifier and pClamp software (Molecular Device, Sunnyvale, CA). The sequence of 7 holding steps was repeated 10 times and the currents for each holding potential were averaged. The two most depolarized and hyperpolarized steps were usually removed from analysis. A small biphasic voltage pulse (5 mV positive, –5 mV negative, 10 ms each) was inserted 250 ms before the intracortical stimulus to measure input resistance (R_{in}). Series resistances (R_{series}) were measured using the membrane test feature in pClamp 9. Series resistance was not compensated online but offline (see next section). Experiments in which the resistance changed by >15% were rejected. In most experiments, the synaptic responses were evoked in the presence of 50 μM D(–)-2-amino-5-phosphonopentanoic acid (APV, Tocris Bioscience, Ellisville, MO). For measuring the inhibitory reversal potential, in addition to APV, 10 μM 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(F) quinoxaline (NBQX) (Tocris Bioscience, Ellisville, MO) was added to bath solution to block glutamate receptors. For measuring excitatory reversal potential, in addition to APV, 50 μM picrotoxin (PTX) (Tocris) was added to bath solution to block GABA_A receptors. In some experiments, we compared the conductance measured with or without APV.

Calculation of conductances

The method we used here is based on the continuous measurement of conductance dynamics during stimulus-evoked synaptic response. This method was primarily described *in vivo* on cat cortex (Borg-Graham et al., 1998; Monier et al., 2003) and was then applied on rat primary auditory cortex *in vivo* (Wehr and Zador, 2003), on slices of rat visual cortex (Le Roux et al., 2006), ferret prefrontal and occipital cortex (Shu et al., 2003; Haider et al., 2006), rat barrel cortex (Higley and Contreras, 2007) and mice barrel cortex (Cruikshank et al., 2007). The excitatory (E) and inhibitory (I) conductance underlying the mixed synaptic responses were extracted by using the equations that were previously described by Cruikshank *et al.* (Cruikshank et al., 2007). In this study, synaptic current (I_{synaptic}) was determined for each holding potential (3 potentials were used for the analysis, Fig. 1A1&A2). We plotted synaptic I-V curve at each time point and fitted these plots with linear regression. Based on the slopes and voltage-intercepts, we calculated synaptic conductance (G_{syn}) and reversal potential (E_{syn}), respectively. Continuous conductance and reversal

potential waveforms were constructed (Fig. 1D). Holding potentials were corrected for a calculated liquid junction potential of 12 mV (Fig. 1B & C), which was similar to the previous studies (Agmon et al., 1996). Excitatory synaptic reversal potential (E_e) = 0 mV. Inhibitory synaptic reversal potential (E_i) = -60 mV. G_{syn} was then decomposed into an excitatory synaptic conductance (G_e) and an inhibitory synaptic conductance (G_i). G_e and G_i values were also plotted as continuous waveforms (Fig. 1E). For validating our methods, we also measured G_e and G_i when the excitatory or inhibitory synapses were blocked by 10 μ M NBQX (Fig. 1F) or 50 μ M PTX (Fig. 1G), respectively.

Western blot (WB)

Before extracting the fresh tissue from P30 mice, the location of barrel cortex was studied and validated with cytochrome C or VGLUT2 staining with approximately 80% tissue composed of barrel cortex. The location was determined based on stereotaxic coordinates described by Franklin and Paxinos (Franklin and Paxinos, 2008). At P30, Bregma was localized and marked with a small coronal cutting. Then, two cuttings parallel to the Bregma cutting were made, of which 0.6 mm rostral of Bregma and 1.65 mm caudal to the Bregma cut. Next, two cuts (2 and 3.5 mm from the midline, measured at the level of the caudal horizontal cut, respectively) were made between the previous cuts dividing the brain laterally, with both cuts made perpendicular to the surface of the cortical curvature. After the area was defined, the part of cortex was taken out and cleaned in 0.01M PBS with 1:100 Protease inhibitor before homogenization. Location of barrel cortex in other age groups was modified from the P30 mice based on the size of the rostral-caudal distance from olfactory bulb to the lambda (~8.5 mm at P30). Next, freshly dissected barrel cortex from GAD67-GFP mice in several age groups was immediately homogenized in an ice-chilled RIPA buffer (1X formulation and consisting of 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS. Thermo Fisher Scientific Inc, Rockford, IL 61105 USA), supplemented with protease inhibitors. Samples of homogenates [40ug of protein; protein concentrations were determined by using the Bradford method (Bio-Rad, Richmond, CA)] were separated by a 4–15% graded SDS-PAGE. Proteins were then transferred onto PVDF membrane, and the membrane was incubated with a variety of antibodies (see list below) overnight at 4°C, then reacted with a horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000; Thermo Fisher Scientific Inc, Rockford, IL) secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence (Thermo Fisher Scientific Inc, Rockford, IL). Membranes were also probed with anti β -actin antibody (1:1500 dilution; Abcam Inc. Cambridge, MA, USA), which served as an internal standard for protein quantification. Antibodies used in Western Blot analysis: anti-GAD67 (1:2500, Millipore/Chemicon, MAB3406); anti-VGAT (1:50, Millipore/Chemicon, AB5062P), anti-KCC2 (1:100, Upstate), anti-GABAA α 2 (1:1000, Millipore/Chemicon, AB5948), anti-synapsin 1 (1:500, Invitrogen), and anti-VGLUT2 (1:200, Neuromab). The film signals were scanned and band optical densities were measured with Quantity One software. Expression level for each protein was normalized by comparing the density of the protein band with the density of the correlated β -actin band.

Data Analysis

Traces shown in the figures are the average of 10 consecutive responses and all values are expressed as mean $\pm$ SEM. One-way ANOVA was used for multiple group comparisons and Bonferroni post-hoc test was used for the comparisons within groups. Two-tailed Student's *t* test was performed for two group comparisons. Significance was placed at $P < 0.05$.

Results

In this study, four developmental stages (P8, P18, P30 and P60) were chosen to investigate the E/I ratios across four major layers of barrel cortex. The rationales for choosing these particular stages are as the follows: the time courses for maturation of thalamocortical and intracortical neurons and synapses are different, which are reflected by the differences in the layer-specific plasticity (Crair and Malenka, 1995; Kirkwood et al., 1995). The critical period for synaptic plasticity is before P7 in layer IV spiny stellate cells (Crair and Malenka, 1995; Feldman et al., 1998) and P12-17 in layer II/III pyramidal neurons (Stern et al., 2001; Maravall et al., 2004). In addition, intracortical synaptic density increases several-fold at P9-15 (Micheva and Beaulieu, 1996; De Felipe et al., 1997). Therefore, P8 and P18 are correlated with the end of the critical periods for synaptic plasticity for layer IV and layer II/III pyramidal neurons, respectively. The GABA inhibitory system is very immature during the first 2 postnatal weeks, however, the synaptic population abruptly increases between P10 and P15 and reaches adult values by P30 (Luhmann and Prince, 1991; Micheva and Beaulieu, 1996). The recordings from P30 mice reflected the E/I ratio after the maturation of inhibitory network. P60 group was chosen to compare with the adolescent groups to examine the change in the E/I ratio in adult mice.

Synaptic currents and conductance measured with or without APV in the bath perfusion

There is a developmental change in NMDA receptor subunit composition in excitatory neurons from mostly NR1/NR2B containing receptors at neonatal age (~P7) to mixed NR1-NR2A&B-containing receptors at older ages (Monyer et al., 1992; Watanabe et al., 1992; Sheng et al., 1994; Zhang and Sun, 2010). Compared with NR2A, NR2B mediated EPSCs exhibit a 3- to 4-fold slower decay time course (Flint et al., 1997; Vicini et al., 1998), which would cause nonlinearities in the synaptic conductances (Fig. 2), especially in P8 neurons (Fig. 2A1 vs. A2). Therefore we added APV to remove nonlinearities in the synaptic conductances associated with NMDA receptors (Fig. 2). In addition, because cesium was used in the patch pipettes, GABA_B-mediated conductance was also largely reduced (Jiang et al., 1995). Therefore, the E/I ratio was determined largely by AMPA vs. GABA_A conductances.

Developmental decrease in E/I ratio across 4 layers of somatosensory cortex

We measured synaptic currents evoked by local intracortical stimulation recorded under voltage clamp mode at varying holding potentials, and then calculated the underlying E and I conductances (see methods), as well as E/I ratio (Fig. 1A, D & E). The results were reported for 99 cells [P8 (n=32), P18 (n=26), P30 (n=21) and P60 (n=20)] recorded from 34 mice [P8 (n=8), P18 (n=8), P30 (n=10) and P60 (n=8)] (Table 1).

The response of most excitatory neurons across all the layers (II/III, IV and V) of the barrel cortex to the local stimulation began as an excitatory conductance (G_e), followed by an inhibitory conductance (G_i) (Fig. 1E). To test whether there is a significant difference in excitatory-inhibitory delays during development, we measured the latencies between the G_e and G_i peaks based on methods described by Cruikshank et al. (Cruikshank et al., 2007). In this experiment, the separation of E and I (i.e. delay of I from E) ranged between 3.2 to 5.2 ms which is similar to a di-synaptic inhibitory responses (Cruikshank et al., 2007). We found that the excitatory-inhibitory delays were similar across developmental stages in all major layers (Table 1). This suggests that our extracellular stimulus mostly recruited di-synaptic inhibitory responses and there were no significant differences in the response time of the delayed inhibitory di-synaptic responses.

Using minimum stimulus intensity, as well as other intensities (see methods), we measured the E/I ratio in principal neurons across multiple layers of the barrel cortex from four age groups (P8, P18, P30 and P60). At minimum stimulus intensity, we found that there was a significant developmental decrease in the E/I ratio in excitatory neurons located across the four main cortical layers. This decrease in the E/I ratio occurred at a critical period between P8–P18 (Fig. 3&Table 1). For example, the mean E/I ratio of layer IV excitatory neurons was 0.7 ± 0.1 at P8, 0.3 ± 0.1 at P18 ($p < 0.001$ vs. P8), 0.2 ± 0.0 at P30 and P60 (not significant vs. P18, Table 1). A similar decrease in the E/I ratio occurred in principal cells across all major cortical layers (II/III, IV and V), with the exception of layer II/III pyramidal cells, in which the E/I ratio decreased significantly from P18 to P30 (Table 1). We also examined the E/I ratio underlying mixed synaptic responses induced by higher stimulus intensities. We found a similar pattern of developmental decrease in E/I ratios, despite the fact that the E and I conductance values were several folds higher (Fig. 3&Table 1). In addition, the E/I ratio recorded under higher stimulus intensities in layer IV decreased significantly from P18 to P30 (Table 1), presumably due to recruitment of more distal axonal collaterals by higher stimulus intensities.

The focus of this study is relative contribution of excitatory and inhibitory conductance to the mixed synaptic conductance of a local circuit. Therefore it is important to examine how consistent the E/I ratio are relative to the stimulation sites. Next, we examined E/I ratio induced at different stimulation sites in P60 mice ($n=8$ mice). We used two stimulation electrodes: one was placed 100–200 μm underneath the recorded neurons (stimulator 1), the other was placed 100–200 μm on the right side of the same recorded neurons (stimulator 2). For example, when we were recording from layer IV neurons, stimulator 1 was placed in layer V in 100–200 μm distances from the recorded neurons; stimulator 2 was placed in the same layer (IV) 100–200 μm on the right side of the recorded neurons. The two stimulators were turned on and off in turns (the order was randomized to avoid any bias) and the same neuron's responses to the two stimulations were recorded at minimum, 5% over minimum and 15% over minimum stimulation intensities. Total 20 neurons from layer II/III ($n=7$), layer IV ($n=6$) and layer V ($n=7$) were recorded. We found that there was no statistical difference either under minimum or 15% over minimum stimulation intensities (Fig. 4). This indicates that in this study the change in the stimulation sites did not lead to different E/I ratio values.

Mechanisms underlying developmental changes in E/I ratio

To study the synaptic mechanisms underlying the postnatal developmental change in the E/I ratio, we first compared the E and I conductances across the four age groups. As shown in Table 1, I induced by minimum intensity was enhanced by more than two folds from P8 to P18 and maintained a similar value at P30 and P60 (Table 1 and Fig. 3). In contrast, there were no significant changes in E. Under higher stimulus intensities, although the absolute values of I and E were several times larger, the same trend was observed (Table 1 and Fig. 5). These data suggest that the change in the E/I ratio is due to postnatal net increase in I, not E conductance.

Because interneurons tends to innervate locally (McBain and Fisahn, 2001; Monyer and Markram, 2004; Kubota et al., 2007), the observed postnatal decrease in the E/I ratio recorded at two fixed stimulus intensities (minimum and 15% over minimum) could be due to selective recruitment of inhibitory axons. If this is the case, we may underestimate the contribution of excitatory axons that were not recruited by the stimulus. To test if this was the case, we measured E and I evoked by a variety of stimulation intensities (minimum, 5% over minimum, and 15% over minimum), and then calculated the E/I ratio. We combined the data from all the excitatory neurons from all major layers at P8 ($n=28$), P18 ($n=16$), P30 ($n=21$) and P60 ($n=20$) (Fig. 5). We found that as the stimulus intensity increased, both the

E and I conductances increased significantly to a similar magnitude (Fig. 5C vs. D). The E/I ratio was highly constant within an age group under all stimulation intensities in all layers (Fig. 5B and Table 1), with an exception occurring at layer II/III under higher (15%) stimulus intensities. Overall, these data indicate that the E/I ratio reported here is determined by synaptic properties of the local circuits, and that E/I ratio change over postnatal period reflect changes in intrinsic properties of local circuits across development, in particular, the inhibitory system.

To further study these intrinsic properties of the local inhibitory circuits across development, we measured both putative monosynaptic inhibitory synaptic responses in layer IV and total synaptic protein expressions in the entire barrel cortex. We recorded minimally induced monosynaptic IPSCs, as well as spontaneous IPSCs, from the layer IV spiny stellate cells and star pyramidal neurons. The results were reported for 60 cells [P6 (n=10), P8 (n=12), P16 (n=8), P18 (n=10), P26 (n=8) and P30 (n=12)] from 28 mice [P6 (n=4), P8 (n=4), P16 (n=4), P18 (n=4), P26 (n=6) and P30 (n=6)]. As shown in Fig. 6, the pattern of changes in the amplitude of monosynaptic IPSCs was similar to the changes in the I conductance extracted from mixed synaptic responses (Fig. 6 vs. Fig. 3). Furthermore, the time course of the decrease in paired pulse ratio and coefficient of variance (CV) of the evoked IPSCs matched closely with the increase in IPSC amplitude (Fig. 6B). These results indicate that a drastic postnatal net increase in the number of local inhibitory synapses may be responsible for the increase in I (Stevens, 1989; Regehr et al., 2009).

To further elucidate the changes in excitatory and inhibitory system across all layers of the barrel cortex, we extracted fresh tissue from the entire barrel cortex and examined synaptic protein levels with Western Blot (WB, see methods). The results were reported for 35 brain samples from 35 mice, 5 brain samples (5 mice) for each age group. As shown in Fig. 7, many proteins involved in GABA transmission (KCC2, GABAA receptor $\alpha 2$ subunits, GAD67 but not VGAT) showed significant increase during the postnatal period (Fig. 7). For example, the relative protein expression level for GAD67, versus β -actin, was 0.2 ± 0.2 at P8, 1.7 ± 0.1 at P18 ($p < 0.01$ vs. P8), 2.0 ± 0.3 at P30 and 2.0 ± 0.1 at P60 (both $p > 0.05$ vs. P18). Similar protein expression pattern was found in KCC2, GABAA receptor $\alpha 2$ subunits. The protein expression level for VGAT was 1.2 ± 0.1 at P8, 1.7 ± 0.1 at P18 ($p > 0.05$ vs. P8), 1.5 ± 0.2 at P30 and 1.6 ± 0.2 at P60 (both $p > 0.05$ vs. P18). In contrast, the protein expression levels for the synapsin 1, a vesicle associated protein (Paggi and Petrucci, 1992; Greengard et al., 1993), were 1.4 ± 0.1 at P8, 1.7 ± 0.3 at P18, 2.2 ± 0.1 at P30 and 3.7 ± 0.1 at P60, which only showed a modest increase in the same developmental period ($p > 0.05$, P8 vs. P30) (Fig. 7). Interestingly, the protein expression level for presynaptic glutamate markers (VGLUT2) increased significantly at P18 (2.8 ± 0.2 at P18 vs. 0.7 ± 0.1 at P8, $p < 0.01$), but significantly decreased at P30 (1.0 ± 0.2 at P30 vs. P18, $p < 0.05$).

In addition, by plotting developmental expression levels (from P6 to P60) of different proteins against each other and fitting the data with linear or Boltzmann equations (Origin 6.1, OriginLab Corporation, Northampton, MA), we found that the developmental expression of inhibitory synaptic proteins correlated very well with other inhibitory synaptic proteins (Fig. 8A-E), with an exception of GABAA receptor $\alpha 2$ subunits vs. VGAT (Fig. 8F), but not with excitatory proteins (VGLUT2) or synapsin 1 (Fig. 8G-N). There was no correlation of protein expression levels between VGLUT2 and synapsin 1 (Fig. 8O).

We next compared the protein expression levels of different proteins with the amplitude of eIPSCs and found that the expression of inhibitory proteins (e.g. GAD67, KCC2 and VGAT) (Fig. 9A-C), with an exception of GABAA receptor $\alpha 2$ subunits (Fig. 9D), but not excitatory proteins (VGLUT2) or synapsin 1 (Fig. 9E&F), correlated very well with the amplitude of eIPSCs. Therefore, the results from WB and monosynaptic IPSCs recordings

support our data regarding the predominant enhancement of I over E conductance. These data together suggest that the entire inhibitory circuits (presynaptic and postsynaptic) are intrinsically co-regulated, give rise to a sharp increase in inhibitory conductance during a short critical period from P8 to P18.

Discussion

By simultaneously measuring excitatory and inhibitory conductances underlying locally induced mixed synaptic responses, we demonstrate that there is a >1 fold decrease in E/I ratio, occurred largely in a critical period between P8-P18, in principal neurons across four cortical layers. The change in the E/I ratio is attributable to a disproportional net increase in inhibitory conductance (Fig. 3 & Table 1). To best of our knowledge, this is the first report of E/I ratio measured in neocortical principal neurons across major postnatal developmental stages *in vitro*.

A few factors affecting E/I ratio in developing neurons

Although in this study the NMDA receptor activity was blocked, the importance of NMDA receptor in the development of neural network needs to be emphasized. NMDA receptor activation plays a crucial role in the maturation of excitatory circuitry (Durand et al., 1996; Liao et al., 1999; Zhu and Malinow, 2002) and the formation of the critical period (Kirkwood and Bear, 1994; Feldman et al., 1999; Barth and Malenka, 2001; Erisir and Harris, 2003; Daw et al., 2007; Zhang and Sun, 2010) during brain development. The maturation of glutamatergic synapse is indicated by an increase in the ratio of AMPA receptor-mediated currents relative to NMDA receptor-mediated currents (Crair and Malenka, 1995; Hsia et al., 1998; Lu et al., 2001; Ye et al., 2005). There is evidence that NMDA receptors play an active part in regulating the synaptic AMPA receptor activity at many levels, including gene transcription, protein synthesis and degradation of proteins that interact with AMPA receptors (Adesnik et al., 2008; Hall and Ghosh, 2008; Hall et al., 2007; Luthi et al., 2001). In the early postnatal period, the low AMPA receptor to NMDA receptor ratio is partly due to the negative expression of GluR subunits controlled by NMDA receptor NR2B subunit-mediated signaling (Hall et al., 2007). The developmental increase in the NR2A/NR2B ratio of synaptic NMDA receptors in principal/excitatory neurons (Hestrin, 1992; Monyer et al., 1994; Sheng et al., 1994; Li et al., 1998; Stocca and Vicini, 1998; Nase et al., 1999; Liu et al., 2004) and in GABAergic interneurons (Zhang and Sun, 2010) is concomitant with the development of neural circuits and the increase in the ratio of AMPARs to NMDARs (Crair and Malenka, 1995; Hsia et al., 1998; Lu et al., 2001; Ye et al., 2005). In more mature neurons (P18 and P30), blocking of NMDA receptors eliminated the nonlinearities associated with NMDA receptor-mediated conductance without significant change in the E/I ratio (Fig. 2B1 vs. B2 and C1 vs. C2), which indicate that non-NMDA receptors, such as AMPA receptors, mediate the vast majority of fast excitatory synaptic transmission [for review, see (Dingledine et al., 1999)]. In P8 neurons, the synaptic conductance associated with NMDA receptors caused nonlinearities, which made the calculation of synaptic conductance difficult (Fig. 2A1 vs. A2). It worth mentioning that compared with layer IV and V, layer II/III pyramidal neurons have more silent synapse (data not shown), due to lacking of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Isaac et al., 1995; Liao et al., 1995; Rumpel et al., 1998; Petralia et al., 1999; Malinow et al., 2000). Like the formation of cortical layers (Rakic, 1982; Takahashi et al., 1999), synapse formation also follows an inside-out gradient. Although synaptogenesis in rats occurs primarily in deep layers up to P6, most neocortical synapses are formed in the upper layers after P8 (Blue and Parnavelas, 1983; Zielinski and Hendrickson, 1992). Silent synapses in layer II/III pyramidal neurons may reflect their later development. With the silent synapses, the blockage of NMDA receptors eliminated the excitatory conductance and

brought the E/I ratio to zero, which was responsible for the smaller E/I ratio values in layer II/III pyramidal neurons at P8 (Table 1).

GABA_A receptors are primarily permeable to Cl⁻ and HCO₃⁻ anions. The Cl⁻ gradients are determined by two specific potassium-chloride co-transporters, NKCC1 and KCC2. The expression of these molecules is developmentally regulated (for example, Fig. 7) (Farrant and Kaila, 2007). It is reported that the GABA_A receptor-mediated transmission is depolarizing in neonatal cortex and may contribute to excitation rather than inhibition (Ben-Ari et al., 1989; Owens and Kriegstein, 2002; Rivera et al., 2005; Fiumelli and Woodin, 2007). For example, barrel cortex GABAergic transmission is depolarizing in the first postnatal week (Agmon et al., 1996; Daw et al., 2006). However recent work demonstrates that GABAergic interneurons are rapidly recruited in a feed forward circuit at the end of the first postnatal week, which coincides with a switch from depolarizing to hyperpolarizing GABA_A receptor-mediated responses, producing a rapid development of fast hyperpolarizing inhibition (Daw et al., 2007). Our earliest developmental group is P8. At this age, the GABA_A receptor-mediated conductance is already hyperpolarizing.

The decrease in E/I ratio is correlated with a disproportional increase of inhibitory conductance

Current literature provides a wealth of information related to postnatal development of excitatory and inhibitory synapses, respectively. During early postnatal stages (<P10) the inhibitory postsynaptic response to electrical stimulation is absent or rare (Komatsu and Iwakiri, 1991; Luhmann and Prince, 1991; Agmon and O'Dowd, 1992; Burgard and Hablitz, 1993) and often manifests as long-lasting polysynaptic barrages (Agmon et al., 1996). However, in rodents, the total number of GABAergic synapses (Blue and Parnavelas, 1983; Miller, 1986; Micheva and Beaulieu, 1997; Kobayashi et al., 2008) and the magnitude of the evoked GABAergic responses (Morales et al., 2002; Kobayashi et al., 2008) increased during development. Recent work in visual and barrel cortex shows that this process is experience-dependent (Morales et al., 2002; Chattopadhyaya et al., 2004; Chandrasekaran et al., 2005; Jiao et al., 2006). Our electrophysiological data demonstrated that the amplitude of synaptically evoked IPSCs increases during development, accompanied by a decrease in both CV and paired pulse ratio (Fig. 6). At most synapses, the change in CV is determined by average release probability and the number of presynaptic release sites, which can be used to measure changes in presynaptic function (Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Bolshakov and Siegelbaum, 1994). Paired pulse ratios can also detect presynaptic changes in synaptic efficacy (Zucker, 1989). The decrease in the CV and paired pulse ratio of IPSCs during development reflects the increase in the presynaptic neurotransmitter release probability (Schulz et al., 1994; Choi and Lovinger, 1997; Kirischuk et al., 2002).

In contrast, excitatory synaptic response can be elicited in neonatal neocortical neurons (Kriegstein et al., 1987; Agmon and O'Dowd, 1992; Burgard and Hablitz, 1993; Kim et al., 1995). However, the transmitter release probability of excitatory neurons (Frick et al., 2007; Feldmeyer and Radnikow, 2009) and the amplitude of excitatory postsynaptic potentials (Reyes and Sakmann, 1999; Oswald and Reyes, 2008) decrease during development, presumably resulting from dramatic changes in the intrinsic and synaptic properties of neurons (Oswald and Reyes, 2008). In addition, the switch of AMPA receptor to GluR2+ subunits in excitatory neurons during postnatal development reduces neuronal excitotoxicity (Kumar et al., 2002; Brill and Huguenard, 2008). Our own data using WB measurement of inhibitory proteins and direct measurement of monosynaptic-evoked IPSCs are consistent with these earlier findings (see Fig. 6–9). Interestingly, the expression of presynaptic glutamate marker (VGLUT2) increased significantly at P18 and then decreased significantly at P30 (Fig. 7). The significant decrease in the protein expression level at P30 for VGLUT2

may be due to the switch from VGLUT2 to VGLUT1 expression, which was observed both *in vivo* (Freneau, Jr. et al., 2004; Wojcik et al., 2004) and *in vitro* (Ni et al., 1995). Thus our data, together with these earlier studies, support the idea that a ‘mismatch’ in the strength of local E and I occurs during postnatal development, which gives rise to a decreased E/I ratio.

Earlier in development, the high excitability of excitatory neurons has advantage in response to low-input currents (Oswald and Reyes, 2008). The reduced intrinsic excitability in older pyramidal neurons may help with enforcement of temporal fidelity by somatic feed-forward inhibition (Pouille and Scanziani, 2001). In addition, GABAergic circuits control the engagement of activity-dependent synaptic modification by controlling excitation (Kirkwood and Bear, 1994; Hensch et al., 1998). The delay in maturation of inhibition may help define a critical period for activity-dependent plasticity to occur in early development (Knudsen and Knudsen, 1990; Kirkwood et al., 1995; Hensch et al., 1998; Huang et al., 1999).

The discrepancies between E/I ratio measured *in vivo* and *in vitro*

In adult cortices, the balance of excitation and inhibition determines the form of cortical activities, such as up and down state (Haider and McCormick, 2009) and circuit amplifications (Shu et al., 2003; Murphy and Miller, 2009; Wehr and Zador, 2003; Wehr and Zador, 2005). Despite the importance of E/I balance (Wilent and Contreras, 2004; Pouille et al., 2009), examples of direct and quantitative measurement of the E/I ratio across development is unavailable.

Recent work indicates that there is a layer-specific difference in somatic E/I ratio in somatosensory cortex pyramidal neurons. For example, simultaneous recording of layer II/III and layer V pyramidal neurons during light stimulation, the E/I ratio was significantly larger in layer V than layer II/III pyramidal neurons (Adesnik and Scanziani, 2010). In our study we found that under the minimum stimulation, the E/I ratio was larger in layer V pyramidal neurons than other layers at P30 (Table 1); however, the difference was not statistically significant. The difference between these two studies might be due to the different stimulation protocol. In addition, there are discrepancies between the E/I ratio measured *in vivo* and *in vitro*. For example, the E/I ratio induced by a wide range of auditory stimulus is around 1 in auditory cortex *in vivo* (Wehr and Zador, 2003; Wehr and Zador, 2005). In 2–4-month-old ferrets, the E/I ratio also approaches 1 in the visual cortex during upstate (Haider et al., 2006). Whisker oscillation induced E/I ratio ranged from 0.1 to 2 in layer IV of barrel cortex *in vivo* (Heiss et al., 2008). However, in several *in vitro* studies, locally or thalamically induced E/I ratio is around 0.25 at an age of ~20 postnatal days in the visual cortex (Le Roux et al., 2009; Moreau et al., 2010) or somatosensory (Cruikshank et al., 2007). In addition, Monier and colleagues report similar E/I values in the visual cortex recorded *in vivo* (0.3) and *in vitro* (Monier et al., 2008). Our data are similar to the E/I values in these earlier *in vitro* studies measured within a similar age range.

The mechanisms underlying the differences between *in vitro* and *in vivo* results are unclear and require further investigation. One possible explanation is that different stimulation paradigm like those tested *in vivo* (auditory stimulus) and *in vitro* (local or thalamic stimulus) engage different circuits. The second possibility is that the local stimulation may activate more di-synaptic or even poly-synaptic responses, which may explain the longer separation between excitatory and inhibitory peaks (Cruikshank et al., 2007). In barrel cortex, local circuits are organized in columns whose postnatal development, local axonal projections between excitatory and inhibitory neurons have been thoroughly investigated and are shown to be well preserved in a so-called TC slice preparation (Agmon and Connors, 1991; Stern et al., 2001; Feldmeyer et al., 2002; Bureau et al., 2004; Bannister et al., 2005; Sun et al., 2006; Cruikshank et al., 2007; Pouille et al., 2009). Despite the caveat

that using the brain slice preparation has been minimized by modifying slice cutting angle and thickness, the E/I ratio measured *in vitro* is still very different from those measured *in vivo*. In experiments where mixed synaptic conductances were induced by natural stimulus (Shu et al., 2003; Wehr and Zador, 2003; Haider et al., 2006), it is likely that only a small fraction of local circuit neurons are involved. Higher E/I ratio (close to 1) measured in these circumstances indicate limited recruitment of inhibition. In contrast, in slice preparations like the ones used in this study, our local stimulus activates the majority of cells, as well as all axons located within ~150 μm from the site of extracellular stimulating electrode (our unpublished observations). Therefore the E/I ratio value reported in this study represent near capacity of relative total weight of excitatory and inhibitory synapses. It is highly likely that a different E/I ratio value is elicited by a specific stimulus paradigm that engage a subsets of local circuits. Under *in vitro* conditions, we demonstrate here that in a given developmental period, the E/I ratio at each layer of the barrel cortex was maintained at a very similar value, even when we altered the intensity of stimulus to induce a wide range of E and I conductance. This result suggests that the E/I ratios are tightly regulated, despite the fact that components of local circuits undergo a large change during the postnatal period. These data also suggest that when the interneurons are properly recruited, the local inhibitory circuit has overwhelming capabilities of shunting recurrent excitatory circuits. Further studies are required to reveal the important intrinsic genetic or epigenetic factors that determine the value of the E/I ratio at different developmental stages.

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Figure 1. Separation and validation of excitatory and inhibitory conductances

A1, Actual recording of postsynaptic current (I_{recorded}) evoked by local stimulation. Voltage clamped at three holding potentials (-10, -40, -60 mV). Traces are means of ten consecutive sweeps. **A2**, Synaptic currents (I_{syn}) reconstructed by subtracting baseline currents from the traces in **A1**. **B1**, Reconstructed I_{syn} of pharmacologically isolated excitatory synaptic currents (in PTX and APV) recorded at different holding potentials (+30 ~ -50 mV). **B2**, Plot of I_{syn} vs. holding potential and linear regression at time of maximal conductance. **C1**, Reconstructed I_{syn} of pharmacologically isolated inhibitory synaptic currents (in NBQX and APV) recorded at different holding potentials (+30 ~ -70 mV). **C2**,

Plot of I_{syn} vs. holding potential and linear regression at time of maximal conductance. **D**, Continuous plots of synaptic conductance (G_{syn}) and reversal potential (E_{syn}). **E**, Continuous plots of G_{syn} , excitatory (G_{e}) and inhibitory (G_{i}) components of the total G_{syn} . Inset, the normalized G_{e} and G_{i} . G_{e} peaked 4.2 ms before G_{i} (dashed lines). **F**, The G_{e} was completely eliminated after blocking AMPA receptors (NBQX) and NMDA receptors (APV). **G**, The G_{i} was completely eliminated after blocking GABA_A receptors (PTX) and NMDA receptors (APV).

Figure 2. Synaptic currents and conductances with or without APV in the bath perfusion
 Left panel was the reconstructed I_{syn} (baseline currents subtracted) recorded at three different holding potentials (-10, -40, -60 mV). Right panel was the separation of excitatory and inhibitory conductances. **A1, B1 & C1**, Synaptic currents and conductances without APV at P8, P18 and P30, respectively. **A2, B2 & C2**, Synaptic currents and conductances with APV.

Figure 3. Developmental change in E/I ratio under minimum and 15% over minimum stimulation conditions

A1, B1, C1, Continuous plot of G_{syn} , G_e and G_i (under minimum stimulation intensity) from layer II/III (**A1**), layer IV (**B1**) and layer V (**C1**) excitatory neurons at four developmental stages (P8, P18, P30 and P60, separated by dashed lines). **A2, B2, C2**, Continuous plot of G_{syn} , G_e and G_i (under 15% over minimum stimulation intensity) from layer II/III (**A2**), layer IV (**B2**) and layer V (**C2**) excitatory neurons at four developmental stages (P8, P18, P30 and P60, separated by dashed lines).

Figure 4. The E/I ratio values remain the same at different stimulation sites

A1-C1, Continuous plot of G_{syn} , G_e and G_i induced under minimum stimulation intensity by stimulator 1 (Stim. 1) and stimulator 2 (Stim. 2) at layer II/III (**A1**), layer IV (**B1**) and layer V (**C1**) from P60 excitatory neurons. **A2-C2**, Continuous plot of G_{syn} , G_e and G_i induced under 15% over minimum stimulation intensity. **D**, Mean E/I ratio (G_e/G_i) values across four layers induced by Stim. 1 (vertical, open bars) and Stim. 2 (horizontal, black bars). **E**, Mean G_e values. **F**, Mean G_i values.

Figure 5. The E/I ratio values remain the same at different stimulation intensities

A, Continuous plot of G_{syn} , G_e and G_i at three different stimulation intensities: minimum (**A1**), 5% over minimum (**A2**) and 15% over minimum (**A3**) from a P8 excitatory neuron. **B**, Mean E/I ratios (G_e/G_i) under different stimulation intensities from P8, P18, P30 and P60 neurons (pooled data from layer II/III, IV, and V). **C**, Mean G_e values under different stimulation intensities. **D**, Mean G_i values under different stimulation intensities. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (One-way ANOVA, Bonferroni Post Hoc) in this and following graphs.

Figure 6. Postnatal maturation of inhibitory synaptic transmission in layer IV

A, Representative traces of monosynaptic eIPSCs [minimally evoked IPSCs as indicated by arrow heads) and spontaneous IPSCs were recorded in layer IV spiny stellate cells in barrel cortex from mice age ranged from P8-P30. **B**, Amplitudes (left), coefficient of variance (CV, middle), and paired pulse ratio (right) of eIPSCs recorded from different postnatal ages. The results were reported for 60 cells [P6 (n=10), P8 (n=12), P16 (n=8), P18 (n=10), P26 (n=8) and P30 (n=12)] from 28 mice [P6 (n=4), P8 (n=4), P16 (n=4), P18 (n=4), P26 (n=6) and P30 (n=6)].

Figure 7. Postnatal maturation of inhibitory and excitatory network in the somatosensory cortex
A, WB of selective markers for inhibitory (GAD67, VGAT, KCC2, GABAAR α 2), excitatory (VGLUT2) and synapsin 1 in lysate obtained from barrel cortical tissues. β -actin level for each tissue sample was used as standard to normalize protein loading. **B**, Normalized (vs. β -actin) protein expressions in lysates derived from the barrel cortical tissues of different postnatal ages. The results were reported for 35 brain samples from 35 mice, 5 brain samples (5 mice) for each age group.

Figure 8. Analysis of protein expressions

A-F, Expressions of different inhibitory markers were plotted against other inhibitory markers. **G-N**, Expressions of different inhibitory markers were plotted against excitatory markers. **O**, Expression of excitatory markers was plotted against each other. Solid lines: linear [$Y = A + B * X$], dark grey or Boltzmann $\{y = (A1 - A2) / [1 + e^{(x - x0)/dx}] + A2$, light grey} fittings.

Figure 9. Analysis of protein expression and electrophysiologically recorded putative eIPSCs
A-D, Expressions of different inhibitory markers were plotted against the amplitude of minimally induced IPSCs. **E&F**, Expressions of different excitatory markers were plotted against the amplitude of minimally induced IPSCs. Solid lines: linear (dark grey) or Boltzmann (light grey) fittings.

Table 1
The properties of conductance under minimum and 15% over minimum stimulation conditions.

Layer	Age	n	Input Resistance (mΩ)	Minimal stimulation				15% over minimal stimulation			
				G _e (nS)	G _i (nS)	G _e /G _i	Separation of E&I peaks (ms)	G _e (nS)	G _i (nS)	G _e /G _i	Separation of E&I peaks (ms)
II/III	P8	9	387.1 ± 55.4*	0.2 ± 0.1	0.4 ± 0.1**	0.5 ± 0.0***	4.1 ± 1.1	0.5 ± 0.1	1.7 ± 0.6***	0.4 ± 0.1*	4.6 ± 1.2
	P18	9	220.8 ± 26.0	0.3 ± 0.0	0.8 ± 0.2	0.4 ± 0.0*	4.7 ± 0.7	1.0 ± 0.2	3.1 ± 0.4	0.3 ± 0.0	4.3 ± 0.7
	P30	7	266.6 ± 25.8	0.4 ± 0.2	1.6 ± 0.5	0.2 ± 0.1	4.9 ± 1.5	1.8 ± 0.5	7.3 ± 2.0	0.3 ± 0.1	3.5 ± 1.3
	P60	7	285.3 ± 43.2	0.4 ± 0.2	1.8 ± 0.5	0.2 ± 0.0	4.4 ± 1.2	1.8 ± 0.5	7.9 ± 2.0	0.2 ± 0.0	3.3 ± 1.3
	P8	13	432.2 ± 42.8*	0.2 ± 0.0	0.3 ± 0.1***	0.7 ± 0.1***	4.3 ± 1.1	0.6 ± 0.2	1.1 ± 0.2***	0.6 ± 0.1**	3.4 ± 0.4
IV	P18	9	279.1 ± 34.7	0.3 ± 0.1	1.0 ± 0.2	0.3 ± 0.1	4.1 ± 0.5	2.2 ± 0.6	4.7 ± 1.1	0.5 ± 0.0*	4.2 ± 0.6
	P30	8	232.9 ± 34.1	0.2 ± 0.0	1.3 ± 0.2	0.2 ± 0.0	4.9 ± 0.9	1.0 ± 0.2	6.5 ± 1.9	0.2 ± 0.0	3.3 ± 1.0
	P60	6	294.9 ± 50.7	0.2 ± 0.0	1.1 ± 0.2	0.2 ± 0.0	4.3 ± 0.9	1.1 ± 0.3	6.8 ± 0.2	0.2 ± 0.1	3.9 ± 1.0
	P8	10	386.9 ± 28.7*	0.2 ± 0.1	0.3 ± 0.1***	0.8 ± 0.1**	5.8 ± 1.3	0.4 ± 0.1	0.8 ± 0.1***	0.7 ± 0.2*	5.2 ± 0.9
V	P18	8	228.9 ± 13.5	0.3 ± 0.1	1.1 ± 0.2	0.3 ± 0.0	3.4 ± 0.3	0.9 ± 0.2	2.9 ± 0.5	0.3 ± 0.1	2.7 ± 0.5
	P30	6	247.7 ± 10.4	0.2 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	4.7 ± 1.6	0.9 ± 0.3	3.9 ± 0.9	0.3 ± 0.1	3.2 ± 1.6
	P60	7	263.3 ± 41.4	0.2 ± 0.0	0.9 ± 0.1	0.2 ± 0.0	4.5 ± 1.5	0.9 ± 0.1	3.9 ± 0.2	0.2 ± 0.0	3.6 ± 1.1

Data expressed in mean ± S.E.M. One-way ANOVA was used for multiple group comparisons and Bonferroni post-hoc test was used for the comparisons within groups. Significance was placed at $P < 0.05$.

* $P < 0.05$,

** $P < 0.01$ and

*** $P < 0.001$