Developmental Reduction of Asynchronous GABA Release from Neocortical Fast-Spiking Neurons

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Delayed asynchronous release (AR) evoked by bursts of presynaptic action potentials (APs) occurs in certain types of hippocampal and neocortical inhibitory interneurons. Previous studies showed that AR provides long-lasting inhibition and desynchronizes the activity in postsynaptic cells. However, whether AR undergoes developmental change remains unknown. In this study, we performed whole-cell recording from fast-spiking (FS) interneurons and pyramidal cells (PCs) in prefrontal cortical slices obtained from juvenile and adult rats. In response to AP trains in FS neurons, AR occurred at their output synapses during both age periods, including FS autapses and FS-PC synapses; however, the AR strength was significantly weaker in adults than that in juveniles. Further experiments suggested that the reduction of AR in adult animals could be attributable to the rapid clearance of residual Ca\(^{2+}\) from presynaptic terminals. Together, our results revealed that the AR strength was stronger at juvenile but weaker in adult, possibly resulting from changes in presynaptic Ca\(^{2+}\) dynamics. AR changes may meet the needs of the neural network to generate different types of oscillations for cortical processing at distinct behavioral states.

Keywords: asynchronous release, Ca\(^{2+}\) transient, development, fast-spiking interneuron, inhibitory synapse

Introduction

The neocortical network consists of interweaved excitatory and inhibitory neurons (Markram et al. 2004). Activation of inhibitory interneurons mainly causes membrane potential (V\(_m\)) hyperpolarization and thus inhibition in their target cells (Glickfeld et al. 2009). Among different types of interneurons, the parvalbumin-containing fast-spiking (FS) neuron that can discharge nonadapting high-frequency action potentials (APs) is the most prevalent interneuronal cell type in the neocortex (Kawaguchi and Kubota 1997; Markram et al. 2004). FS neurons mainly innervate the perisomatic region of pyramidal cells (PCs) and control the timing of postsynaptic APs (Miles et al. 1996). Moreover, activation of these interneurons plays important roles in maintaining rhythmic oscillations (Cardin et al. 2009; Sohal et al. 2009) and regulating behavior states (Fuchs et al. 2007; Lee et al. 2012).

Previous studies showed that neurotransmitter release from FS neurons is both synchronous and asynchronous. Synchronous release of GABA occurs at FS neuron terminals within a narrow time window (~1 ms) after each AP (Sabatini and Regehr 1999); however, asynchronous release (AR) takes place in a wide time window (up to hundreds of milliseconds) after AP generation, particularly in response to high-frequency discharges (Hefft and Jonas 2005; Daw et al. 2009; Manseau et al. 2010; Jiang et al. 2012). In hippocampus, in comparison with parvalbumin-containing FS interneurons, cholecystokinin-expressing non-FS interneurons show much stronger asynchronous GABA release in response to a burst of APs (Hefft and Jonas 2005; Daw et al. 2009). In neocortex, however, recent studies revealed that after a burst of APs neocortical FS neurons show prolonged AR at their output synapses, including FS autapses (synapses onto themselves), FS-to-FS and FS-to-PC synapses (Manseau et al. 2010; Jiang et al. 2012; Ma and Prince 2012). This prolonged asynchronous GABA release causes long-lasting inhibition and reduction of the spiking precision in postsynaptic neurons (Hefft and Jonas 2005; Manseau et al. 2010). A recent study showed that AR occurs in both human and rat neocortical FS neurons, and its strength is upregulated in epileptic tissues (Jiang et al. 2012). Considering the key role of GABAergic transmission mediated by FS interneurons in shaping network activity and regulating network function (Tamas et al. 2000; Freund 2003; Bartos et al. 2007), one can speculate that changes in the strength of asynchronous GABA release could also contribute to the regulation of network activities.

At early postnatal developmental stages, FS neurons undergo morphological changes, including extension of neurites, proliferation of boutons, and formation of synapses (Chattopadhyaya et al. 2004; Huang et al. 2007). This maturation process could be controlled by neuronal activity and sensory inputs (Chattopadhyaya et al. 2004). Furthermore, synaptic properties were refined to ensure a fast and precise synaptic transmission, by showing shortened synaptic delay and increased synaptic efficiency (Doischer et al. 2008; Pangratz-Fuehrer and Hestrin 2011; Sauer and Bartos 2011). These studies mainly focused on developmental changes in synchronous GABA release from FS neurons; however, it remains unclear whether AR is subjected to a developmental regulation.

In this study, we compared the strength of AR and synchronous release from neocortical FS neurons in juvenile and adult animals. We found that, in both age periods, AR occurred at output synapses of FS neurons. Interestingly, AR strength was substantially weaker in adult in comparison with juvenile animals. This developmental reduction of AR strength may result from a rapid recovery of Ca\(^{2+}\) transients in axonal terminals in adults.

Materials and Methods

Ethics Statement

The use and care of animals complied with the guidelines of the Animal Advisory Committee at the Shanghai Institutes for Biological Sciences.

Slice Preparation

Most of the experiments were performed in 2 groups of SD rats, the juvenile group (P15–19, 45–51 g) and adult group (P50–55, 265–305 g). In a subset of experiments, we also performed recordings from P32
to P34 rats for AR strength comparison (Fig. 5). Cortical slices were obtained from these animals with standard slicing procedures. In brief, the animal was first anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and sacrificed with decapitation. The brain was then dissected out and immersed into ice-cold oxygenated sucrose ACSF in which the NaCl was substituted with equiosmolar sucrose and dextrose was reduced to 10 mM. Coronal slices (350 μm in thickness) from prefrontal cortices were cut in this ice-cold sucrose ACSF and maintained in an incubation chamber at 35.5 °C. After incubation for ~1 h, the slices were then transferred into a submerge-style recording chamber and perfused with regular ACSF containing (in mM): 126 NaCl, 2.5 KCl, 2 MgSO4, 2 CaCl2, 26 NaHCO3, 1.25 Na2HPO4, 25 dextrose (315 mOsm, pH 7.4). Neurons in the slice were visualized with an infrared-differential interference contrast (IR-DIC) microscope. The temperature during the electrophysiological recording was maintained stable around 35.5 °C.

In some experiments (Supplementary Fig. 4), we prepared prefrontal slices from B13 parvalbumin (PV)-EGFP transgenic mice (juvenile group: P15–21; adult group: P55–57), in which PV-containing neurons could be visualized under fluorescent microscope due to the expression of EGFP (Dumitriu et al. 2007). We compared the autaptic AR frequencies in PV-positive FS cells at the 2 ages.

**Paired Recording**

We performed whole-cell recording from cortical neurons with a Multi-clamp 700B amplifier (Molecular Devices). Usually, FS-PC paired recordings were obtained in adjacent neurons with intersomatic distance of ~20 μm, no larger than 50 μm. Voltage and current signals were filtered at 10 kHz and sampled at 20 kHz with Micro 1401 mKII data acquisition system and Spike 2 software (Cambridge Electronic Design, UK). The impedance of patch pipettes was about 5–7 MΩ. Internal solution for whole-cell recording contained (in mM): 71 KC1, 72 Kgluconate, 2 MgCl2, 10 HEPES, 0.025 BAPTA, and 2 Na2ATP (pH 7.2 with KOH, 288 mOsm). With this internal solution, the calculated reversal potential for Cl− was ~−15 mV, and thus, the IPSPs were depolarizing potentials and IPSPs were inward currents at Vm; levels more negative than ~−15 mV. Alexa Fluor 488 (50 μM) and biocytin (0.2%) were added to the internal solution for visualizing the morphology of recorded neurons. In these experiments, trains of APs with varying numbers (2–40 APs) and frequencies (50–200 Hz) were evoked by trains of step-current injections (in current-clamp mode) or voltage commands (in voltage-clamp mode). The occurrence of AR was examined by 40-AP train stimulation (200 Hz). Unless otherwise stated, properties of PT-AR induced by 20 APs at 200 Hz were analyzed and illustrated in figures. Liquid junction potential (~9 mV) has not been corrected for the Vm shown in the text and figures.

Picrotxin (PTX, GABAA receptor antagonist, Tocris), EGTA-AM (membrane permeable Ca2+ chelator, Invitrogen) were applied through bath perfusion.

**Two-Photon Ca2+ Imaging**

Calcium imaging was performed with a 40x (NA 0.8) water immersion objective on an upright laser-scanning microscope (BX-61W1, Olympus). Two-photon excitation was achieved with a mode-locked Ti:S laser (Chameleon Vision II, Coherent) running at a wavelength of 810 nm (repetition rate: 80 MHz; pulse width: 140 fs). We recorded FS neurons with a pipette solution similar to that of paired recording but containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl2, 26 NaHCO3, 1.25 Na2HPO4, 25 dextrose (315 mOsm, pH 7.4). Alexa Fluor 594 (50 μM) was applied through a Ti:S laser (Chameleon Vision II, Coherent) running at a wavelength of 810 nm (repetition rate: 80 MHz; pulse width: 140 fs). We recorded FS neurons with a pipette solution similar to that of paired recording but containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl2, 26 NaHCO3, 1.25 Na2HPO4, 25 dextrose (315 mOsm, pH 7.4). Neurons in the slice were visualized with an infrared-differential interference contrast (IR-DIC) microscope. The temperature during the electrophysiological recording was maintained stable around 35.5 °C.

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**Data Analysis**

In order to quantitatively analyze the strength of post-train AR (PT-AR), PT-AR duration and events were calculated as reported previously (Jiang et al. 2012). Because individual synaptic currents have more rapid rising slope than baseline fluctuations, current traces were first transformed into slope for detection of spontaneous event. Synaptic events were identified if their rising slopes were greater than a slope threshold. We then obtained the PT-AR frequency with a bin size of 50 ms within a time window of 2 s immediately after the AP train. Because stochastic synaptic events (EPSCs/IPSCs) from other presynaptic cells could also occur during recording, we subtracted the mean frequency of these spontaneous events occurred during a period of 4 s before the train stimulation from the frequency of PT-AR events to minimize the potential effects of spontaneous events on the count of AR events. To measure the PT-AR duration, we first identified the time of the last synaptic event after which PT-AR frequency decreased to the baseline frequency. The PT-AR duration was then measured as the time period from AP train to the last synaptic event. We also obtained the total number of PT-AR events occurred during this period. These parameters (PT-AR duration, frequency and total number) were used for measurement of AR strength in most of the figures (Figs 1, 2, 4, and 5 and Supplementary Fig. 4).

Deconvolution analysis was also used to evaluate the amount of synchronous AR, as previously described (Hefft and Jonas 2005; Jiang et al. 2012). In brief, we first used an IPSC template to identify individual IPSCs occurred during the PT-AR period, and then chose the largest IPSC and fitted its rising and decay phase with linear and exponential functions, respectively. This resultant trace was considered as a template for deconvolution; release rate = F(IPSC trace)/F (quanta), in which F is the discrete Fourier transform (Hefft and Jonas 2005; Daw et al. 2009; Jiang et al. 2012). The release rate was then filtered (5–10 trials) using Gaussian-window FIR filter. The amount of release during (Train) and after the train (PT-AR) was measured as the integrated area of the release rate. Based on these analyses, the sensitivity of synchronous release and AR to EGTA was evaluated in Figure 3 and Supplementary Figure 1. In the EGTA-AM experiment (Fig. 3D), the progressive changes of Train and PT-AR were illustrated to show the time course of drug effect. Plots of quanta number (bin size: 5 ms) as a function of time was utilized to show the decay process of AR following the train. The time constant of this decay phase was estimated by exponential fitting, and the changes in time constant during drug application were plotted in Figure 3F. To minimize the contribution of residual GABA in the synaptic cleft to PT-AR, we used the decay time constant of unitary IPSC to predict the decay of summed train IPSCs and subtracted the train-IPSC decay component from the total post-train charge (Supplementary Fig. 3). The AP threshold was defined as the voltage when the derivation of Vm (dVm/dt) reaches 20 mV/s. The AP amplitude was measured as the voltage difference between AP threshold and its peak. The AP half-width was the duration measured at half amplitude. The AP area was the integrated voltage above the AP threshold.

Values are given as mean ± SEM and error bars in figures also indicate SEM. Significance of differences was assessed by student’s t-test or
frequency discharges (Fig. 1A). These events were inhibitory postsynaptic responses (IPSPs or IPSCs) because they could be blocked by the bath application of 50 μM picrotoxin (PTX). Individual events could be frequently distinguished from the outlasting IPSP (or IPSC) barrages. Consistent with previous studies (Bacci et al. 2003; Jiang et al. 2012), the tightly coupled IPSCs, which reflected the synchronous GABA release in autaptic connections, could be observed after single APs and blocked by PTX (Fig. 1B). We subtracted the current traces in the presence of PTX from those in control condition (control – PTX, Fig. 1B) and then measured the kinetics of individual autaptic IPSCs (Table 1).

To examine the dependence of AR strength on stimulation intensity, we varied the AP numbers and frequencies. Due to the difficulties in distinguishing AR events during the high-frequency AP train, we only examined the properties of posttrain AR (PT-AR) in this study. As shown in Figure 1C, the PT-AR duration and total number of events progressively increased with increasing AP numbers or frequencies (P < 0.05 for both, 2-way ANOVA). Taken together, we observed robust AR in FS autapses in juvenile rats, similar to that in human cortex (Jiang et al. 2012).

### AR in FS-PC Synapse in Juvenile Rats

We next investigated whether postsynaptic excitatory neurons receive AR from FS neurons. Among 451 FS-PC pairs tested, we found 103 FS-to-PC (22.8%) and 18 PC-to-FS (4.0%) connected pairs, and 12 (2.7%) bidirectionally connected pairs. In 98 of 121 FS-PC connections (81.0%), trains of APs in the presynaptic FS neurons could trigger both synchronous and asynchronous IPSCs in postsynaptic PCs (Fig. 2A–C).

Further analysis demonstrated that the AR strength depended on the stimulation intensity. Group data from 37 FS-PC pairs showed that the PT-AR duration and total number of events increased with increasing number and frequency of APs (P < 0.001, 2-way ANOVA, Fig. 2D). Cumulative frequency distribution of FS autaptic and FS-PC synaptic connections showed no significant difference in PT-AR strength between the 2 types of connections (P = 0.28 and 0.06 for PT-AR duration and events, respectively, Fig. 2E). Similarly, the PT-AR frequency showed no significant difference either (Fig. 2F). Consistently, in 17 FS-PC pairs that exhibited both autaptic and FS-PC synaptic AR, no significant differences in the duration and total events of PT-AR were observed in the 2 types of connections (Fig. 2G,H). These results differ from a recent finding in human (5–69 years old) and adult rat (P52–62) cortical tissue that demonstrated substantially stronger AR in FS autapses as compared with FS-PC synapses (Jiang et al. 2012), possibly due to the difference in age periods. Together, our results indicate that, in juvenile rats, AR also occurred in FS-PC connections, and the AR strength was similar to that in FS autaptic connections.

### Ca^{2+} Dependence of AR

Calcium accumulation in presynaptic terminals during AP trains is responsible for AR in hippocampus (Hefft and Jonas 2005) and neocortex (Manseau et al. 2010; Jiang et al. 2012); we therefore investigated the role of background or residual Ca^{2+} in mediating the occurrence of AR in prefrontal cortex of juvenile rats. In the presence of EGTA-AM (100 μM), a membrane permeable Ca^{2+} chelator, FS autaptic AR, and FS-PC connections were blocked by the bath application of 50 μM picrotoxin (PTX). Trace subtraction (control–PTX) revealed unitary IPSCs (bottom). (C) Group data show the dependence of PT-AR duration (top) and event number (bottom) on AP number and frequency.
synaptic AR were significantly reduced after the drug application (Fig. 3A–D). Release rate was calculated by deconvolution as described in previous reports (Hefft and Jonas 2005; Jiang et al. 2012). The integrated areas of release rate during and after AP train were used to measure the quanta released during (Train) and after (PT-AR) AP train (also see Materials and Methods section). The PT-AR in FS autaptic connections was decreased to 19.0 ± 11.1% of control ($P < 0.01$, $n = 5$, Fig. 3A, B). In FS-PC synaptic connections, the peak amplitude of the first IPSC (IPSC$_1$), the total release during the train (Train)}
and the PT-AR were decreased to 58.9 ± 9.6, 40.3 ± 6.9, and 9.4 ± 3.4% of control, respectively (∗P < 0.001, n = 8, Fig. 3C, D). 

In order to characterize the time course of PT-AR, the release rate was binned (bin size: 5 ms) and fitted with a single exponential function. The time course of PT-AR was progressively shortened after the application of EGTA-AM (τ = 81.3, 70.0, 44.5, 30.5 ms at 0, 2, 5, 10 min after EGTA-AM application, Fig. 3E). The normalized group data also showed that the PT-AR decayed faster after EGTA-AM application (Fig. 3F).

As the actual concentration of EGTA inside the presynaptic terminals was unknown with EGTA-AM perfusion, we then performed experiments using internal solutions containing 2 mM EGTA (n = 8). Similar to the effect of bath EGTA-AM (Fig. 3C, D), the presence of EGTA in patch pipettes also substantially decreased the strength of both synchronous and asynchronous GABA release (Supplementary Fig. 1). These results were consistent with the finding that EGTA accelerates the initial decay phase of Ca²⁺ transients (Helmchen and Tank 2005). Together, our results showed that the occurrence of AR in juvenile prefrontal cortex was dependent on the residual Ca²⁺ level in presynaptic terminals after high-frequency firing.

Comparing AR Strength in Juvenile and Adult Rats
We next examined the difference of AR strength in juvenile and adult rats. In adult rats (P50–55), among 160 FS-PC pairs tested, we found 31 FS-to-PC (19.4%) and 4 PC-to-FS (6.7%) connected pairs, and 2 (1.3%) bidirectionally connected pairs. The kinetics of unitary IPSCs in adult rats were measured and compared with those from juvenile animals. As shown in Table 1, the CV and paired pulse ratio (PPR) of IPSC amplitude was significantly increased, while the peak amplitude and decay time constant of IPSCs were decreased in adult animals. The changes in CV and PPR reflected a reduced release probability in presynaptic vesicles (Sola et al. 2004; Ma and Prince 2012). The reduced synaptic strength might result from increased complexity of circuit wiring (Chattopadhyaya et al. 2004) and homeostatic regulation of synaptic transmission (Turrigiano and Nelson 2004). Although there was a reduction in unitary strength (first IPSCs), we found no significant change in peak amplitudes of 2nd–20th IPSCs evoked by APs at 200 Hz (Supplementary Fig. 2). Furthermore, we observed that the integrated charge of PT-AR was significantly decreased in adult tissue (∗∗P < 0.01), while the integrated charge of train
IPSCs only showed a slight decrease at a significance level of \( P = 0.08 \) (Supplementary Fig. 3). These results indicate that the overall synaptic efficiency in response to multiple APs was not significantly changed, and the decrease in the unitary strength may result from the reduced release probability. The acceleration of decay time constant of IPSCs in adult was consistent with previous reports (Galarreta and Hestrin 2002; Doischer et al. 2008; Sauer and Bartos 2011), presumably resulting from changes in membrane properties (Doischer et al. 2008), expression of presynaptic Ca\(^{2+}\) channels (Iwasaki and Takahashi 1998; Iwasaki et al. 2000), and expression patterns of various GABAA receptors (Dunning et al. 1999; Hutcheon et al. 2000; Doischer et al. 2008).

Among 148 FS neurons tested, 53 (35.8\%) showed autaptic AR; and 28 of 31 (90.3\%) FS-PC pairs exhibited obvious synaptic AR in response to high-frequency firing in FS neurons (40 APs at 200 Hz). Consistent with recent findings in human and adult rat cortical tissues, the AR in FS autaptic connections was significantly stronger than that in FS-PC synaptic connections (\( P = 0.02 \) and 0.001 for PT-AR duration and events, respectively; K-S test; \( n = 53 \) FS neurons with autapses and 24 FS-PC pairs, Fig. 4A,B). However, this result was different from that in juvenile rats (Fig. 2), suggesting a differential developmental modification between the 2 types of connections.

The dependence of AR strength on AP number and frequency was preserved in adult animals (Fig. 4A). However, we found that AR strength in both FS autapses and FS-PC synapses in adult rats was dramatically decreased as compared with juvenile rats (Fig. 4A,B). For FS autaptic connections, the PT-AR duration and number of events in adults were 58.4 ± 5.9 ms and 4.0 ± 0.5, respectively, significantly less than those in juveniles (161 ± 11 ms and 11.9 ± 1.2; \( P < 0.001 \) for both parameters, K-S test). Similarly, for FS-PC synaptic connections, significantly weaker AR was observed in adults. The PT-AR duration and number of events in adults were 40.7 ± 5.9 ms and 2.1 ± 0.4, respectively, whereas in juveniles, these values were 141 ± 14 ms and 10.4 ± 1.9 (\( P < 0.001 \) for both parameters, K-S test). Similar results were obtained when the number of APs was increased to 40. The PT-AR frequency in FS autapses and FS-PC synapses also showed dramatic difference between juvenile and adult animals (Fig. 4C). To further narrow down the time window during which the developmental reduction of AR occurred, we performed similar recording from FS cells in an intermediate age group.
We found that the PT-AR frequency in this group of rats was significantly lower than that in juveniles (P15–19), but similar to that in adults (P50–55) (Fig. 5). These results suggest that a great reduction of AR occurred during an age period between P19 and P32, a critical period for the development of medial prefrontal cortex (Makinodan et al. 2012).

Figure 5. Comparison of AR strength in FS autapses from 3 ages. (A) Example recordings showing FS autaptic AR from P15–19 (juvenile), P32–34 (Intermediate), and P50–55 (Adult) rats. (B) PT-AR frequencies showed significant difference between P32–34 and P15–19 rats ($P < 0.001$ within 200 ms after 20-AP trains and 50–200 ms after 40-AP trains), but no significant difference between P32–34 and P50–55 rats (except the data point of 100–150 ms after 40 APs). For 20-AP trains, plots for juvenile and adult animals (same as in Fig. 4C) are shown for comparison.
Previous studies demonstrated that the amount of AR correlates with the synaptic strength (Manseau et al. 2010; Jiang et al. 2012); therefore, AR differences between the 2 age periods may reflect changes in synaptic strength. Indeed, we found a significant reduction in the average amplitude of unitary IPSCs, that is, synaptic strength (Fig. 4D). However, when we normalized the PT-AR by synaptic strength, the AR differences between adults and juveniles were still observed (Fig. 4E). In addition, the PT-AR duration showed a weak but significant correlation with the synaptic strength in juvenile (r = 0.38, P = 0.03) but not in adult rats (r = 0.06, P = 0.78). The inconsistent correlation may result from developmental changes in presynaptic release machinery, including the size of readily releasable pool of vesicles, the expression pattern of Ca2+ channels and sensors.

To exclude the possibility that we sampled different subgroups of FS cells at different ages, we next performed recordings only from PV-expressing FS neurons in B13 PV-EGFP transgenic mice (Dumitriu et al. 2007). The strength of autaptic AR in these GFP-positive neurons was examined in juvenile (P15–21) and adult mice (P55–57). Similarly, we observed a dramatic decrease in PT-AR frequency (Supplementary Fig. 4), suggesting that the developmental AR reduction is a common phenomenon in FS cells, including PV-positive cells.

Together, we found a substantial reduction in AR in adult animals as compared with juveniles. As AR in FS output synapses provides long-lasting inhibition (Hefft and Jonas 2005) and regulates the probability and precision of spiking in postsynaptic cells (Manseau et al. 2010), the differences in AR strength in juvenile and adult FS neurons may reflect the differential contribution of asynchronous GABA release to the control of firing probability in their target cells and the distinct network dynamics at different age periods.

### Differences in Presynaptic Ca2+ Dynamics

Previous reports showed that AR may share the same Ca2+ source with synchronous release, and require a high level of background Ca2+ after AP bursts (Hefft and Jonas 2005; Jiang et al. 2012). We therefore sought to investigate the differences in Ca2+ dynamics at presynaptic terminals in juvenile and adult animals.

We combined whole-cell recording and two-photon Ca2+ imaging in these experiments. We loaded the FS neurons with Ca2+ indicator Fluo-5F and fluorescent dye Alexa Fluor 594 through patch pipettes. The presynaptic boutons were identified according to previous reports (Koester and Sakmann 2000; Goldberg et al. 2005). Fluorescence signals at these boutons were acquired while AP trains (200 Hz) with varying numbers of APs (2–60) were elicited by somatic current injection (Fig. 6A–C). The Ca2+ transients in adult rats showed substantially faster decay than those in juvenile rats (Fig. 6C). Group data (n = 61 boutons from 15 juvenile FS neurons and 73 boutons from 11 adult FS neurons) showed that the peak amplitude and the decay time constant progressively increased with increasing number of APs (Fig. 6D,E and Supplementary Fig. 5), indicating that the residual Ca2+ levels were elevated and prolonged. This could explain the dependence of AR strength on the number of presynaptic APs (Figs 1C, 2D, and 4A).

In comparison with juvenile rats, the peak amplitudes of Ca2+ transients were significantly greater (Fig. 6D) and the decay time constants were dramatically shorter in adult rats (P < 0.001 for all comparisons; Fig. 6E and Supplementary Fig. 5). The distribution of decay time constant also confirmed this dramatic change (Fig. 6F). The decay time constant of Ca2+ transients reflects the time needed for Ca2+ clearance; a longer time constant, therefore, indicates that the Ca2+ concentration remains high for a longer period of time. The slow Ca2+ dynamics in juvenile rats may lead to an elevated and prolonged level of background Ca2+ in presynaptic terminals of FS neurons and thus result in stronger asynchronous GABA release.

Taken together, our results indicate that AR occurs at all output synapses of FS neurons at the ages we examined, and interestingly the AR strength is more robust at juvenile ages but weaker in adults, possibly due to changes in the clearance of accumulated Ca2+ in presynaptic boutons. Considering that asynchronous GABA release causes long-lasting inhibition and decrease in spiking precision in postsynaptic neurons (Hefft and Jonas 2005; Manseau et al. 2010), we speculate that the occurrence of AR at neocortical inhibitory synapses and the developmental reduction of AR may have distinct functional impacts on neuronal signaling and contribute to cortical processing.

### Discussion

Our results indicate that AR occurs at output synapses of FS neurons including FS autapses and FS-PC synapses, and continues to function in adulthood. Interestingly, we found a dramatic decrease in AR strength from juvenile to adult ages. Further Ca2+ imaging experiments suggested that this decrease may result from rapid Ca2+ kinetics in the presynaptic terminal after spiking. Stronger AR observed at developmental stages implicated that AR may play more important roles in shaping the neuronal and network activities during cortical development.

### Developmental Changes of Synapses From FS Neurons

During early developmental stages, the excitatory and inhibitory synapses undergo continuous modification by the neuronal activity (Bear and Malenka 1994; Zucker and Regehr 2002; Dan and Poo 2004; Turrigiano and Nelson 2004) and sensory inputs (Yashiro et al. 2005; Tyler et al. 2007). Although most of studies were performed in young animals, it was
believed that synapses could be modified across the whole life-span (Creutzfeldt and Hegelund 1975). Because of the uniformity in morphology and microcircuits, the maturation and refinement of excitatory synapses between PCs were intensively studied (Hsia et al. 1998; Wasling et al. 2004; Sametsky et al. 2010). However, only a few studies focused on the

Figure 6. Different kinetics of Ca^{2+} transients in FS terminals in juvenile and adult rats. (A) Calcium imaging from boutons of a FS neuron in a juvenile rat. (Aa) projection of the recorded FS neurons (red, Alexa Fluor 594). (Ab) the boxed area in (Aa). The dashed arrow indicates the path of laser scanning. Note the 2 boutons that were scanned. (Ac) \( V_m \) responses to step-current injections (−100 and 400 pA, 500 ms). (A) Similar to (A) but for a FS neuron in an adult rat. Arrowheads indicate the axon initial segment. (C) Calcium transients (Ca^{2+} indicator: Fluo-5F, 200 μM in patch pipette) in boutons indicated in (A) and (B). Top, raw data for 20 APs at 200 Hz. The dashed line indicates the onset of the AP train (20 APs, bottom). Middle, the corresponding Ca^{2+} signals. The exponential fits (red lines) and the decay time constants (\( \tau \)) were indicated. Bottom, APs evoked by a train of brief current injections. (D) Plot of the peak amplitudes of Ca^{2+} transients (ΔG/R) in FS neurons from juvenile and adult rats. (E) Plot of the decay time constant of Ca^{2+} transients (\( P < 0.001 \) for all comparisons). (F) Distribution of the decay time constants of Ca^{2+} transients evoked by 2, 20, and 40 APs (black: juvenile; red: adult). Group data (D–F) were obtained from 61 boutons of 15 FS neurons (juvenile) and 73 boutons from 11 FS neurons (adult). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \).
development of inhibitory synapses (Huang et al. 2007). Previous studies (Chattopadhyaya et al. 2004; Doischer et al. 2008; Pangratz-Fuehrer and Hestrin 2011; Sauer and Bartos 2011) revealed dramatic changes in synchronous release from inhibitory synapses. FS neurons developed abundant neurites during the first 3 weeks (Chattopadhyaya et al. 2004; Doischer et al. 2008), leading to increased probability of synaptic connection in FS-FS pairs and FS-PC pairs (Doischer et al. 2008; Pangratz-Fuehrer and Hestrin 2011). The active and passive membrane properties also undergo substantial alterations, allowing faster transduction of electrical signals (Doischer et al. 2008; Pangratz-Fuehrer and Hestrin 2011). For synchronous release, the increase in synaptic strength and decrease in synaptic failure can ensure high precision and efficiency of synaptic transmission in FS output synapses (Doischer et al. 2008; Pangratz-Fuehrer and Hestrin 2011). However, it remains unclear whether AR is subjected to developmental changes.

In this study, we compared the strength of both synchronous release and AR from FS neurons between juvenile and adult brain. We found that both types of neurotransmitter release were significantly decreased in adult animals (Fig. 4A–D). These changes may reflect the modification of synaptic properties during later development. A recent study showed a positive correlation between the synaptic strength and the AR strength (Jiang et al. 2012). However, here we only found a weak linear correlation between PT-AR duration and synaptic strength in FS-PC pairs in juvenile animals. Normalization of the PT-AR duration by synaptic strength in FS-PC pairs still revealed a significant difference between juvenile and adult animals (Fig. 4E), suggesting that the mechanism for alterations of AR may differ from that of synchronous release.

Previous studies showed that both synchronous release and AR depend on Ca\textsuperscript{2+} influx through presynaptic Ca\textsuperscript{2+} channels (Hefft and Jonas 2005). Prolonged elevation of Ca\textsuperscript{2+} concentration at presynaptic terminals is required for the occurrence of AR, possibly resulting from a long perfusion distance of Ca\textsuperscript{2+} from the location of activated channels to the active zone (Hefft and Jonas 2005; Hestrin and Galaretta 2005). The reduced AR in adult animals could be attributable to shortened perfusion distance. Indeed, the coupling between Ca\textsuperscript{2+} channels and synaptic vesicles is tighter in mature calyx of held (Wang et al. 2008). In hippocampus, pharmacological experiments revealed that P/Q-type Ca\textsuperscript{2+} channels were localized closely (nanodomain coupling) with Ca\textsuperscript{2+} sensors in a type of inhibitory synapse with weak AR, whereas N-type Ca\textsuperscript{2+} channels were localized distantly (microdomain coupling) from Ca\textsuperscript{2+} sensors in another type of inhibitory synapse with strong AR (Hefft and Jonas 2005; Eggermann et al. 2011). A longer diffusion distance of Ca\textsuperscript{2+} allows a buildup of Ca\textsuperscript{2+} concentration in the presynaptic terminal, and leads to slower decay phase of Ca\textsuperscript{2+} transients and thus stronger AR. In contrast, tight coupling between Ca\textsuperscript{2+} channels and sensors results in weaker AR. Therefore, the diffusion distance can determine the AR strength. Previous studies reported that the N-type Ca\textsuperscript{2+} channels at presynaptic terminals were gradually replaced by P/Q-type Ca\textsuperscript{2+} channels during development (Iwasaki and Takahashi 1998; Iwasaki et al. 2000). The increased contribution of P/Q-type Ca\textsuperscript{2+} channels and the associated nanodomain coupling between the Ca\textsuperscript{2+} source and sensor might cause a decrease in the AR strength in adult FS neurons. However, whether the Ca\textsuperscript{2+} diffusion distance is subjected to developmental changes remains to be further examined.

Mechanisms Underlying Developmental AR Reduction

In our experiments, we found that in comparison with juveniles the decay time constant of Ca\textsuperscript{2+} transients was significantly shorter in adult animals (Fig. 6C,E,F), consistent with the decrease in AR strength in adults. These results may reflect a change in Ca\textsuperscript{2+} diffusion distance at different age periods. Moreover, the reduction in the decay time constant of Ca\textsuperscript{2+} transients can result from changes in the efficiency of Ca\textsuperscript{2+} clearance (Yasuda et al. 2004), a process dependent on Ca\textsuperscript{2+} extrusion via membrane pumps (e.g., Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger) and Ca\textsuperscript{2+} uptake by presynaptic endoplasmic reticulum and mitochondria. Indeed, an upregulation of Ca\textsuperscript{2+} extrusion systems has been observed during development and neuronal maturation (Li et al. 2000; Kip et al. 2006). In addition to the briefer calcium transients in the presynaptic terminals, a reduction in the number of synapses and release probability may also contribute to the reduced AR strength in adult tissue. Analysis on the unitary IPSCs revealed a decrease in peak amplitude and an increase in CV (see Table 1), suggesting a reduction in number of functional synapses in adult tissue. This reduction may not result in shorter PT-AR duration because each synaptic contact may have similar time course of AR after the train of spikes; however, less synaptic contacts may cause a decrease in the frequency of PT-AR events, similar to the effect on spontaneous "mini" PSCs. We also observed an increase in CV and PPR in adult tissue when compared with juvenile tissue (see Table 1), suggesting a reduction in release probability. Previous findings in nucleus accumbens slices indicated that the synchronous release and AR may share the same readily releasable pool of vesicles in presynaptic terminals (Hjelmstad 2006; but see Hagler and Goda 2001 and Otsu et al. 2004 in cultured hippocampal cells). Lowering the release probability could substantially reduce AR to a degree similar to the change in overall release probability. The amount of AR correlated well with the size of initial response (unitary IPSC) during the spike train (Hjelmstad 2006). Similar correlation was observed in output synapses of FS neurons in neocortical slices (Manseau et al. 2010; Jiang et al. 2012). Together, the reduction in number of functional synapses and release probability may also contribute to the reduced AR strength in adult tissue.

Calcium buffers inside a neuron play important roles in regulating Ca\textsuperscript{2+} dynamics. Interneurons express various types of Ca\textsuperscript{2+} binding proteins (Markram et al. 2004), including parvalbumin (PV), calbindin and calretinin. Previous findings showed that the decay kinetics of Ca\textsuperscript{2+} transients in cerebellar stellate cells in P21 mice are faster than that in P11 mice, and this change is accompanied with an increase in the proportion of PV-positive cells (Collin et al. 2005), indicating a role of endogenous PV in regulating Ca\textsuperscript{2+} dynamics. Interestingly, this study also suggested that PV might accelerate the initial decay phase but cause an additional slow phase in Ca\textsuperscript{2+} transients in response to AP trains, thus enhancing AR in inhibitory synapses. However, a recent finding demonstrated an opposite effect of PV on AR, by showing that PV-negative cells exhibited stronger AR (Manseau et al. 2010). These inconsistent results may result from differences in PV concentration in individual cells (Eggermann and Jonas 2012). Whether alterations in the expression level of Ca\textsuperscript{2+}-binding proteins could play a role in regulating the AR strength requires further examination.

The reduced AR strength in adults could also result from changes in the expression pattern of various Ca\textsuperscript{2+} sensors. Manipulating the expression level of various Ca\textsuperscript{2+} sensors could
differentially regulate the strength of synchronous release and AR (Sun et al. 2007; Yao et al. 2011). Importantly, the expression levels of synaptotagmin isoforms undergo changes during development (Berton et al. 1997), possibly contributing to the reduced AR strength in adult animals. In addition, developmental alteration of ion channel expression can lead to changes in AP waveforms, which may determine the amount of Ca$^{2+}$ influx during APs and regulate synaptic transmission (Geiger and Jonas 2000; Shu et al. 2006). Here, we also observed a developmental alteration of AP waveform, that is, decrease in AP half-width and integrated voltage area (Supplementary Fig. 6), suggesting a role of AP waveform in regulating presynaptic Ca$^{2+}$ dynamics and asynchronous GABA release.

**Physiological Significance**

In early developmental stages, GABAergic transmission plays important roles in regulating synapse formation and structural refinement of neural network (Ben-Ari 2002). For instance, reduction of GABAergic transmission could significantly delay the critical period of ocular dominance (Hensch et al. 1998; Hensch 2005), which peaks at third postnatal week and ends by fifth week. Accelerated GABAergic innervation and inhibition caused early termination of the critical period for ocular dominance plasticity (Huang et al. 1999). More investigations suggest that different inhibitory circuits contribute differentially to this cortical plasticity, by showing a crucial role of perisomatic inhibition during the critical period (Fagiolini et al. 2004; Katagiri et al. 2007). The AR changes in prefrontal cortex during development may also contribute to the regulation of neuronal wiring and the development of functional circuits. Interestingly, our results indicate that the greatest reduction of AR strength occurred between P20 and P32, the critical period for oligodendrocyte maturation in prefrontal cortex and related behaviors including sociability and working memory (Makinodan et al. 2012). However, it remains unclear whether asynchronous GABA release at critical period has impacts on cortical plasticity.

FS neurons form intensive electrical and chemical connections with other FS neurons, and also innervate a large population of PCs (Fino and Yuste 2011; Packer and Yuste 2011; Pangratz-Fuehrer and Hestrin 2011). Because of this high connectivity with neighboring cells, FS neurons can synchronize the cortical network and generate rhythmic oscillations (Tamas et al. 2000; Freund 2003; Bartos et al. 2007). Manipulating the activity of FS neurons could selectively regulate the gamma oscillations in the barrel cortex (Cardin et al. 2009) and alter the theta phase of spikes in hippocampal place cells (Royer et al. 2012). As shown previously (Miles et al. 1996), precise neurotransmitter release from FS neurons determines the spike timing of their target cells; however, prolonged asynchronous GABA release from FS neurons could decrease the probability and precision of postsynaptic spikes, and thus desynchronize the neuronal network (Manseau et al. 2010). Previous modeling studies (Voegtlin and Martinez 2007; Volman et al. 2011) showed that the synchronization of neural network was decreased when the inhibitory transmission became asynchronous. Taken together, we speculate that the reduction of AR in adult animals may promote the synchronization of cortical network at appropriate frequencies and contribute to the proper functioning of the cortex; changes in AR strength may cause an alteration of rhythmic oscillations and consequently affect the behavioral performance. We reported recently that the AR strength is substantially enhanced in both human and rat epileptic neocortical tissues (Jiang et al. 2012). This upregulation of AR may play a role in regulating epileptiform activity but, on the other hand, it can disrupt normal cortical oscillations that are critical for cognitive functions. The reduction of AR in adult animals is therefore important for normal brain functioning; in contrast, stronger AR at early developmental stages may prevent hypersynchronization of the cortical network.

It has been reported that the neuronal network showed spontaneous or sensory-driven oscillations in anesthetized animals (Ylinen et al. 1995; Jones et al. 2000) and epileptic human patients (Staba et al. 2002). These oscillations could reach a frequency of 100–600 Hz; importantly, FS cells can generate APs with frequencies up to 500 Hz and follow these fast oscillations (Ylinen et al. 1995; Jones et al. 2000), indicating a role of FS cells in shaping these high-frequency oscillations. Considering that AR could be evoked when FS cells discharge at frequencies larger than 100 Hz, we speculate that under certain physiological and pathological conditions asynchronous GABA release may regulate the firing behavior of their postsynaptic targets and network function.

Taken together, we demonstrated a developmental decrease in AR strength of FS neurons from juvenile to adult rats, and this reduction could result from the acceleration of Ca$^{2+}$ decay phase in adult FS neurons. Given the key role of GABAergic inhibition induced by FS neurons in regulating circuit maturation and neuronal synchronization, the reduction in asynchronous GABA release from FS neurons during development may differentially modulate the functional organization and performance of neural networks at distinct behavioral states.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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**References**


