Strength and Diversity of Inhibitory Signaling Differentiates Primate Anterior Cingulate from Lateral Prefrontal Cortex

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The lateral prefrontal cortex (LPFC) and anterior cingulate cortex (ACC) of the primate play distinctive roles in the mediation of complex cognitive tasks. Compared with the LPFC, integration of information by the ACC can span longer timescales and requires stronger engagement of inhibitory processes. Here, we reveal the synaptic mechanism likely to underlie these differences using *in vitro* patch-clamp recordings of synaptic events and multiscale imaging of synaptic markers in rhesus monkeys. Although excitatory synaptic signaling does not differ, the level of synaptic inhibition is much higher in ACC than LPFC layer 3 pyramidal neurons, with a significantly higher frequency ($\sim 6\times$) and longer duration of inhibitory synaptic currents. The number of inhibitory synapses and the ratio of cholecystinin to parvalbumin-positive inhibitory inputs are also significantly higher in ACC compared with LPFC neurons. Therefore, inhibition is functionally and structurally more robust and diverse in ACC than in LPFC, resulting in a lower excitatory: inhibitory ratio and a greater dynamic range for signal integration and network oscillation by the ACC. These differences in inhibitory circuitry likely underlie the distinctive network dynamics in ACC and LPC during normal and pathological brain states.

Key words: electron microscopy; *in vitro* slice; inhibitory neurons; prefrontal cortex; ultrastructure; whole-cell patch clamp

Significance Statement

The lateral prefrontal cortex (LPFC) and anterior cingulate cortex (ACC) play temporally distinct roles during the execution of cognitive tasks (rapid working memory during ongoing tasks and long-term memory to guide future action, respectively). Compared with LPFC-mediated tasks, ACC-mediated tasks can span longer timescales and require stronger engagement of inhibition. This study shows that inhibitory signaling is much more robust and diverse in the ACC than in the LPFC. Therefore, there is a lower excitatory: inhibitory synaptic ratio and a greater dynamic range for signal integration and oscillatory behavior in the ACC. These significant differences in inhibitory synaptic transmission form an important basis for the differential timing of cognitive processing by the LPFC and ACC in normal and pathological brain states.

Introduction

The lateral prefrontal cortex (LPFC) and anterior cingulate cortex (ACC) in primates comprise the frontal executive control network that enhances task-relevant and suppresses task-irrelevant information to guide behavior (for review, see Knight et al., 1999; Miller and Cohen, 2001; Barbas et al., 2002; Rushworth et al., 2011). The LPFC continuously updates information

in “working memory” for the task at hand by rapidly encoding and transiently storing sensory–motor information (for review, see Levy and Goldman-Rakic, 2000; Fuster, 2001; Petrides, 2005). As cognitive demands increase (i.e., there are a higher number of temporally distinct variables to consider) the ACC becomes engaged (Johnston et al., 2007; Kaping et al., 2011). The ACC encodes conflict, error, and motivational signals, accessing information from both short- and long-term memory to guide behaviors that span both rapid and longer timescales (MacDonald et al., 2000; Ito et al., 2003; Murray et al., 2014; Kolling et al., 2016; Stoll et al., 2016; for review, see Bush et al., 2000; Botvinick, 2007; Lee et al., 2007; Schall and Boucher, 2007). The synaptic and network mechanisms underlying these key differences in processing dynamics remain poorly understood.

Excitatory and inhibitory synaptic networks determine the spatial and temporal dynamics of signal enhancement or suppression during a task (for review, see Vogels and Abbott, 2009). Excitatory pyramidal neurons in layers 2–3 are integrally involved in corticocortical communication and differ in structure

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and function depending on cortical area (Elston, 2002; Amatrudo et al., 2012). How local and long-range synaptic inputs are integrated across pyramidal neuron dendritic trees in distinct cortical areas in primates is unknown. Inhibitory neurons, which are highly diverse in neurochemistry, morphology, and physiology, target distinct morphological compartments of pyramidal neurons (for review, see DeFelipe, 1997; Markram et al., 2004; Kubota et al., 2016). Calbindin-positive (CB^+) double-bouquet and Martinotti cells target distal dendrites to provide nuanced inhibitory regulation of electrotonically distant inputs (for review, see DeFelipe, 1997), whereas fast-spiking parvalbumin-positive (PV^+) basket cells provide rapid and powerful inhibitory control by targeting somata and proximal dendritic segments (Williams et al., 1992; González-Burgos et al., 2005; Kubota et al., 2015). Cholecystokinin-positive (CCK^+) interneurons also target somata and proximal dendrites, but fire at lower rates and generate synaptic currents with slower kinetics compared with PV^+ neurons (for review, see Freund and Katona, 2007; Bartos and Elgueta, 2012). Inhibitory neurons regulate the gain and dynamic range of excitatory signals, set the time window for reception of inputs, and are crucial for brain oscillations (for review, see Buzsáki et al., 2007; Kepecs and Fishell, 2014). At a behavioral level, inhibitory mechanisms suppress irrelevant signals and control the temporal dynamics of relevant ones (Rao et al., 2000; Wang et al., 2004; Wilson et al., 2012), capacities that differ between ACC and LPFC. The ACC plays a more critical role than the LPFC in goal-directed behaviors dependent on robust inhibition of irrelevant or erroneous signals (MacDonald et al., 2000; Ito et al., 2003; Matsumoto and Tanaka, 2004; Johnston et al., 2007; Watanabe, 2007; Chen et al., 2008; Shen et al., 2015; for review, see Badre and Wagner, 2004; Lee et al., 2007). Coupling of theta and gamma oscillations, which are dependent on inhibition (Kopell et al., 2010), have distinct temporal dynamics in the ACC and LPFC during behavioral tasks (Tsujimoto et al., 2010; Voloh et al., 2015; Stoll et al., 2016).

The detailed properties of networks underlying excitatory: inhibitory (E:I) synaptic balance and oscillatory behavior within the primate ACC and LPFC are largely unknown. Disruption of E:I balance in these areas due to selective loss of PV^+ basket cells is a core pathological feature of schizophrenia (for review, see Benes, 2000; Cotter et al., 2002; Vogels and Abbott, 2007; Allen et al., 2008; Eisenberg and Berman, 2010). Here, we show that inhibition differs markedly in the two areas, with a higher magnitude and diversity of synaptic inhibition in ACC compared with LPFC. These differences in inhibitory circuitry likely underlie the distinctive network dynamics in ACC and LPC during normal and pathological brain states.

Materials and Methods

Experimental subjects. Adult rhesus monkeys (*Macaca mulatta*; 11.9 $\pm$ 1.5 years old; range = 5–20 years) were used for studies of pyramidal neurons (5 male, 9 female), immunohistochemical studies (3 male, 1 female), and ultrastructural studies (4 female). All monkeys were part of a larger program of studies on brain and cognition. Monkeys were obtained from the Yerkes National Primate Research Center (RRID: SCR_001914) at Emory University (Atlanta, GA) and housed individually in the Laboratory Animal Science Center at Boston University School of Medicine; both facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, with animal research conducted in strict accordance with guidelines of the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Perfusion and preparation of acute slices. Preparation of acute slices for recordings was performed as described previously (Amatrudo et al., 2012). After sedation with ketamine hydrochloride (10 mg/ml) and deep anesthetization with sodium pentobarbital (to effect, 15 mg/kg, i.v.), animals were perfused through the ascending aorta with ice-cold Krebs–Henseleit buffer containing the following (in mM): 6.4 Na_2HPO_4 , 1.4 Na_2PO_4 , 137 NaCl, 2.7 KCl, 5 glucose, 0.3 $CaCl_2$, and 1 $MgCl_2$, pH 7.4 (Sigma-Aldrich). A block of tissue (10 mm³) from the LPFC was taken from the caudal half of the ventral bank of the principal sulcus, Walker's area 46 (Walker, 1940) based on the map of Barbas and Pandya (1989), which is equivalent to area 9/46 in the map of Petrides and Pandya (for review, see Petrides et al., 2012). A second block of tissue of the same size was removed from the ACC within the rostral part of area 24 in the ventral bank of the cingulate sulcus (Barbas and Pandya, 1989; Morecraft et al., 2012; for review, see Petrides et al., 2012). Both blocks were removed from the left hemisphere and sectioned into 300- μ m-thick coronal slices in ice-cold Ringer's solution containing the following (in mM): 26 $NaHCO_3$, 124 NaCl, 2 KCl, 3 KH_2PO_4 , 10 glucose, and 1.3 $MgCl_2$, pH 7.4 (Sigma-Aldrich) with a vibrating microtome. Slices were immediately placed into room temperature, oxygenated (95% O_2 , 5% CO_2) Ringer's solution and equilibrated for 1 h.

Standard tight-seal, whole-cell patch-clamp recordings with simultaneous biocytin filling were obtained from layer 3 (L3) pyramidal cells, as described previously (Amatrudo et al., 2012). The series resistance ranged from 10 to 15 M Ω and was not compensated. Series and access resistance were both monitored frequently during the 15 min recording period and if a change was $>10\%$, then the cell was discarded. Bessel filter frequency was 10 kHz and sampling frequency was at 7 kHz for voltage-clamp recordings and 12 kHz for current-clamp recordings. All recordings were performed at room temperature ($\sim 26^\circ C$).

Potassium methane sulfonate-based solution containing the following (in mM): 122 KCH_3SO_3 , 2 $MgCl_2$, 5 EGTA, and 10 NaHEPES, with 1% biocytin, pH 7.4 (Sigma-Aldrich) was used as internal solution in electrodes (3–6 M Ω resistance). Data were acquired using HEKA EPC-9 or EPC-10 patch-clamp amplifiers interfaced with PatchMaster software and analyzed using FitMaster software (HEKA Elektronik).

Assessment of electrophysiological properties. A series of 200 ms depolarizing and hyperpolarizing current steps were used to determine passive membrane properties [resting membrane potential (V_r), input resistance (R_n), and passive membrane time constant (τ)] and single action potential (AP) properties (threshold, amplitude, rise time, fall time, and duration at half-maximal amplitude, measured from the first AP elicited in the current-clamp series), as described previously (Amatrudo et al., 2012). R_n was calculated as the slope of the best-fit line of the voltage-current linear relationship. τ was assessed by fitting a single exponential function to the membrane potential response to a -10 pA hyperpolarizing current step. Rheobase was determined as the minimum current required to evoke a single AP during a 10 s depolarizing current ramp (0–200 pA). Repetitive AP firing was assessed using a series of 2 s hyperpolarizing and depolarizing current steps (-170 to $+380$ pA using either 20 or 50 pA increments).

Spontaneous EPSCs and IPSCs were recorded for a minimum 2 min at a holding potential of -80 mV for EPSCs and -40 mV for IPSCs. In a few slices, EPSCs were fully blocked by the AMPA receptor antagonist CNQX (10 μ M) and IPSCs were fully blocked by bicuculline methiodide (10 μ M). Analyses of synaptic event data were performed using MiniAnalysis software (Synaptosoft), with event detection threshold set at maximum root mean squared noise level (5 pA). Recordings of synaptic events were assessed for frequency (Hz), mean amplitude (pA), and area under the curve (pAms). The kinetics (rise and decay time constants and half-width in milliseconds) of events was determined from single exponential fits to averaged waveforms (all events in a given cell). The numbers of postsynaptic current traces averaged to obtain mean traces were the following: for IPSCs, ~ 30 events were averaged for LPFC and ~ 200 for ACC neurons; for EPSCs, ~ 400 events were averaged for neurons from both areas. To increase the n for the analyses of IPSCs, data from a subset of LPFC neurons ($n = 10$ cells from 4 animals) studied in (Amatrudo et al., 2012) were included in the current dataset and reanalyzed.

For inclusion in electrophysiological analyses, neurons were required to have a resting membrane potential of ≤ -55 mV, a stable access resistance, an AP overshoot (>0 mV), and the ability to fire repetitive APs in response to prolonged depolarizing current steps.

Processing of filled neurons and immunohistochemical methods. To visualize recorded cells filled with biocytin, 300 μm slices were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, incubated for 2 h in 1% Triton X-100 (in 0.1 M PBS), followed by 48 h in streptavidin–Alexa Fluor 488 (diluted 1:500 in 0.1 M PBS; Invitrogen). To label inhibitory terminals closely apposed to filled neurons, microwave-assisted immunohistochemistry of vesicular GABAergic transporter (VGAT; 1:400, rabbit polyclonal, catalog #AB5062P, RRID:AB_2301998; Millipore) was used in 300 μm slices, as described previously (Medalla and Luebke, 2015). Slices were incubated for 2 h in 50 mM glycine (at room temperature, RT), treated with 10 mM sodium citrate buffer (pH 8.4 at 50–60°C, water bath) for antigen retrieval, and preblocked for 2 h in 5% normal goat serum (NGS) and 5% bovine serum albumin (BSA) with 0.1% Triton X-100. Slices were then incubated in the primary antibody diluted in 1% NGS, 0.2% acetylated BSA (BSA-c, Aurion), and 0.1% Triton X-100 for 6–7 d at 4°C with mild agitation, with 4 interspersed 10 min 150 W microwave sessions at 20°C (Biowave; Ted Pella) to assist in tissue penetration. Slices were then incubated in a goat anti-rabbit secondary F(ab')₂ IgG conjugated to Alexa Fluor 546 (red) over 4 d at 4°C, with 2 microwave sessions (150 W at 20°C, 10 min each run).

To study VGAT colocalization with PV or CCK, we stained VGAT (1:400, guinea pig polyclonal, catalog #131 004, RRID:AB_887873; Synaptic Systems), PV (1:2000, mouse monoclonal, catalog #235, RRID:AB_10000343; Swant), and CCK8 (1:2000, rabbit polyclonal, catalog #20078, RRID:AB_572224; ImmunoStar), together with DAPI, in 30 μm sections from brains perfused with 4% paraformaldehyde, cryoprotected, frozen, and sectioned as described previously (Rosene et al., 1986). Anti-PV was labeled using an Alexa Fluor 488-conjugated goat anti-mouse F(ab')₂ IgG secondary (1:200; Invitrogen). Anti-VGAT was labeled using a biotinylated goat anti-guinea pig F(ab')₂ IgG secondary (1:200; Jackson ImmunoResearch Laboratories), which was subsequently incubated in Alexa Fluor 633-conjugated Streptavidin (1:200; Invitrogen) overnight at 4°C. Anti-CCK was labeled using an HRP-conjugated goat anti-rabbit IgG secondary (1:200, 2 h; Jackson ImmunoResearch), which was subsequently reacted with Alexa Fluor 568-conjugated tyramide (in 0.005% H₂O₂ for 30 min at RT; Invitrogen catalog #T20949). Control experiments in which the primary antibody was omitted showed no labeling. Sections were mounted on glass slides and coverslipped with Prolong anti-fade medium (Invitrogen).

Confocal imaging and analyses of filled neurons and VGAT⁺ appositions. Neurons with complete somata and dendritic fills with no cut branches in the proximal third of the apical dendritic tree were included in morphological analyses. Image stacks were acquired using a Zeiss 510 or 710 confocal laser-scanning microscope. For imaging entire neurons to assess whole-cell dendritic and spine topology, stacks were acquired using a 40 $\times$ /1.3 numerical aperture (NA) oil-immersion objective (210 μm working distance; Plan-Apochromat; Zeiss) at a resolution of $0.1 \times 0.1 \times 0.3$ μm per voxel. Image stacks were then deconvolved using AutoQuant (Media Cybernetics), tiled using Volume Integration and Alignment System (VIAS) software (Rodriguez et al., 2003), and neurons were reconstructed in 3D using NeuronStudio (RRID:SCR_013798; Rodriguez et al., 2006). Spines across entire arbors were marked manually and classified into subtypes and by size, as described previously (Medalla and Luebke, 2015). Spines with a head width of <0.6 μm were classified as either thin or filopodia, with thin spines having a neck length ≤ 3 μm and filopodia having a neck length >3 μm . Spines with a head width of ≥ 0.6 μm were classified as mushroom shaped. Spines lacking a neck were classified as stubby shaped. Densities of total spines and of each subtype were calculated as the number of spines per micron of dendritic length. The resulting measured spine head widths for each cell were then used to assess the size of spines. K-means cluster analysis, an unsupervised learning algorithm that defines k centroids (clusters), which was performed using SPSS (version 19, RRID:SCR_002865; IBM) defined a cutoff point to separate the population of spines into two different clusters: “large” (spine head width ≥ 0.52) and “small” spines (spine head width <0.52

μm). Sholl analyses with concentric spheres placed at 20 μm increments from the center of the soma (Sholl, 1953) were used to determine dendritic and spine parameters of apical and basal arbors as a function of distance from the center of the soma.

For assessment of filled spines and dendrites apposed to VGAT⁺ boutons, a second series of image stacks were acquired via dual-channel confocal imaging at a resolution of $0.04 \times 0.04 \times 0.2$ μm per voxel, using a 100 $\times$ /1.3 NA or 63 $\times$ /1.4 NA oil-immersion objective (UPlan-FL, Olympus, or Plan-Apochromat, Zeiss) and the argon 488 nm (green channel) together with either a 561 nm diode (red channel), a helium–neon 543 nm excitation laser (red channel), or a helium–neon 633 nm excitation laser (far-red channel). For each neuron, confocal stacks within 100 μm from the tissue surface were acquired to image one complete basilar dendritic branch and the main apical trunk followed to the end of one complete distal apical dendritic branch. Image stacks were deconvolved (as above) and tiled and analyzed using NeuroLucida (RRID:nif-0000-10294; MBF Bioscience).

Appositions between the neurons and VGAT⁺ puncta were classified as follows: apposed to dendritic shafts, spine head, or neck of each subtype, soma, or axon initial segment (AIS). An apposition was identified as a spatial overlap between the saturated red and green signal greater than >2 pixels in the x – y ($\sim >0.1 \times 0.1$ μm), >2 optical slices in the z ($\sim >0.4$ μm) for thin dendrites and axons, or >4 optical slices in the z ($\sim >0.8$ μm) for highly saturated segments of thick dendrites and somata. Densities of appositions were calculated as the number per micron of dendritic length. Sholl analyses of each branch (10 μm spheres) were used to determine the shaft or spine apposition density as a function of distance from the soma. Appositions on the AIS were quantified as the number per micron of the first 50 μm of axon proximal to the soma. Appositions on somata were quantified as the number per surface area of somata. Profiles of somata were automatically traced section by section and the surface area of somata was calculated using NeuroLucida software as a series of cylindrical sections capped by the end profiles (surface area per cylinder = perimeter of the profile $\times$ distance to the next profile).

Assessment of VGAT label density and colocalization with inhibitory neuron markers in the neuropil. Confocal image stacks of thin 30 μm sections labeled for VGAT and inhibitory markers were acquired (63 $\times$ /1.3 NA, oil-immersion; $0.07 \times 0.07 \times 0.2$ μm voxel) from 1–2 fields within layer 2–3 neuropil of ACC area 24 and LPFC area 46 from 4 animals, as described above. In each section of the image stack, the density of VGAT labeling in the neuropil was assessed using the particle analysis function of ImageJ (RRID:SCR_003070; Schneider et al., 2012) and quantified as the number of labeled puncta and fractional area covered (as a fraction of the total sampled site) by labeled puncta in each confocal stack ($n = 4$ for each area). To assess the density of perisomatic VGAT⁺ label, profiles of pyramidal somata with clear nucleoli identified with DAPI staining surrounded by pyramidal-shaped cytoplasm and apical dendrites visualized by autofluorescence were traced in every other optical slice using ImageJ ROI manager and particle analysis was conducted. Perisomatic VGAT⁺ density per cell was expressed as the number of particles and fractional area of VGAT⁺ label divided by the cylindrical surface area of each soma examined (perimeter of profiles examined $\times$ distance between profiles; end caps were not counted because most neuronal somata were not fully reconstructed).

To assess VGAT⁺ puncta dual labeled with CCK⁺ or PV⁺, an ImageJ colocalization plug-in was used to mark colocalized pixels across the confocal stack, as described previously (Medalla and Luebke, 2015). Particle analysis of colocalized points in the neuropil and around pyramidal somata was used as above. A second manual counting method was used by a second observer; pyramidal somata were traced in 3D across the entire stack and VGAT⁺ puncta with/without PV or CCK colocalization were marked manually using Reconstruct software (RRID:SCR_002716; Fiala, 2005). The following subsets of VGAT⁺ puncta were identified and counted as follows: (1) VGAT⁺ only, (2) VGAT⁺ PV⁺, and (3) VGAT⁺ CCK⁺. The density and proportion of each subset of perisomatic VGAT⁺ bouton population were quantified per soma. A total of $n = 38$ somata in ACC and $n = 41$ somata in LPFC from 4 animals were assessed.

3D serial electron microscopy and reconstruction of synapses. Excitatory and inhibitory synapses in L3 neuropil of ACC ($n = 4$ tissue blocks from

2 cases) and LPFC ($n = 4$ tissue blocks from 4 cases) were assessed with electron microscopy using archived tissue blocks from the collection of Dr. Alan Peters (Boston University School of Medicine, Boston, MA). Monkeys used only for ultrastructural studies were perfused with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate or phosphate buffer, pH 7.4, as described previously (Peters et al., 2008). The brains were then postfixed for at least 1 week at 4°C in 2% paraformaldehyde and 2.5% glutaraldehyde in the same buffer used for the perfusion. Small blocks of tissue taken from ACC area 24 and LPFC area 46 were osmicated, dehydrated in increasing gradients of alcohol, infiltrated with propylene oxide, and embedded in Araldite resin, as described previously (Peters et al., 2008). Data for LPFC were partially derived from a previously published dataset (Medalla and Luebke, 2015). Embedded blocks were precision trimmed (average block face area = $150 \times 500 \mu\text{m}$) using an ultramicrotome (Ultracut; Leica) and a diamond trim tool (Diatome) to include the entire depth of L3 ($\sim 250 \mu\text{m}$ from the pia to $\sim 500 \mu\text{m}$ deep) for serial sectioning.

Ultrathin serial sectioning, grid staining, and scoping of serial sections were as described previously (Medalla and Luebke, 2015). To analyze synapses on dendrites (presumably more distal) in the L3 neuropil, 1–2 fields devoid of cell bodies were photographed throughout 30–60 serial sections at 25,000 $\times$ magnification using a JEOL JEM 1011 at 80 kV with a digital camera (Gatan). Serial images were aligned and calibrated using Reconstruct (RRID:SCR_002716; Fiala, 2005) and synapses in the series were counted using stereological methods (Fiala and Harris, 2001). Synapses and postsynaptic sites were identified based on classic criteria (Peters et al., 1991): a dense postsynaptic density (PSD), wide synaptic cleft, and round presynaptic vesicles for an asymmetric synapse and an indistinct PSD, narrow synaptic cleft, and pleomorphic presynaptic vesicles for a symmetric synapse. The subset of synapses completed in the series were reconstructed in 3D and imported in 3D Studio Max (Autodesk) for additional rendering. An average of $n = 55$ synapses was reconstructed per case in ACC (mean sampled volume = $127 \mu\text{m}^3$) and $n = 108$ per case in LPFC (mean volume = $465 \mu\text{m}^3$).

To analyze inhibitory synapses on somata and proximal apical dendrites of pyramidal neurons, 10–15 neurons pyramidal neurons per area were identified based on classic criteria: the presence of a clear nucleolus, pyramidal-shaped soma, and a clear apical dendrite (Peters et al., 1991). Each pyramidal neuron was photographed serially at low magnification (3000 $\times$) and examined for synapses in every three sections. Synapses were counted stereologically and photographed serially in every other section at high magnification (20,000 $\times$). The number of total symmetric synapses per surface area of cell body or apical dendrite was calculated for each pyramidal neuron.

Statistical analyses and computational simulations. All data are expressed as mean $\pm$ SEM, plotted as vertical scatter plots of individual data points in Microsoft Excel, or plotted as box-and-whisker plots in MATLAB (R2016, RRID:SCR_001622; The MathWorks). For each variable, differences between the groups were assessed using independent-samples Student's t test. For comparing VGAT⁺ optical density measured from multiple sampling sites in each area and each case, a two-factor nested ANOVA in SPSS (version 19, RRID:SCR_002865; IBM) was used to determine that results were not dependent on sampling site. Relationships between variables were determined using linear and nonlinear regression analyses conducted in SPSS or MATLAB. Significance was set at $\alpha = 0.05$ for all statistical tests.

A MATLAB-based model of pyramidal-interneuron network gamma (PING) was used to simulate how inhibition can affect oscillations in ACC and LPFC, as described previously (Kopell et al., 2010 and available on ModelDB (RRID:SCR_007271, Accession #138421; <http://senselab.med.yale.edu/ModelDB>). The model included 80 excitatory (E) and 20 inhibitory (I) cells, with intrinsic properties based on standard Hodgkin-Huxley equations as detailed in previous studies (for review, see Kopell et al., 2010). The network is all-to-all connected, that is, with probabilities of connection, p_{IE} , p_{EE} , p_{EI} , and $p_{II} = 1$. Each synaptic connection is characterized by a gating variable associated with the presynaptic neuron dependent on synaptic current rise and decay time constants, as described mathematically in Kopell et al. (2010). Total synaptic input from neuron i to neuron j was modeled by adding the term $g_{ij} \cdot s_i(t) \cdot (V_{\text{rev}} - V_j)$

to the right side of the equation for the membrane potential V_j of neuron j . Here, g_{ij} denotes the maximal conductance associated with the synapse, s_i the gating variable associated with neuron i , and V_{rev} the synaptic reversal potential. Excitatory currents were presumed to be AMPA mediated (rise time constant $\tau_R = 0.1$ ms, $V_{\text{rev,E}} = 0$ mV) and inhibitory currents were presumed to be GABA mediated ($\tau_R = 0.1$ ms, $V_{\text{rev,I}} = -80$ mV). For both E and I synaptic connections, the decay time constants (τ_D) were varied according to empirical data obtained here. The model code included a MATLAB parameter file in which values were specified to run the simulation. Parameter values used in our simulations here matched those that elicited weak PING oscillations in Kopell et al. (2010). Namely, maximal conductances were $g_{EI} = 0.5$, $g_{IE} = 1.5$, and $g_{II} = 0.5$ (all mS/cm²). Model E neurons received external inputs as a deterministic current of 1.25 mA/cm² plus Poisson-distributed stochastic inputs with a frequency of 20 Hz. Model I neurons only received input through the network itself. Previous mathematical analysis and computational experiments (Börger and Kopell, 2005; Gloveli et al., 2005; Tort et al., 2007; Kopell et al., 2010) have shown that the frequency of oscillations in the PING model is a decreasing function of IPSC τ_D and g_{IE} ; the periodicity of rhythm is linearly dependent on decay time constants of IPSCs and logarithmically dependent on g_{IE} .

Results

Dendritic topology of L3 pyramidal neurons

Somatodendritic morphology, which influences the capacity for synaptic integration and filtering (Spruston, 2008), was assessed in ACC and LPFC L3 neurons (Fig. 1), the somata of which were located within 250–500 μm from the pia. Mean soma-to-pia distance (ACC, $426 \pm 29 \mu\text{m}$; LPFC, $379 \pm 31 \mu\text{m}$; $p = 0.26$, Student's t test) and soma surface area (ACC, $488 \pm 105 \mu\text{m}^2$; LPFC, $389 \pm 68 \mu\text{m}^2$; $p = 0.42$) were similar in the two areas. Neurons in ACC and LPFC did not differ in mean total, apical, and basal dendritic length or branch number (Fig. 1A–D), but basilar branch density was higher in LPFC than in ACC neurons ($p = 0.00002$; Fig. 1E). The dendritic span of basal arbors was wider in ACC than in LPFC neurons, having greater horizontal ($p = 0.00005$) and vertical extents ($p = 0.0002$; Fig. 1B,F). For apical arbors, horizontal ($p = 0.04$), but not vertical ($p = 0.78$) extent, was significantly wider in ACC than LPFC neurons (Fig. 1B,F). Sholl analyses (Sholl, 1953) revealed that dendritic lengths of ACC neurons were significantly longer than LPFC neurons in selected distal apical (160, 300, and 320 μm) and basal (140–180 μm from the soma) dendritic segments ($*p < 0.05$; Fig. 1G), whereas complexity (branch points) did not differ (Fig. 1G). The dendritic arbors of L3 pyramidal neurons in ACC are modestly but significantly larger than those of LPFC neurons.

Excitatory synaptic responses and spines in ACC versus LPFC pyramidal neurons

Spontaneous EPSCs of L3 pyramidal neurons in ACC and LPFC (Fig. 2A,B) did not differ in frequency, amplitude, or kinetics (Fig. 2A,B). However, the density of dendritic spines—the sites of excitatory synapses—was $\sim 1.3\times$ higher on ACC neurons than on LPFC neurons ($p < 0.001$; Fig. 2D). Analyses of spine subtypes revealed a significantly higher number, density, and proportion of thin spines ($*p < 0.03$; Fig. 2C,D) and a lower proportion of stubby spines ($*p = 0.01$, apical; $*p = 0.009$, basal; Fig. 2D) in ACC versus LPFC neurons. The relative distribution of spines along the extent of dendritic arbors did not differ in neurons from ACC and LPFC (Fig. 2E,F, top), but total spine numbers in select distal apical (160 and 320 μm from the soma) and basal (140–180 μm) dendritic segments were higher in ACC versus LPFC neurons ($*p < 0.03$; Fig. 2E). Sholl analyses of spine distribution by subtype revealed that, compared with LPFC neu-

Figure 1. Dendritic architecture of ACC versus LPFC pyramidal neurons. **A**, Top, Medial and lateral surfaces of the rhesus monkey brain showing locations of sampling sites in ACC and LPFC. CC, Corpus callosum; Cg, cingulate sulcus; A, arcuate sulcus; P, principal sulcus. Axes: D, dorsal, V, ventral, R, rostral, C, caudal. Bottom, x - y , y - z , and x - z maximum projections of confocal image stacks of representative L3 pyramidal neurons. **B**, Representative 3D reconstructions of L3 pyramidal neurons in ACC ($n = 10$) and LPFC ($n = 10$). **C–F**, Mean $\pm$ SEM (open symbols) and individual data (filled symbols) for dendritic length (**C**), number of branch points (**D**), branch points per dendritic length ($*p = 0.00002$, t test; **E**), and horizontal and vertical extents of apical ($*p = 0.04$, t test) and basal ($*p = 0.00005$, 0.0002) arbors of ACC and LPFC L3 neurons (**F**). **G**, Sholl analyses of dendritic length (top; p -values, t test: apical $*p = 0.03$, 0.04 , 0.02 ; basal $*p = 0.0009$, 0.0007 , 0.046) and number of dendritic branch points (bottom) as a function of distance from the soma. Data are represented as mean $\pm$ SEM. Scale bars: **A**, top, 1 cm, bottom, 100 μ m; **B**, 100 μ m.

rons, ACC neurons had a greater number and density of thin spines and a lower number and density of stubby spines across the proximal–distal extent of both apical and basal arbors ($*p < 0.05$; Fig. 2E). The size of spine heads was smaller across the proximal–distal extent of basal ($*p < 0.01$; Fig. 2G) and, to a lesser extent, apical arbors of ACC neurons compared with LPFC neurons ($\#p = 0.06$, $*p = 0.05$; Fig. 2G). Separating spines into large versus small (<0.52 μ m, determined by cluster analysis; Medalla and Luebke, 2015) showed that ACC neurons have a higher proportion of small spines compared with LPFC neurons ($*p < 0.04$; Fig. 2H).

Higher density but smaller size of excitatory synapses in ACC versus LPFC

Most excitatory asymmetric synapses in L3 neuropil were located on dendritic spines (~ 81 – 89%), with the rest located on dendritic shafts likely belonging to aspiny/sparsely-spiny interneurons (Fig. 3A). Asymmetric axospinous synapses in ACC neuropil were $\sim 2.2\times$ more dense than in LPFC ($p = 0.0003$; Fig. 3B), but the mean surface area of PSDs was $\sim 42\%$ smaller in ACC ($p = 0.004$; Fig. 3D). Consistent with PSD area findings, the volume of spines of ACC synapses were $\sim 50\%$ smaller than in LPFC ($p = 0.001$; Fig. 3D). There was a significant leftward shift

Figure 2. Similar EPSC properties but distinct spine morphologies in ACC versus LPFC neurons. **A**, Left, Representative traces of spontaneous EPSCs recorded from ACC neurons ($n = 17$ from 4 cases) and LPFC neurons ($n = 18$ from 6 cases); $V_{\text{hold}} = -80$ mV. Right, Averaged EPSC waveforms normalized to peak. **B**, Box-and-whisker plots of EPSC properties. **C**, Confocal x-y projection image of distal apical branches from ACC and LPFC neurons and schematic of spine subtype criteria (t, thin; m, mushroom; s, stubby; f, filopodia). **D**, Mean ($\pm$ SEM) spine number, spine density (total spines and by subtype), and proportion of spines by subtypes on apical (top) and basal (bottom) dendritic arbors. (t test, spine number: apical, $^{\#}p = 0.06$, $^*p = 0.03$; basal $^*p = 0.03$, 0.008 ; spine density: apical $^*p = 0.0009$, 0.0001 ; basal $^*p = 0.001$, 0.00004 ; proportion: apical $^*p = 0.013$, 0.01 ; basal $^*p = 0.012$, 0.009). **E**, Sholl analyses of mean ($\pm$ SEM) spine numbers of total (apical $^*p = 0.03$, 0.05 ; basal $^*p = 0.02$, 0.01 , 0.03), thin (apical $^*p = 0.04$, 0.05 , 0.02 , 0.0003 , 0.01 , 0.03 ; basal $^*p = 0.05$, 0.05 , 0.03 , 0.007 , 0.009 , 0.03), mushroom (basal $^*p = 0.02$, 0.01) and stubby (apical $^*p = 0.003$, 0.04 , 0.03 , 0.054 ; basal $^*p = 0.05$) spines as a function of distance from the soma across the entire apical and basal arbors. **F–H**, Sholl analyses of mean density (number of spines/dendritic length) of total spines (basal 80–120 μ m, $^*p = 0.04$, 0.04 , 0.05 ; **F**), spine head width (apical $^*p = 0.05$, $^{\#}p = 0.06$; basal $^*p = 0.002$, 0.0008 , 0.001 , 0.01 ; **G**), and proportion of large spines (spine width ≥ 0.52 μ m, bottom) determined through cluster analyses (apical $^*p = 0.01$, 0.02 , 0.0007 , 0.04 , 0.02 , 0.03 ; basal $^*p = 0.04$, 0.02 , 0.03 , 0.04 ; **H**). Scale bar in **C**, 2.5 μ m.

Figure 3. Distinct properties of excitatory synapses in L3 neuropil of ACC versus LPFC. **A**, Electron micrographs showing excitatory boutons (green) with perforated (dark blue) versus nonperforated (light blue) asymmetric synapses on dendritic spines (Sp) in ACC (serial sections A1 and A2) and LPFC (A3). Note morphology compared with inhibitory boutons (red) forming symmetric synapses (red arrows) on dendritic spines and shafts. **B**, Numerical density (Nv) of total (t test, $*p = 0.0007$), spine- ($*p = 0.0003$), and shaft-targeting asymmetric synapses in ACC ($n = 3$) versus LPFC ($n = 4$ tissue blocks) neuropil. **C**, Proportions of asymmetric axospinous synapses with perforated PSD (perf), with spine SA/SER ($*p = 0.02$), or with accompanying inhibitory synapse (inh syn). **D**, Mean PSD areas and spine volumes of axospinous synapses (t test: PSD area $*p = 0.004, 0.009, 0.03, 0.01, 0.01$; spine volume $*p = 0.001, 0.004, 0.01, 0.0002, 0.02$). **E**, Cumulative distributions of PSD areas and spine volumes of axospinous synapses. Data in **B–E** are represented as mean $\pm$ SEM. **F**, Scatter plot and linear regression of PSD area versus bouton volume (left) and of bouton volume versus number of vesicles (right, $p < 0.01$). **G**, 3D reconstructions showing top views of perforated and nonperforated PSDs. **H**, 3D reconstructions of presynaptic bouton, PSD and spine of axospinous synapses. Scale: **A**, $0.5 \mu\text{m}$; **G**, $0.1 \mu\text{m}^3$; **H**, $0.5 \mu\text{m}^3$.

(higher frequency of small synapses and spines) of cumulative distribution of synapse and spine size in ACC relative to that in LPFC [Kolmogorov–Smirnov (K-S) test, $p < 0.01$; Fig. 3E]. In both areas, most axospinous synapses (~ 63 – 78%) were nonperforated and the proportion of perforated synapses did not differ (ACC ~ 22 – 32% , LPFC ~ 32 – 38% ; Fig. 3C). The proportion of spines with spine apparatus (SA) or smooth endoplasmic reticulum (SER), a structure associated with calcium reuptake efficacy (for review, see Bourne and Harris, 2008) was higher in LPFC (60–81%) than in ACC (46–63%, $p = 0.02$; Fig. 3C). Subpopulations of axospinous excitatory synapses (e.g., with perforated PSDs, SA, or SER and/or with an accompanying inhibitory synapse) were each larger in LPFC than in ACC ($*p < 0.02$; Fig. 3D). In both ACC and LPFC, PSD area correlated positively with spine volume (data not shown) and with presynaptic bouton volume (ACC, $r = 0.54$; LPFC, $r = 0.62$; $p < 0.01$; Fig. 3F,H). Bouton volume in turn correlated positively with number of synaptic vesicles (ACC, $r = 0.81$; LPFC, $r = 0.81$; $p < 0.01$; Fig. 3F,H).

More frequent, larger, and longer duration inhibitory synaptic currents in ACC versus LPFC neurons

In contrast to EPSCs, IPSCs were very different in ACC and LPFC pyramidal neurons (Fig. 4A,B). The mean frequency of spontaneous IPSCs was $\sim 6\times$ higher in ACC than in LPFC neurons ($p = 0.0005$; Fig. 4B). Moreover, IPSCs of ACC neurons also exhibited larger mean amplitude ($p = 0.01$) and area ($p = 0.003$), and longer mean decay time constant (τ , $p = 0.001$) and half-width ($p = 0.00002$) than those of LPFC neurons. Therefore, the population distribution of events based on frequency (interevent interval) was left shifted, whereas those based on decay times were right shifted in ACC relative to LPFC neurons (K-S test, $p < 0.01$; Fig. 4C). Although mean rise times did not differ between the two areas ($p = 0.13$), the population distribution of event rise times was also right shifted in ACC relative to LPFC neurons (K-S test, $p < 0.01$; Fig. 4C).

Higher density of VGAT⁺ inhibitory boutons apposed to ACC versus LPFC neurons

Quantification of inhibitory boutons immunostained with VGAT⁺ apposed to labeled L3 pyramidal neurons in ACC versus LPFC revealed that most VGAT⁺ appositions were on dendritic shafts (~ 65 – 75%), with fewer on dendritic spines (Fig. 4D–H). There was a significantly higher density of VGAT⁺ appositions onto apical dendritic shafts in ACC than LPFC neurons ($*p = 0.04$; Fig. 4H), whereas the density of appositions on basal dendrites did not differ. The density of appositions on spines was similar between the two areas, but apical dendrites of ACC neurons had a higher proportion of VGAT⁺ appositions on spine necks versus heads compared with LPFC neurons ($*p = 0.008$; Fig. 4I). In both areas, ~ 13 – 22% of all spines were apposed to VGAT⁺ boutons (Fig. 4J) and these occurred primarily on the most prevalent thin spines. However, thin spines with VGAT⁺ appositions comprised only a small ($\sim 14\%$) proportion of the total population of thin spines (Fig. 4J). In contrast, mushroom spines were selectively targeted, because 27–44% of this subtype had VGAT⁺ appositions (Fig. 4J).

The peak density of VGAT⁺ appositions on dendritic shafts occurred within the first 40 μm from the soma, corresponding to the proximal spine-free areas of pyramidal neuron dendrites, followed by a sharp decline to a steady state in the more distal spiny dendritic segments (Fig. 4K). ACC neurons had a higher density of VGAT⁺ appositions in these proximal segments of apical ($p = 0.047$) and basal ($p = 0.02$) dendrites and a portion of the most

distal apical segments (320–380 and 400 μm from the soma, respectively, $p < 0.02$) than LPFC neurons (Fig. 4K). In neurons from both areas, low densities of VGAT⁺ appositions on spines were found in the proximal ~ 60 μm , increasing to peak at ~ 100 μm and then maintaining a relatively constant density (Fig. 4K). VGAT⁺ appositions on spines of distal basal segments were higher in density on ACC than on LPFC neurons ($*p < 0.01$; Fig. 4K).

Neurochemical diversity of perisomatic inhibitory boutons in ACC versus LPFC

Similar to dendritic compartments, the somata ($p = 0.03$) and AIS ($p = 0.007$) of ACC neurons had a higher density of VGAT⁺ appositions compared with LPFC neurons (Fig. 5A,B). Consistent with data on individual filled neurons, there was a higher optical density of VGAT⁺ puncta surrounding dendritic compartments in the neuropil ($p = 0.001$) and somata ($p = 0.0004$, nested ANOVA) in ACC compared with LPFC (Fig. 5C,D). The higher density of inhibitory inputs onto dendrites in ACC versus LPFC is consistent with the higher proportion of dendritic-targeting CB⁺ inhibitory neurons in ACC than in LPFC (Dombrowski et al., 2001). However, the finding of a higher density of perisomatic inhibitory inputs in ACC compared with LPFC is unexpected because the density of somatic-targeting PV⁺ inhibitory neurons is lower in ACC than in LPFC, as shown here qualitatively (Fig. 5E) and as shown quantitatively in previous studies (Dombrowski et al., 2001). Given that pyramidal neuron density is also lower in ACC than in LPFC (Dombrowski et al., 2001), the PV-to-pyramidal neuron ratio is similar between ACC and LPFC, but each PV⁺ neuron may issue more boutons to each pyramidal neuron. Alternatively, there could be other sources of perisomatic inhibition in ACC that are absent or present at lower density in LPFC. To address these possibilities, we investigated the specific sources of VGAT⁺ perisomatic inhibition in ACC and LPFC.

Consistent with the VGAT⁺ particle analyses, the density of VGAT⁺ boutons apposed to L3 pyramidal neuron somata was $\sim 1.5\times$ greater in ACC than LPFC (nested ANOVA, $p = 0.00006$; Fig. 5F). Next, we assessed the density and proportion of these VGAT⁺ perisomatic boutons double labeled with PV⁺ or CCK⁺, which label two different populations of somatic-targeting interneurons (Bartos and Elgueta, 2012; Fig. 5E). The density of perisomatic VGAT⁺ PV⁺ boutons per pyramidal neuron was similar in the two areas ($p = 0.4$), whereas the density of perisomatic VGAT⁺ CCK⁺ boutons was higher in ACC than LPFC ($p = 0.0005$; Fig. 5F), consistent with previous qualitative observations (Oeth and Lewis, 1990). Of the total perisomatic VGAT⁺ boutons on each ACC pyramidal neuron, $\sim 45\%$ were colocalized with PV⁺ and $\sim 32\%$ with CCK⁺. In LPFC, PV⁺ input made up a significantly higher proportion of perisomatic VGAT⁺ appositions compared with ACC ($p = 0.000003$), with $\sim 72\%$ of VGAT⁺ perisomatic boutons colocalized with PV⁺ and only $\sim 20\%$ with CCK⁺ (Fig. 5G). Concomitantly, the proportion of perisomatic CCK⁺ boutons was lower in LPFC than in ACC ($p = 0.0002$). Perisomatic boutons that colocalized with neither CCK nor PV were observed in $\sim 23\%$ of the VGAT⁺ appositions in ACC, but only $\sim 8\%$ in LPFC. Due to the high labeling efficacy of PV and CCK used here and in other studies (Oeth and Lewis, 1990; DeFelipe, 1997) and the fact that we observed highly consistent regional specificity in labeling across cases, these VGAT⁺/PV[−]/CCK[−] appositions likely represent a third population of perisomatic input that, similar to CCK⁺ input, is more abundant in ACC than in LPFC.

Figure 4. Distinct properties of IPSCs and VGAT⁺ input onto ACC versus LPFC pyramidal neurons. **A**, Left, Representative traces of spontaneous IPSCs recorded from ACC ($n = 30$ neurons from 7 cases) and LPFC neurons ($n = 25$ from 10 cases); $V_{\text{hold}} = -40$ mV. Right, Averaged IPSC waveforms normalized to peak. **B**, Box-and-whisker plots of IPSC properties (* p -values, t test: frequency $p = 0.0005$, amplitude $p = 0.01$, decay time constant (τ) $p = 0.001$, area $p = 0.003$). **C**, Cumulative distributions of properties of IPSCs per neuron. Data are represented as mean $\pm$ SEM. **D**, Reconstruction (left, red) and confocal image (right) of apical and basal dendrites subsampled to quantify VGAT⁺ appositions. **E–G**, Single optical plane (**E**) and optical sections in series (**F**, **G**) showing VGAT⁺ appositions (arrows) on dendritic shafts, spine head, and neck. **H**, Density of VGAT⁺ appositions (apps/ μm) on dendritic shafts and spines of ACC ($n = 10$) versus LPFC ($n = 9$) neurons (* $p = 0.04$); mean $\pm$ SEM (open symbols) and data from individual neurons (filled) are shown. **I**, Proportion of VGAT⁺ spine appositions on spine head and neck compartments ($p = 0.008$). **J**, Proportion of spines (total and by subtype) with VGAT appositions on apical (Ap) and basal (Bas) arbors. **K**, Sholl analyses of VGAT⁺ appositions on shafts and spines as a function of distance from the soma. Data in **I–K** are represented as mean $\pm$ SEM. Scale bars: **D**, 100 μm ; **E–G**, 5 μm .

Figure 5. Diverse sources of perisomatic inhibitory input onto ACC versus LPFC L3 pyramidal neurons. **A**, Right, Maximum x - y projection of confocal image stack (5 optical stacks) of filled neuron and VGAT⁺ label in ACC. Left, Insets (**A1**, **A2**) at high magnification; dual-channel (red–green) and single-channel (red) images of single optical planes show VGAT⁺ appositions on the proximal dendrite, soma, and AIS (arrows). **B**, Density of VGAT⁺ appositions on somata (apps/ μm^2 soma area; ACC, $n = 9$ neurons; LPFC $n = 8$, $*p = 0.003$, t test) and AIS (apps/ μm length axon; ACC, $n = 7$; LPFC $n = 9$; $*p = 0.007$, t test) of filled ACC versus LPFC neurons; mean $\pm$ SEM (open symbols) and individual data points (filled) are shown. **C**, Optical density of VGAT⁺ label in L3 neuropil quantified as the fractional area with VGAT⁺ label in fields devoid of cell bodies ($*p = 0.001$, nested ANOVA; $n = 15$ fields from 3 cases for each area). **D**, Optical density of VGAT⁺ label surrounding randomly selected pyramidal somata in L3, quantified as VGAT⁺ labeled area/soma area ($*p = 0.0004$, nested ANOVA), and number of VGAT⁺ particles/soma area ($*p = 0.0000002$, nested ANOVA; ACC, $n = 38$ somata from 3 cases; LPFC $n = 35$ somata from 3 cases). Data in **C** and **D** are represented as mean $\pm$ SEM. **E**, Confocal x - y projection images (each with 10 optical slices) of VGAT⁺, CCK⁺, PV⁺, and neuronal somata (DAPI, blue) label. **F**, Density of total perisomatic VGAT⁺ appositions ($*p = 0.00006$) and VGAT⁺ appositions colocalized with CCK⁺ ($*p = 0.0005$) and PV⁺ in ACC ($n = 36$) versus LPFC ($n = 38$ somata from 4 cases); mean $\pm$ SEM (open symbols) and individual data points per neuron (filled) are shown. **G**, Mean proportion of perisomatic VGAT⁺ colocalized with CCK⁺ and PV⁺. Scale bars in **A** and **E**, $5 \mu\text{m}$.

Higher density of inhibitory synapses in ACC versus LPFC

These confocal microscopy analyses of inhibitory appositions onto pyramidal neurons represent only an estimate of synaptic input. Therefore, we used electron microscopy to quantify the density of inhibitory synapses in ACC versus LPFC neuropil. The density of inhibitory symmetric synapses was $\sim 3.3 \times$ higher in L3 neuropil of ACC than in LPFC (207 ± 49 vs $64 \pm 2 \times 10^6$ synapses/ mm^3 ; $p = 0.04$, t test; Fig. 6A–C), consistent with the

VGAT⁺ confocal data. However, the mean PSD surface area of symmetric synapses was similar between the two areas ($p = 0.68$; Fig. 6D). The majority of symmetric synapses targeted dendritic shafts (~ 69 – 89% in ACC; 75 – 88% in LPFC), with the remainder targeting spines; both synapse types were present at higher densities in ACC than in LPFC neuropil ($*p < 0.05$; Fig. 6C). Symmetric synapses on shafts were similar in size, whereas symmetric synapses on spines had larger PSDs in LPFC compared

Figure 6. Distinct properties of inhibitory synapses in L3 neuropil and somata of ACC versus LPFC. **A**, Electron micrographs of axon terminals (At) each forming a symmetric synapse (red arrows), one on a dendritic shaft (A1) and the other on a spine (Sp, A2) also receiving an asymmetric synapse (blue arrow). **B**, 3D reconstructions of symmetric and asymmetric synapses on the shaft (left) and spine (right) of spiny dendrites. **C**, Numerical density (Nv) of total ($*p = 0.04$), spine- ($*p = 0.04$), and shaft- ($*p = 0.05$, t test) targeting symmetric synapses in ACC ($n = 3$) versus LPFC ($n = 4$ tissue blocks) neuropil. **D**, Cumulative distribution of PSD areas of symmetric synapses. **E, F**, Left panels, Low-magnification electron micrograph of ACC and LPFC pyramidal neuron somata and apical dendrites (green) showing locations (insets) of axosomatic symmetric boutons (red). Right panels, Insets are shown at higher magnification (**E1**, **E2**, **F1**, **F2**) to visualize the details of At's and symmetric PSDs (arrows). **G**, 3D reconstruction of sampled region of a pyramidal neuron where symmetric synapses were quantified. **H**, Density of symmetric synapses on somata ($*p = 0.004$) and proximal apical trunk (first 50 μm ; $\#p = 0.059$) of pyramidal neurons in ACC ($n = 9$) and LPFC ($n = 8$). Data are represented as mean $\pm$ SEM. Scale: **A**, 0.5 μm ; **B**, 0.5 μm^3 ; **E, F**, 5 μm low magnification, 0.5 μm high magnification; **G**, 2.5 μm^3 .

with ACC ($p = 0.02$; Fig. 6D). The density of symmetric synapses on somata and proximal apical trunks of L3 pyramidal neurons in the two areas was assessed (Fig. 6E–H), revealing a higher density in both compartments of ACC neurons than LPFC neurons ($*p = 0.004$, proximal apical $\#p = 0.059$; Fig. 6H, somata).

E:I ratio and physiological properties of ACC versus LPFC neurons

We further investigated how functional differences in inhibitory signaling in the two prefrontal areas relate to excitatory signaling and intrinsic biophysical properties of pyramidal neurons. There were no significant relationships between EPSC and IPSC frequency or area (Fig. 7A) or any other synaptic variable (data not

shown). Due to the higher frequency and larger area of IPSCs in ACC, the mean E:I ratio (EPSC to IPSC frequency $\times$ area) was significantly lower in ACC than in LPFC ($*p = 0.02$, t test; Fig. 7A), indicative of a higher inhibitory tone in ACC.

To determine whether the specific population of ACC neurons with robust inhibition share similar intrinsic electrophysiological features, we further examined relationships between the properties of IPSCs (frequency and decay time constant) and intrinsic membrane and firing properties. Assessments of intrinsic membrane properties revealed that the mean passive membrane time constant (τ_m) of ACC neurons was $\sim 1.6\times$ longer than that of LPFC neurons ($p = 0.0000005$, t test; Fig. 7B, C). V_r of pyramidal neurons in the ACC (-64.4 ± 0.6 mV) and LPFC

Figure 7. Relationship of inhibitory synaptic properties with excitatory synaptic and intrinsic membrane properties. **A**, Relationship of IPSC with EPSC properties of ACC and LPFC L3 neurons. Right, Scatter plots of IPSC versus EPSC frequency (top) and area (bottom) of individual neurons. Left, Box-and-whisker plot of E:I ratio. **B**, Box-and-whisker plots of intrinsic membrane and single AP firing properties in ACC ($n = 50$, from 6 cases) versus LPFC ($n = 34$, from 5 cases) neurons (* p -values, t test: τ_{in} $p = 0.0000005$, R_n $p = 0.13$, rheobase $p = 0.00005$, AP amplitude, $p = 0.00003$; AP duration $p = 0.0000007$). **C**, Difference in τ_{in} shown in hyperpolarizing voltage responses to -30 pA current injection. **D**, Voltage responses to current ramp stimulus; rheobase of ACC neuron (dotted line) lower than LPFC neuron. **E**, Superimposed AP waveforms showing differences in amplitude and duration (dotted line). **F**, Repetitive AP firing responses to $+120$ pA 2 s current pulse. **G**, Left, Mean firing rates ($\pm$ SEM) in response to a series of depolarizing current steps. Right, Linear relationships ($p < 0.01$) of input resistance versus firing rate (at $+130$ pA) in both areas and of τ_{in} versus firing rate in LPFC, but not in ACC, neurons. **H**, Normalized population histograms and cumulative distributions (insets) of τ_{in} and IPSC frequency. **I**, Scatter plots of IPSC frequency and decay time constant versus intrinsic membrane properties. Significant negative linear correlations of IPSC frequency with passive membrane τ_{in} and with R_n (Figure legend continues.)

(-64.3 ± 0.8 mV; $p = 0.9$) and R_n ($p = 0.13$) did not differ significantly. Compared with LPFC neurons, ACC neurons displayed a significantly lower rheobase ($p = 0.00005$; Fig. 7*B,D*). Individual action potentials in ACC neurons had slightly lower amplitude ($p = 0.00003$) and slower kinetics (AP duration; $p = 0.0000007$) compared with those in LPFC neurons (Fig. 7*B,E*). However, neurons in the two areas were similar in terms of repetitive AP firing frequency responses to prolonged depolarizing current injections, especially at the lower current steps between 80 and 180 pA ($p = 0.2$ – 0.8 ; Fig. 7*F,G*). Firing frequency was positively correlated with R_n in neurons from both areas, but firing frequency was positively correlated with τ_m only in LPFC neurons ($p < 0.05$ for all correlations; Fig. 7*G*).

The population distributions of neurons based on IPSC frequency and τ_m showed a positively skewed unimodal distribution in LPFC neurons for both variables, with a high proportion of neurons having low IPSC frequency and short τ_m (Fig. 7*H*). In contrast, ACC neurons showed a somewhat bimodal distribution with higher variability and larger range, specifically due to second peak in the proportion of neurons with higher IPSC frequency and also longer τ_m (Fig. 7*H*). Cumulative histograms of τ_m and IPSC frequency for individual neurons revealed a significant rightward shift of the ACC population curve relative to that of LPFC (K-S test, $p < 0.01$).

Scatter plots of IPSC frequency versus τ_m and versus R_n show that ACC and LPFC neurons formed two largely nonoverlapping groups, with ACC neurons forming a cluster with relatively higher IPSC frequency, longer τ_m , and higher R_n compared with LPFC neurons (Fig. 7*I*, top). IPSC frequency was negatively correlated with τ_m ($r = 0.42$, $p < 0.05$) and R_n ($r = 0.48$, $p < 0.05$) in ACC neurons; neurons with higher IPSC frequencies tend to have shorter τ_m and lower R_n (Fig. 7*I*). A similar trend was observed for LPFC neurons, but the correlation was weaker and not significant. A significant positive correlation was found between IPSC decay time constant and τ_m across all neurons ($r = 0.47$, $p < 0.05$), in which neurons with long IPSC decay time constants also exhibited long τ_m (Fig. 7*I*, bottom). Neither IPSC frequency nor IPSC decay time constant showed significant correlations with rheobase or repetitive AP firing properties (data not shown).

Relationship between inhibitory synaptic physiology and morphology

We then assessed whether functional differences in inhibitory signaling are related to structural differences in inhibitory input onto pyramidal neurons in the two prefrontal areas. There was a positive exponential relationship between IPSC frequency and VGAT⁺ apposition density (Fig. 7*J*), specifically on the apical dendrites ($r = 0.7$, $p < 0.01$) and on the AIS ($r = 0.82$, $p < 0.01$). Within-group regression analyses confirmed this relationship specifically in the population of ACC neurons ($r = 0.8$, $p < 0.05$), but not in LPFC neurons, all of which had low IPSC frequencies and low VGAT⁺ apposition densities. Consistent with the relationship between IPSC properties and τ_m , regression analyses in ACC neurons revealed a negative correlation between VGAT⁺ apposition density on apical

dendrites and τ_m that best fit an exponential function (for ACC, $r = 0.71$, $p < 0.05$; Fig. 7*J*). No significant relationships were found between any IPSC property and VGAT⁺ apposition density on basal arbors, somata, or proximal dendrites.

Distinct temporal dynamics of inhibition in ACC and LPFC

Given that fast and slow IPSCs have distinct effects on network behavior (Bartos et al., 2002; Kopell et al., 2010), we examined the proportion of fast (decay times = 2–14 ms) and slow (>14 to 70 ms) IPSCs in each cell (Fig. 8*A,B*). The mean proportion of IPSCs with slow decay times was higher in ACC compared with LPFC neurons (37 ± 5 vs $16 \pm 4\%$, $p < 0.001$) and their mean maximal decay time was also greater (~70 vs 30 ms; Fig. 8*B*). These findings are consistent with our anatomical results showing that, compared with LPFC, neurons in the ACC receive denser inputs from both dendritic-targeting interneurons and somatic-targeting CCK⁺ interneurons, which have been reported to elicit long-duration IPSCs in rodents (Bartos and Elgueta, 2012). Conversely, inhibitory currents in L3 pyramidal neurons in LPFC on the other hand, were characterized by fast decay kinetics, consistent with a higher proportion of inputs from PV inhibitory neurons, which elicit short duration IPSCs (Bartos and Elgueta, 2012). The schematic shown in Figure 8*C* summarizes the key differences in the anatomical and functional characteristics of inhibitory inputs onto L3 pyramidal neurons in the two prefrontal areas. The higher density of inhibitory synapses and higher frequency of IPSCs, as well more numerous IPSCs with both larger areas and longer decay times, all contribute to a higher total inhibitory conductance (g_i) in ACC compared with LPFC pyramidal neurons.

The strength and temporal dynamics of inhibition have significant effects on network oscillatory behavior. A PING computational model that simulates gamma oscillations in the hippocampus predicts that the period of the oscillatory rhythm depends predominantly on the decay time constant of the GABA_A-mediated inhibitory synapses (Börger and Kopell, 2005; Gloveli et al., 2005; Tort et al., 2007; Kopell et al., 2010). Using this model, we tested whether the different IPSC properties in ACC versus LPFC pyramidal neurons alone were capable of altering the frequency of network oscillatory behavior (Fig. 8*D,E*). Setting IPSC decay time constant to the means for LPFC (12 ms) and ACC (17 ms), together with the EPSC decay time constant set to the mean value for each area (6.4 ms for both), resulted in synchronous firing of pyramidal neurons at frequencies of ~31 Hz in LPFC and ~24 Hz in ACC, both within the mid-beta to low-gamma range (for review, see Benchenane et al., 2011; Fig. 8*D*). To model the combined effect of the longer IPSC decay time and 6× higher frequency in ACC neurons, we also scaled the total maximal conductance of inhibitory synapses to excitatory neurons (g_{IE}) by 6× (Fig. 8*E*). These conditions reduced ACC oscillation frequency to ~14 Hz in the low-beta range. Without the increase in g_{IE} , the model predicted that IPSC decay times of 50–70 ms are required to obtain oscillations within the 5–10 Hz in the theta- α range (Fig. 8*F*). In LPFC pyramidal neurons, the maximum decay times of spontaneous IPSCs was ~30 ms (<1% of events), whereas in ACC pyramidal neurons, a significant proportion of IPSCs had longer decay times, falling between 30 and 70 ms (~9%). Therefore, together, the empirically observed differences in inhibition and network modeling predict that slow oscillations are generated more readily in ACC networks than in LPFC.

(Figure legend continued.) for ACC neurons and a significant positive linear correlation of IPSC decay time constant with passive membrane τ_m were found ($p < 0.05$). *J*, Scatter plots showing positive exponential relationship of VGAT⁺ apposition density versus IPSC frequency for all neurons and a negative correlation of VGAT⁺ apposition density on apical dendrites versus membrane τ_m for ACC neurons only ($p < 0.05$).

Figure 8. Distinct temporal properties of inhibitory microcircuits in ACC and LPFC. **A**, Examples of average trace waveforms of fast (decay times 2–14 ms) and slow (14–70 ms) IPSCs normalized to peak in ACC and LPFC. **B**, Mean population distribution of IPSC decay times in LPFC (light gray, translucent) superimposed on ACC distribution (dark gray) to show overlap and relative proportion (pie chart inset) of fast and slow IPSCs in ACC and LPFC neurons. Data are represented as mean $\pm$ SEM. **C**, Schematic diagram summarizing key differences in the anatomical and functional properties of inhibitory inputs onto L3 pyramidal neurons in the two areas. The higher density of inhibitory synapses, higher IPSC frequency, larger area, and longer decay all contribute to a higher total g_i in ACC compared with LPFC neurons. **D–F**, Simulations of the effects of IPSC kinetics and conductance on network oscillatory behavior based on a PING model (DB accession #138421; Gloveli et al., 2005; Tort et al., 2007; Kopell et al., 2010). **D**, Simulated AP spike rasters of 20 inhibitory neurons (red, bottom) and 80 excitatory pyramidal neurons (black, top) showing oscillatory activity as a result of setting IPSC decay time constants to the mean for LPFC (12 ms) versus the mean for ACC (17 ms; EPSC decay is at 6.4 ms for both). **E**, Prolonging IPSC decay time (17 ms) and scaling g_i by $6\times$ reduces oscillation frequency to ~ 14 Hz; **F**, and setting IPSC decay time between 50 and 70 ms (example shown at 60 ms) can elicit very slow oscillations of ~ 5 –10 Hz within the theta- α range.

Discussion

LPFC neurons integrate and transiently store ongoing sensory-motor information during cognitive tasks (for review, see Fuster, 2001). The ACC is subsequently recruited to help allocate attention and flexibly gate behavioral responses based on additional conflicting, erroneous, and motivational signals (for review, see Paus, 2001; Rushworth et al., 2011). Integration of signals by the ACC spans both short and long behavioral timescales (Kolling et al., 2016; Stoll et al., 2016) and requires strong engagement of inhibitory processes (Shen et al., 2015). We propose that robust and diverse inhibitory inputs endow the ACC circuit with flexibility in integration and gating of output signals based on con-

stantly changing cognitive demands and temporal dynamics during behavior.

Distinct inhibitory modulation of ACC versus LPFC neurons: implications for synaptic balance and temporal dynamics

The E:I ratio in ACC is significantly lower than in LPFC due to the greater frequency and size of IPSCs but similar EPSC properties in ACC versus LPFC neurons. There is a higher density of both inhibitory and excitatory synaptic inputs onto ACC neurons, which would predict a higher frequency of both IPSCs and EPSCs. That this is not the case suggests a higher level of shunting of excitatory by inhibitory currents (for review, see Markram et

al., 2004; Kubota et al., 2016) and a higher degree of filtering of distal excitatory inputs in the larger ACC neurons (Rall, 1964). The lower E:I ratio in ACC neurons does not lead to reduced excitability, however, because these neurons exhibit a lower rheobase and similar steady-state AP firing rates compared with those in LPFC. This demonstrates that E:I ratio, although it may differ broadly, does not necessarily lead to altered firing frequency *in vitro* (Barral and D Reyes, 2016; Denève and Machens, 2016).

Inhibitory synapses impinging on pyramidal neuron somata are favorably situated to influence AP generation (for review, see Freund and Katona, 2007; Kubota et al., 2015). We found that ACC neurons possess a higher density and diversity of perisomatic inhibitory boutons and synapses compared with LPFC neurons. The density of PV⁺ boutons is the same in the two areas, but the density of CCK⁺ boutons is higher in the ACC. Therefore, the proportions of perisomatic PV⁺ and CCK⁺ inputs differ dramatically, with ratios of ~1:1 versus ~3:1 PV:CCK in ACC and LPFC, respectively. PV⁺ and CCK⁺ inhibitory neurons have very different physiological properties and also play distinct roles in network oscillatory dynamics in the hippocampus of rodents (Klausberger et al., 2005). PV⁺ fast-spiking interneurons fire at much higher frequencies than CCK⁺ interneurons (for review, see Freund and Katona, 2007; Lewis et al., 2011; Bartos and Elgueta, 2012). GABA_A receptors found at PV⁺ synapses possess the $\alpha 1$ subunit, which produce IPSCs with fast decay kinetics (Klausberger et al., 2002; Szabadics et al., 2007; Doischer et al., 2008). In contrast, GABA_A receptors at CCK⁺ synapses possess the $\alpha 2$ subunit, resulting in slower IPSC decay kinetics (Klausberger et al., 2002). Moreover, CCK⁺ neurons have a higher rate of asynchronous GABA release that prolongs the overall inhibitory response (Hefft and Jonas, 2005). In summary, kinetically rapid PV⁺ neurons are well suited for rapid input–output transformations and for the generation of fast-gamma oscillations (Bartos et al., 2002; for review, see Kopell et al., 2010; Buzsáki and Wang, 2012). Kinetically slower CCK⁺ neurons are capable of mediating long-lasting inhibition and slower theta-type oscillations (for review, see Bartos and Elgueta, 2012). Because “fast” PV⁺ inputs predominate on LPFC neurons, IPSC decay times are nearly all rapid; in contrast, because “fast” PV⁺ and “slow” CCK⁺ inputs are similar in density on ACC neurons, the range of IPSC decay times is broader and includes a significant proportion (9%) of very slow events. In summary, the near equivalent distribution of PV and CCK inhibition in ACC is consistent with a microcircuit suited for both rapid and long-lasting inhibition and diverse timescales for signal integration.

Slower inhibitory dynamics in ACC versus LPFC neurons: implications for network behavior

Distinct inhibitory networks underlie temporal dynamics of signal processing *in vivo* (Wang et al., 2004; Wilson et al., 2012), which differs between ACC and LPFC (Johnston et al., 2007; Voloh et al., 2015; Stoll et al., 2016). The correlated fluctuation of spontaneous AP activity decays more slowly in the ACC than in the LPFC *in vivo*, indicating that the intrinsic timescales of signal processing differ in the two areas (Murray et al., 2014). Our finding of higher inhibitory tone and longer membrane time constant in ACC neurons likely contribute to lengthening the time window for signal summation in this brain area.

The oscillatory frequencies of cortical networks are dependent on the timescale of inhibitory events (Börgers and Kopell, 2005; Vierling-Claassen et al., 2010). Computational modeling of a pyramidal interneuron network (Gloveli et al., 2005; Tort et al.,

2007; Kopell et al., 2010) predicts that the prolonged time course of IPSCs in ACC will generate slower oscillatory activity in this region (~24 Hz, mid-beta) compared with LPFC (~31 Hz, low-gamma/high-beta). Incorporation into the model of the 6-fold higher frequency of IPSCs in ACC further reduces the oscillation frequency to ~14 Hz in the low-beta range. Gamma and beta oscillations associated with rapid sensory–motor processing and attentional allocation have been observed in both LPFC and ACC *in vivo* (Rothé et al., 2011; for review, see Engel and Fries, 2010; Benchenane et al., 2011; Helfrich and Knight, 2016). The ACC also processes information that occurs at slower timescales, such as integration of past reward context (Kolling et al., 2016). The ACC and LPFC have coordinated but distinct contributions to theta-gamma phase-amplitude coherence during attentional tasks, with slower theta activity being more prominent in ACC than in LPFC (Tsujimoto et al., 2010; Voloh et al., 2015). Indeed, unlike the LPFC, the ACC can access motivational signals from long-term memory through strong connections with the medial temporal lobe, where theta activity is robust (Paz et al., 2008; Takehara-Nishiuchi et al., 2011; Jutras et al., 2013; for review, see Barbas et al., 2013; Likhtik and Paz, 2015). Moreover, compared with LPFC, the ACC receives denser cholinergic innervation (Mesulam et al., 1984; Lewis, 1991; Ghashghaei and Barbas, 2001), an important neuromodulator of theta oscillations (for review, see Heys et al., 2012). Our data further suggest that theta can be induced more readily in ACC than in LPFC at the local circuit level based on the following: (1) the higher density of CCK⁺ boutons in ACC compared with LPFC (in the rodent hippocampus, CCK and PV neurons play key roles in setting the theta cycle; Klausberger et al., 2005) and (2) the higher proportion of very slow IPSCs with 50–70 ms decay times, which, if selectively activated, are predicted to reduce oscillation frequency to 5–10 Hz in the theta- α range. These prolonged inhibitory events likely result from asynchronous GABA release by inhibitory neurons, a mechanism associated with theta activity in rodents (Klausberger et al., 2005). Whether this same mechanism applies to monkey ACC will be important to assess in future empirical and computational studies. In the rodent cortex, long-lasting inhibition of pyramidal neurons by non-fast-spiking interneurons is necessary to drive low-frequency oscillations (Vierling-Claassen et al., 2010). In addition to inputs from fast-spiking PV⁺ interneurons, we show that ACC pyramidal neurons receive a higher proportion inputs from “slow,” non-fast-spiking interneuron types (dendritic-targeting and CCK⁺ perisomatic-targeting) compared with those in LPFC. These two temporally distinct sources of inhibition endow the ACC network with a large dynamic range of synaptic timing and network oscillations. These circuits are well suited for the highly integrative processing in ACC that spans both rapid and long behavioral timescales, allocating attention and monitoring reward outcomes and error to guide ongoing and future action (Ito et al., 2003; Johnston et al., 2007; Kolling et al., 2016); review (Lee et al., 2007; Rushworth et al., 2011).

The ACC–PFC network and inhibitory neurons are selectively vulnerable in schizophrenia (for review, see Allen et al., 2008; Lewis et al., 2011). Multiple lines of evidence suggest that GABA neurotransmission from PV⁺ neurons is decreased, whereas that from CCK⁺ neurons is increased, resulting in a reduced ratio of PV:CCK inhibition in schizophrenia (for review, see Benes, 2000; Reynolds et al., 2001; Curley and Lewis, 2012). Our findings predict that diminished PV⁺ inhibition associated with schizophrenia will remove strong perisomatic inhibition almost completely in LPFC, but only partially in ACC neurons, because the abun-

dant inhibitory drive from CCK neurons will remain intact or even be enhanced within ACC. These region-specific alterations in inhibitory circuitry can result in robust disinhibition of the LPFC, whereas ACC circuits remain relatively inhibited. Moreover, inhibition of ACC activity can lead to further LPFC disinhibition through weakening of the ACC pathway poised to engage inhibition within LPFC (Medalla and Barbas, 2009). A weakening of ACC relative to LPFC activity is consistent with the hypoactivation of the ACC and concomitant hyperactivation of the LPFC observed in schizophrenic patients (Kerns et al., 2005; Allen et al., 2008; Fornito et al., 2009; Leicht et al., 2010; Bersani et al., 2014; for review, see Honey and Fletcher, 2006; Allen et al., 2008). The diverse intrinsic features of excitatory and inhibitory circuits reported here provide a compelling basis to test hypotheses on prefrontal areal dynamics and behavior in normal and neuropathological states.

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