Intraglomerular inhibition shapes the strength and temporal structure of glomerular output

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Shao Z, Puche AC, Liu S, Shipley MT. Intraglomerular inhibition shapes the strength and temporal structure of glomerular output. J Neurophysiol 108: 782–793, 2012. First published May 16, 2012; doi:10.1152/jn.00119.2012.—Odor signals are transmitted to the olfactory bulb by olfactory nerve (ON) synapses onto mitral/tufted cells (MCs) and external tufted cells (ETCs). ETCs, in turn, provide feedforward excitatory input to MCs. MC and ETCs are also regulated by inhibition: intraglomerular and interglomerular inhibitory circuits act at MC and ETC apical dendrites; granule cells (GCs) inhibit MC lateral dendrites via the MC–GC–MC circuit. We investigated the contribution of intraglomerular inhibition to MC and ETCs responses to ON input. ON input evokes initial excitation followed by early, strongly summating inhibitory postsynaptic currents (IPSCs) in MCs; this is followed by prolonged, intermittent IPSCs. The N-methyl-D-aspartate receptor antagonist N-A-aminophosphonovaleric acid, known to suppress GABA release by GCs, reduced late IPSCs but had no effect on early IPSCs. In contrast, selective intraglomerular block of GABA_A receptors eliminated all early IPSCs and caused a 5-fold increase in ON-evoked MC spiking and a 10-fold increase in response duration. ETCs also receive intraglomerular inhibition; blockade of inhibition doubled ETC spike responses. By reducing ETC excitatory drive and directly inhibiting MCs, intraglomerular inhibition is a key factor shaping the strength and temporal structure of MC responses to sensory input. Sensory input generates an intraglomerular excitation-inhibition sequence that limits MC spike output to a brief temporal window. Glomerular circuits may dynamically regulate this input-output window to optimize MC encoding across sniff-sampled inputs.

METHODS

All experiments were performed on olfactory bulb slices obtained from male C57BL6 mice (Charles River, age: 6–8 wk old). Animals were anesthetized with saturated vapor isoflurane and decapitated, and the olfactory bulbs were surgically removed and immediately placed in 4°C oxygenated sucrose-artificial CSF (aCSF) containing (in mM) 26 NaHCO_3, 1 NaH_2PO_4, 3 KCl, 5 MgSO_4, 0.5 CaCl_2, 10 glucose, and 248 sucrose equilibrated with 95% O_2-5% CO_2 (pH 7.38). Horizontal slices (400 μm thick) were cut with a Leica VT1000 vibratome. Slices were incubated in oxygenated aCSF containing (in mM) 124 NaCl, 26 NaHCO_3, 3 KCl, 1.25 NaH_2PO_4, 2 MgSO_4, 2
CaCl$_2$, and 10 glucose equilibrated with 95% O$_2$-5% CO$_2$ (pH 7.38)] at 30°C for 20–30 min and then at room temperature (22°C) in ACSF for at least 1 h before being used. For recording, individual slices were transferred to a recording chamber and perfused with ACSF (as above) at a rate of 2.5 ml/min maintained at a constant 30°C (Bipolar Temperature Controller, Norfolk, VA). MCs were observed with a ×40 water-immersion objective using an Olympus BX51W upright microscope equipped for near-infrared differential interference contrast optics (Olympus Optical). All experimental procedures were carried out in accordance with protocols submitted to and approved by the Institutional Animal Care and Use Committee of the University of Maryland. All drugs used in this study were purchased from Tocris (Ellisville, MO), including α,β-2-amino-5-phosphonovaleric acid (APV), gabazine (SR-95531 hydrobromide), and N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride (QX-314). All other chemicals were purchased from Sigma (St. Louis, MO).

Whole cell (current and voltage) patch-clamp recordings were performed as previously described (Hayar et al. 2001, 2004b, 2005; Heyward et al. 2001; Aungst et al. 2003; Shao et al. 2009). Briefly, recording pipettes were made from thick-wall borosilicate glass with filament (inner diameter: 0.75 mm, Sutter Instrument, Novato, CA) pulled on a P-97 Flaming-Brown puller (Sutter). For current clamp, the internal solution contained (in mM) 120 K-gluconate, 20 KCl, 10 HEPES, 0.2 Na$_2$GTP, and 0.1 BAPTA with 0.02% lucifer yellow (pH 7.3 adjusted with KOH, osmolarity: 287–295 mosM). For voltage clamp, the internal solution contained (in mM) 120 CsMeSO$_4$, 10 QX-314, 10 HEPES, 1 MgCl$_2$, 2.5 Mg$_2$ATP, 0.2 Na$_2$GTP, 0.1 BAPTA, and 10 phosphocreatin with 0.02% lucifer yellow (pH 7.3 adjusted with CsOH, osmolarity: 287–295 mosM). The final pipette tip resistance was 5.0–5.5 MΩ, and seal resistance was routinely >4 GΩ. All data were acquired with pCLAMP 9 software using a MultiClamp 700A amplifier digitized with a Digidata 1322A analog-to-digital board (Axon Instruments) and low-pass filtered online at 2 kHz (voltage clamp, sampling rate: 5 kHz) and 10 kHz (current clamp, sampling rate: 40 kHz). Constant-current electrical stimulation (170-μs duration, PG4000A Digital Stimulator and SIU91 stimulus isolator, Cygnus Technology) was applied with dual-barrel glass microelectrodes (Borosilicate Theta, 5- to 10-μm tip pulled on a P-97 Flaming-Brown puller, filled with ACSF) positioned in the ON layer slightly anterior to the recording site (ON bundles course generally anterior to posterior across the bulb).

MCs were selected for analysis only when they contained an intact apical dendrite with a visible intraglomerular tuft and exhibited membrane bistability in spontaneous activity. Membrane bistability, an intrinsic property of MCs, is sensitive to mechanical damage in slice preparations. MCs close to the cut surface of a slice rarely exhibit membrane bistability, whereas MCs deeper than 50 μm are typically bistable; MCs can lose bistability with time in slices but never regain this property (Heyward 2001). Thus, membrane bistability is indicative of MC “health,” and experiments were performed only on bistable MCs.

ETCs were initially reported in Golgi anatomy studies (Cajal 1911; Macrides and Schneider 1982; Pinching and Powell 1971a, 1971b). Recently, ETCs were rigorously characterized by correlating their morphological and electrophysiological properties (Hayar et al. 2004; Antal et al. 2006). Based on these studies, we defined ETCs by the following criteria: 1) spontaneous intrinsic burst firing that persists even when fast synaptic transmitter receptors are blocked (Hayar et al. 2004; Liu and Shipley 2008), 2) a “pear-shaped” cell body (10- to 15-μm diameter) located in the deep half of the glomerular layer, 3) an apical dendrite with extensively ramified tuft confined to the glomerulus, and 4) the absence of lateral dendrites in the external plexiform layer (EPL). A second type of tufted cell (~30%) has lateral dendrites in the EPL and requires depolarizing current to exhibit bursting (Antal et al. 2006). We recognize cells with these features (Hayar et al. 2004) but term them “superficial tufted cells” to avoid confusion. The present study was restricted to ETCs.

Microinjection of drugs into a target glomerulus was performed using 30-psi pneumatic pressure applied for 2 ms (via a picospritzer, General Valve). Injection pipettes were made from thick-wall borosilicate glass with filament (inner diameter: 0.75 mm) pulled on a vertical pipette puller (6-μm tip opening). These parameters were calibrated to deliver an injection volume of ~20 nl.

Inhibitory postsynaptic currents (IPSCs) were detected on the basis of amplitude and area by having at least a 3:1 signal-to-noise ratio as measured with Minianalysis software (Synaptosoft). In current-clamp recordings, action potentials were detected in pCLAMP and analyzed by Neuroexplorer (NEX Technologies). Comparisons of IPSC-inhibitory postsynaptic potentials evoked by ON stimulation were performed on frequency, amplitude, and area under the curve (charge), as measured by Minianalysis software. IPSCs were detected for each of the multiple sweeps (6–10 sweeps), and the detected IPSCs were averaged from across those sweeps to calculate a perisynaptic time histograms (PSTH) using a custom Excel spreadsheet for each cell. Population PSTHs were calculated by normalizing each cell’s response at each time point to the first bin after ON stimulation in ACSF and averaging each time point and treatment condition across multiple cells to generate the population response. Spontaneous IPSCs were analyzed from a minimum of 2 min of recording and >1,000 IPSCs. Unless otherwise stated, data are represented as means ± SE, with n indicating the number of cells examined. Statistical tests were performed on raw non-normalized data using t-tests, one-way ANOVA, or multivariate ANOVA (MANOVA; following the general linear model and post hoc with the Bonferroni test) to test for statistical significance.

RESULTS

Intralglomerular circuits inhibit MCs and ETCs. GABAergic synapses from GCs onto the lateral dendrites of MCs regulate olfactory bulb output. However, the glomerular layer comprises approximately the same volume as the GC layer, and the number and density of neurons, most of which are inhibitory, is slightly greater in the glomerular layer than the GC layer, suggesting that intralglomerular inhibition may potentially regulate MC activity (Parrish-Aungst et al. 2007). Inhibitory interneurons in the glomerular layer consist of GABAergic PG cells, which primarily innervate a single glomerulus, forming intralglomerular circuits, and dopaminergic/GABAergic short axon cells, which project across multiple glomeruli, forming interglomerular circuits (Aungst et al. 2003; Kiyokage et al. 2010). Dopaminergic/GABAergic short axon cells comprise only ~10% of glomerular interneurons (Parrish-Aungst et al. 2007; Kiyokage et al. 2010). Thus, the major potential source of intralglomerular inhibition is GABAergic PG cells, but little is known of their functional impact on MCs and ETCs.

First, we examined the timing of excitatory and inhibitory currents evoked by sub- and perithreshold ON stimulation in MCs (Fig. 1, A and B). MCs were voltage clamped (~50-mV holding potential) with QX-314 and Cs$_2^+$ in the internal solution; Lucifer yellow was infused to visualize the MC apical dendrite in its target glomerulus (Fig. 1, A and B). A narrow range of minimal current (20.7 ± 0.7 μA, n = 15) sufficed to evoke excitatory postsynaptic currents (EPSCs) in >95% of trials. MCs responded with an initial excitatory deflection consistent with monosynaptic ON→MC input (latency: 2.19 ± 0.07 ms, jitter: 194 ± 16 μs, n = 15; Fig. 1, C–E) (De Saint Jan et al. 2009; Najac et al. 2011). This was augmented by recurrent excitatory input that generates a prolonged inward current, the long-lasting depolarization (LLD; Fig. 1C) (Carlson et al. 2000; Schoppa and Westbrook 2001; Gire
and Schoppa 2008; Urban and Sakmann 2002). As stimulus intensity was decreased, the short latency current dropped out, but in five of nine cells, a longer-latency (4.52 ± 0.64 ms), higher-jitter (1,455 ± 239 μs, n = 5), slow inward current persisted (Fig. 1D). This suggests that at stimulus intensities subthreshold for a given MC, sufficient ETCs in the same glomerulus are engaged to generate longer-latency recurrent excitation in MCs (De Saint Jan et al. 2009; Gire and Schoppa 2009; Najac et al. 2011).

To assess latencies of inhibitory inputs, we recorded ON-evoked IPSCs in MCs voltage clamped at −40 mV (n = 27). At this holding potential, IPSCs generate outward currents clearly distinguishable from inward EPSCs. The mean IPSC onset latency was 6.62 ± 0.09 ms (jitter: 432 ± 49 μs; Fig. 1, F and G). To determine if intraglomerular circuits contribute to this relatively short-latency outward current, we blocked intraglomerular GABA_A receptors by microinjection of gabazine into the glomerulus containing the apical tuft of the recorded MC. Intraglomerular block of GABA_A receptors eliminated early onset ON-evoked IPSPs (Fig. 1F).

**Fig. 1.** Latencies of mitral cell (MC) excitatory and inhibitory responses to olfactory nerve (ON) stimulation. A: schematic of the experimental setup indicating the location of the pipette used to puff gabazine (GBZ). GL, glomerular layer; ONd PGC, ON-driven periglomerular cell (PGC); ETC-driven PGC, external tufted cell (ETC)-driven PGC; EPL, external plexiform layer; MCL, MC layer; GCL, granule cell (GC) layer. B: MC showing a Lucifer yellow (LY) dye-filled apical dendrite, superimposed with a bright field image of the GBZ puff pipette. C: MC responses to ON stimulation with increasing stimulation strength [holding potential (V_h): −50 mV, average of 6–8 sweeps]. In this cell, stimulation intensities below 16 μA elicited no response (purple trace: 10 μA, brown trace: 15 μA); at 16 μA, the cell responded with a slow excitatory ramp into a long-lasting depolarization (LLD; red trace). At 18 μA or greater, a short-latency excitatory postsynaptic current (EPSC) and LLD occurred (blue trace: 18 μA, black trace: 20 μA). D: expanded traces from A showing the latency and onset of ON-evoked MC responses. MCs held at −50 mV responded to suprathreshold ON stimulation with a short-latency EPSC (downward deflection). At 18 μA or greater, a short-latency excitatory postsynaptic current (EPSC) and LLD occurred (blue trace: 18 μA, black trace: 20 μA).
This early onset outward current was due to a compound IPSC that peaked at 11.8 ± 3.5 ms, with 90% of the IPSC integrated area occurring in the first 19.3 ± 2.4 ms (160.3 ± 24.8 pA·ms, n = 7). This fit with a two-parameter exponential predicting a return to baseline at 37.7 ms (curve fit regression R = 0.99). This early compound IPSC was followed by longer-latency, intermittent IPSCs, which generated a smaller integrated area (16.4 ± 2.2 pA·ms, n = 7). The duration of the late IPSC train was 806 ± 133 ms (calculated from the time at which the IPSC integrated area in a rolling 30-ms window returned to 2 SD of baseline, n = 7). Thus, we operationally distinguished two poststimulus time windows for further analysis of inhibition: 0–20 ms, comprising "early" IPSCs, and 21–800 ms, containing "late" IPSCs.

To investigate the contribution of intraglomerular circuits to early and late ON-evoked IPSCs, we puffed gabazine into the glomerulus containing the apical tuft of the recorded MC (Fig. 2). Intraglomerular GABA_A receptor blockade abolished virtually all of the early IPSCs (97 ± 2% reduction in the early IPSC area, P < 0.0001 by MANOVA, n = 4; Fig. 2D) and reduced late IPSCs by 44 ± 9% (P < 0.05 by MANOVA, n = 4). To assess how well gabazine was restricted to the targeted glomerulus, we puffed equal amounts of the drug into an adjacent glomerulus or into the EPL immediately below the target glomerulus. Puffing gabazine into an adjacent glomerulus had no effect on early or late IPSCs (P = 0.9 for early IPSCs and P = 0.4 for late IPSCs by MANOVA, n = 4; Fig. 2E). Gabazine puffed into the EPL below the target glomerulus had no effect on early IPSCs (P = 0.9) but slightly attenuated late IPSCs (11 ± 4% reduction in the late IPSC area, P < 0.05 by MANOVA, n = 4; Fig. 2, F and G). Early IPSCs were eliminated only when gabazine was applied to the glomerulus containing the apical dendrite of the recorded MC.

These results indicate that essentially all early IPSCs and approximately half of the late IPSCs evoked by ON stimulation derive from intraglomerular circuits. We reasoned that most of the remaining late IPSCs reflect GC feedback as they were partially attenuated by gabazine puffed into the EPL. It was not feasible to puff sufficient gabazine into the EPL to block all GC-derived IPSCs without the potential for drug spread into the glomeruli, so we used another strategy to suppress GC input to MCs. In rat bulb slices, GABA release from the GC→MC synapse is dependent on Ca^{2+} influx via activation of the N-methyl-D-aspartate (NMDA) receptor at the MC→GC synapse (Isaacson and Strowbridge 1998; Schoppa et al. 1998; Chen et al. 2000); the NMDA receptor antagonist APV eliminates most MC-evoked IPSCs from GCs. In contrast, APV has little impact on GABA release from PC cells (Hayar et al. 2004b, 2005). We first validated the NMDA receptor dependency of the MC→GC synapse in mouse bulb slices. MCs were step depolarized from −70 to 0 mV in the presence of TTX to evoke recurrent IPSCs. When APV (50 μM) was added to the bath, 79 ± 10% of recurrent IPSCs were eliminated (P < 0.005 by t-test, n = 4; Fig. 3A). The addition of gabazine blocked all IPSCs (not shown).

With GABA release at GC-MC synapses suppressed by bath application of APV (50 μM), ON-evoked early IPSCs in MCs were completely unaffected (7.5 ± 1.4% increase, not significant by MANOVA, n = 4; Fig. 3C), but the late IPSCs were attenuated 55 ± 9% (P < 0.05 by MANOVA, n = 4). This indicates that the MC-GC-MC circuit contributes little, if any, to the early barrage of IPSCs but contributes approximately half of late IPSCs. Lack of APV attenuation of early IPSCs was consistent with reports that PG cell GABA release is relatively independent of NMDA receptor activation (Hayar et al. 2004b, 2005). When gabazine was puffed into the glomerulus containing the apical tuft of the recorded MC with APV still in the bath, early IPSCs were abolished (96 ± 4% reduction, P < 0.0001 by MANOVA, n = 4; Fig. 3D) and late IPSCs suppressed an additional 51 ± 11% from APV alone (P < 0.05 by MANOVA, n = 4; Fig. 3D). After the washout of all drugs (Fig. 3E), a second intraglomerular microinjection of gabazine still abolished early IPSCs (Fig. 3F) and reduced late IPSCs by ~50%, as described above (Fig. 1).

These results demonstrate that early ON-evoked IPSCs derive from intraglomerular circuits. Late IPSCs derive from both intraglomerular and MC→GC→MC circuits. It is important to acknowledge, however, that the relative strengths of inhibition contributed by these two sources cannot be assessed in slice preparations. A major input to GCs consists of excitatory synapses from the olfactory cortex. These inputs generate feedback and possibly tonic inhibition of MCs in vivo, but these connections are severed in slices. In addition, the lateral dendrites of MCs, which are targeted by GC synapses, extend for hundreds of micrometers, and some are inevitably truncated in slices. However, while reduced in slices, GC inputs to lateral dendrites remain and can be activated by direct depolarization of MCs (Fig. 3A). APV strongly attenuates these recurrent IPSCs but has no effect on ON-evoked early IPSCs. Thus, while the relative magnitudes of sensory-evoked inhibition from intraglomerular and GC→MC circuits cannot be inferred from these slice experiments, their dynamics are clearly different: 1) intraglomerular circuits generate rapid onset inhibition that is relatively insensitive to NMDA receptor block but entirely eliminated by local intraglomerular GABAergic block and 2) inhibition from GCs is slower onset and highly sensitive to NMDA receptor block. Thus, MCs are differentially regulated by fast intraglomerular inhibitory inputs that target the apical dendrite and slower GC inputs that synapse on the lateral dendrites.

Intraglomerular inhibition regulates MCs and ETCs. The preceding results show that sensory input generates an excitation-inhibition sequence in MCs and that rapid onset inhibition derives mainly from intraglomerular circuits. Early IPSCs target the intraglomerular dendritic tuft of the MC, which is the site of LLD generation (Carlson et al. 2000; Schoppa and Westbrook 2001; Gire and Schoppa 2008; Urban and Sakmann 2002). Thus, we reasoned that intraglomerular inhibition should attenuate LLDs, and, indeed, when it was blocked by intraglomerular gabazine, both the amplitude and duration of ON-evoked LLDs increased (amplitude: 82.3 ± 13.9 vs. 122.8 ± 12.9 pA with gabazine block, P < 0.01 by t-test, and duration: 2.5 ± 0.4 vs. 2.9 ± 0.5 s with gabazine block, P < 0.05 by t-test, n = 6; Fig. 4). Thus, intraglomerular inhibition attenuates ON-evoked LLD amplitude by 33%, duration by 15%, and integrated area (charge) by 42%. Intraglomerular GABAergic synapses also target ETCs, and ETCs provide feedforward excitation to MCs, contributing to LLD (Carlson et al. 2000; Schoppa and Westbrook 2001; De Saint et al. 2009; Gire and Schoppa 2009; Najac et al. 2011). Thus, intraglomerular inhibition could attenuate LLDs by reducing the output of ETCs, by postsynaptically inhibiting MCs, or both. The previous results show that intraglomerular circuits generate rapid postsynaptic inhibition in MCs. Next, we as-
ON stimulation engages intraglomerular circuits to generate short-latency, summating IPSCs in MCs. A: schematic of the experimental setup indicating different locations of pipettes used to puff GBZ. B: a LY-filled MC superimposed with a bright-field image showing the puff pipette used to deliver GBZ or fluorescent dye into the EPL to assess the spread of the puff. C1, D1, and E1: voltage-clamp recordings ($V_h = -10$ mV; internal solution contained QX-314, Cs$^+$, and LY; bath contains aCSF) of a MC response to ON stimulation. C2, D2, and E2: normalized population peristimulus time histograms (PSTHs) of the IPSC integrated area averaged from four cells. C: control. In aCSF, ON stimulation evoked an initial EPSC followed by a rapid onset barrage of IPSCs and then lower-frequency IPSCs. D: Glomerular injection of 100 μM GBZ abolished early IPSCs and attenuated late IPSCs. E: Injection of GBZ into an adjacent glomerulus had no influence on early or late IPSCs. F1 and G1: voltage-clamp recordings in a separate experiment ($V_h = -10$ mV; internal solution contained QX-314, Cs$^+$, and LY; bath contained aCSF) of a MC response to ON stimulation. F2 and G2: normalized population PSTHs of the IPSC integrated area averaged from four cells. F: control. In aCSF, ON stimulation evoked an initial EPSC followed by a rapid onset barrage of IPSCs and then lower-frequency intermittent IPSCs identical to the previous control cells. G: GBZ injected into the EPL. Early IPSCs were not influenced by the injection of GBZ into the EPL below the glomerulus containing the dendrite of the recorded MC. Late IPSCs were slightly attenuated (11% reduction).
Fig. 3. Intraglomerular and GC inhibition of MCs. A, left: voltage-clamp recording of a MC (internal solution contained high chloride; bath contained 1 μM TTX). Voltage steps from −70 to 0 mV evoked extensive feedback IPSCs. Middle, addition of Dl-amino-5-phosphovaleric acid (APV) to the bath abolished most step depolarization-evoked IPSCs. Right, washout of APV restored evoked IPSCs. B1, C1, D1, E1, F1, and G1: separate experiments with voltage-clamp recording of a MC (Vh = −30 mV; internal solution contained QX-314, Cs2, and LY; bath solution contained aCSF only) showing responses to ON stimulation (5 superimposed sweeps). B2, C2, D2, E2, F2, and G2: normalized population PSTHs for the IPSC integrated area averaged from multiple cells. B: control. In aCSF, ON stimulation evoked an initial EPSC followed by a rapid onset early barrage of IPSCs and longer-latency, lower-frequency IPSCs. C: Bath application of 50 μM APV attenuated late IPSCs with no change of early IPSCs. D: with APV still in the bath, intraglomerular injection of 100 μM GBZ abolished early IPSCs. E: washout of APV and GBZ restored both early and late IPSCs. F: glomerular injection of 100 μM GBZ abolished early IPSCs and reduced late IPSCs by ~45%. G: washout of GBZ restored all IPSCs.
sessed their actions on ETCs. First, we examined the timing of excitatory and inhibitory currents evoked by perithreshold ON stimulation in ETCs voltage clamped at $V_{m}$ to 0 mV (internal Cs$^{2+}$). ON stimulation evoked a short-latency (2.12 ± 0.18 ms), low-jitter (83 ± 10 ms) EPSC ($n$ = 6). This was consistent with previous studies showing monosynaptic excitatory ON input to ETCs (Hayar et al. 2004a, 2004b). The EPSC was followed by a compound IPSC with an onset latency of 6.4 ± 0.5 ms and a jitter of 695 ± 79 ms (Fig. 5A). Bath application of gabazine blocked all spontaneous and ON-evoked IPSCs in ETCs. As ETCs do not have extraglomerular dendrites, they are not targeted by GC synapses. Indeed, when we examined the effect of APV alone, which would block MC excitation of GCs, there were no changes in ON-evoked ETC spiking (2.9 ± 0.2 spikes/stimulus in aCSF vs. 2.8 ± 0.2 spikes/stimulus in APV, not significant by $t$-test, $n$ = 5; Fig. 5, C and D) or spontaneous ETC activity (3.7 ± 1.0 Hz bursting in aCSF vs. 3.4 ± 0.8 Hz bursting in APV, not significant by $t$-test, $n$ = 5).

Thus, we interpret ON-evoked IPSCs to reflect di- or plurisynaptic inhibitory input via either ON→ET→PG circuits, ON→ET→PG→ET circuits, or both (Shao et al. 2009).

Fig. 4. Intraglomerular inhibition reduces MC responses to ON stimulation. A: ON-evoked MC LLDs in control (aCSF, black trace, average of 10 sweeps) and after glomerular injection of 100 μM GBZ (red trace, average of 10 sweeps) into the glomerulus containing the MC apical dendrite. B: plot of ON stimulation-evoked LLD amplitude and duration in aCSF and GBZ.

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Fig. 5. Intraglomerular inhibition of ETCs. A: ETC responses to ON stimulation held at −20 or 0 mV in aCSF (black traces) or GBZ (red traces). ETCs held at −20 mV responded to suprathreshold ON stimulation with a short-latency EPSC (downward deflection) followed by an inhibitory current (upward deflection). ETCs held at 0 mV showed only the prominent upward-deflection IPSC. B: average of five excitatory postsynaptic potentials (EPSPs) in an ETC held at −70 mV after ON stimulation in aCSF (black trace) or GBZ (red trace). $V_m$, membrane voltage. Inset: individual sweeps. C: current-clamp recording in an ETC after ON stimulation in control (aCSF, black trace) and after the addition of 50 μM APV (green trace). D: plot of individual ETC spikes/stimulation in control and after the addition of APV. The gray horizontal bar shows the population mean. E: current-clamp recording in an ETC after ON stimulation in control (aCSF containing 50 μM APV, black trace) and after the addition of 100 μM GBZ (red trace). F: plot of individual ETC spikes/stimulation in control and after the addition of GBZ. The gray horizontal bars show population means.
glomeruli, but these neurons target GCs and PGs not ETCs (Eyre et al. 2008). Taken together, these results indicate that ON excitation of ETCs is regulated by rapid intraglomerular feedback and/or feedforward inhibition by PG cells.

Intraglomerular inhibition should reduce ETC excitability. Figure 5B shows excitatory postsynaptic potentials evoked by ON input in an ETC held below threshold for spiking. When intraglomerular inhibition was blocked, the monosynaptic excitatory postsynaptic potentials were followed by recurrent excitatory inputs. This indicates that inhibition reduces ON-evoked recurrent excitation and suggests that it could limit ETC spike generation. As shown in Fig. 5, C and D, ON stimulation evoked an average of 3.7 ± 0.4 spikes/stimulus. After pharmacological block of intraglomerular inhibition, the same input generated an average of 6.2 ± 0.6 spikes/stimulus (P < 0.0001 by t-test, n = 7; Fig. 5, E and F). Thus, intraglomerular inhibition reduced ETC spike responses to sensory input by nearly one-half. As ETCs provide feedforward excitatory drive to MCs, intraglomerular inhibition would limit ON-evoked ETC excitation of MCs. This is consistent with the finding that block of inhibition by an intraglomerular puff of gabazine increased the amplitude and duration of MC LLDs. Thus, intraglomerular inhibition reduces MC LLDs directly by postsynaptic inhibition of MCs and indirectly by reducing ETC excitatory drive.

These actions of intraglomerular inhibition should limit MC spike generation. MCs responded to perithreshold ON stimulation with a burst of spikes (Fig. 6A). When gabazine was puffed into the glomerulus containing the apical tuft of the recorded MC (n = 10; Fig. 6B), the ON-evoked spike response increased nearly six-fold (582 ± 219%, P < 0.002 by t-test, n = 10; Fig. 6B). This was due in part to a 175 ± 53% increase in spiking the first 100 ms after stimulation (P < 0.05 by t-test). However, the major factor in the overall approximately sixfold increase was a prolongation of the time course of elevated spiking. The ON-evoked MC spike response decayed with a time constant of 93.7 ms (single exponential, 0.95 correlation coefficient), but when intraglomerular inhibition was blocked, the time constant increased nearly 10-fold to 904.2 ms (correlation coefficient: 0.94, P < 0.001). Thus, by reducing ETC excitatory drive and increasing postsynaptic inhibition of MC apical dendrites, intraglomerular inhibition significantly shapes both the strength and the temporal structure of the MC responses to sensory input.

Glomerular circuits tonically inhibit MCs. ETCs spontaneously generate rhythmic, spike bursting, which provides recurring bursts of monosynaptic excitation of PG cells (Hayar et al. 2004a; Shao et al. 2009). This causes intraglomerular GABA release, which activates GABA<sub>B</sub> receptors on ON terminals to produce tonic presynaptic inhibition of sensory input (Shao et al. 2009; Aroniadou-Anderjaska et al. 2000; Pirez and Wachowiak 2008). Thus, we reasoned that ETC-driven GABA release from PG cells should produce tonic postsynaptic inhibition of MCs. To test this, spontaneous IPSCs were recorded in MCs before and after gabazine was puffed into the glomerulus containing its apical dendrite. Intraglomerular microinjection of gabazine reduced the frequency of spontaneous IPSCs by 42% (38.0 ± 8.6 Hz in control vs. 22.3 ± 5.8 Hz with gabazine puff, n = 5, P < 0.05 by t-test; Fig. 7, A–C) and the amplitude by 23% from 24.0 ± 2.8 to 18.5 ± 1.5 pA (n = 5, P < 0.05 by t-test). The decrease in IPSC amplitudes may reflect larger average amplitudes of PG- versus GC-mediated IPSCs in MCs. Upon washout, frequency returned to 97 ± 19% and amplitude returned to 92 ± 12% of control. Thus, in slices, half of all spontaneous IPSCs in MCs are of glomerular origin. This is consistent with spontaneous IPSCs in MCs
reduced by 54% in slices with the glomerular layer cut away (Dong et al. 2007).

Tonic intraglomerular inhibition should regulate MC excitability. If true, then blocking it should increase spontaneous LLDs. Indeed, intraglomerular microinjection of gabazine increased both the amplitude and frequency of spontaneous LLDs (amplitude: $40.8 \pm 2.3$ pA in control vs. $78.2 \pm 7.4$ pA with gabazine puff, $P < 0.01$ by t-test, and frequency: $0.23 \pm 0.05$ Hz in control vs. $0.44 \pm 0.04$ Hz with gabazine, $P < 0.05$ by t-test, $n = 6$; Fig. 7, D–F), but there was no effect on duration ($1.82 \pm 0.12$ s in control vs. $1.83 \pm 0.13$ s with gabazine puff). This suggests that duration may be governed by excitatory mechanisms such as the long-lasting NMDA currents present in MCs (Ennis et al. 1996). The increased amplitude and frequency of spontaneous LLDs caused by intraglomerular GABA$_A$ receptors should increase spontaneous spike generation in MCs. Intraglomerular gabazine puff caused an approximately threefold increase in spontaneous spiking ($1.6 \pm 0.6$ Hz in control vs. $4.6 \pm 1.1$ Hz with gabazine, washout: $1.4 \pm 0.6$ Hz, $P < 0.05$ by t-test, $n = 10$; Fig. 7, G–I). ETC spontaneous spike bursts are generated by intrinsic conductances (Liu and Shipley 2008). Block of all intraglomerular fast synaptic transmission had no impact on ETC bursts per second, but selective block of GABA$_A$ receptors caused an $\sim 20\%$ increase in spikes per burst (Hayar and Ennis 2007). Thus, part of the increased MC excitability after intraglomerular gabazine puff may be due to increased spontaneous ETC drive on MCs in addition to the $\sim 50\%$ reduction in postsynaptic inhibition. These results show that intraglomerular circuits exert tonic inhibition that attenuates LLDs and strongly limits spontaneous spiking in MCs.

**DISCUSSION**

In the neocortex, excitatory “principle” neurons outnumber inhibitory interneurons by $\sim 4$–$5:1$. In the olfactory bulb, this is dramatically reversed, with $5$–$10:1$ inhibitory to principle neurons (Parrish-Aungst et al. 2007). Olfactory bulb inhibitory
neurons comprise two major classes: 1) GCs form serial and reciprocal synapses with the lateral dendrites of the principle output neurons, MCs, and 2) glomerular interneurons form synapses with the apical dendrites of MCs and with a glomerular excitatory neuron, the ETC. While much is known about the functional organization of the MC-GC-MC circuit, our knowledge of inhibition generated by glomerular circuits is limited.

Glomerular circuits transform sensory input into output signals transmitted by MCs to downstream olfactory networks, including the MC-GC-MC circuit. Sensory inputs monosynaptically excite MCs and ETCs, and ETCs provide parallel feedforward excitation that amplifies sensory drive on and promotes synchronous activation of MCs (Hayar et al. 2004b; De Saint Jan et al. 2009; Gire and Schoppa 2009; Najac et al. 2011; Gire et al. 2012). There are two types of glomerular inhibitory neurons: 1) GABAergic PG cells, which innervate a single glomerulus; and 2) dopaminergic/GABAergic short axon cells, which innervate multiple glomeruli. As short axon cells comprise only ~10% of glomerular interneurons (Augnst et al. 2003; Kiyokage et al. 2010; Parrish-Aungst et al. 2007), intraglomerular inhibition derives mainly from PG cells. The present experiments in slices demonstrated that in addition to exciting MCs and ETCs, perithreshold input activates intraglomerular inhibitory circuits. These circuits provide fast feedforward and feedback GABAergic inhibition of MCs and ETCs. Spontaneous activity of MCs and other classes of tufted cells generates tonic inhibition of MCs.

Intraglomerular inhibition regulates MC and ETC excitability. Sensory input generates LLD in MCs but only a brief burst of action potentials during the early phase of the LLD, suggesting that spiking is curtailed by inhibition. Indeed, ON-evoked excitatory currents are followed by inhibition with two distinct temporal phases: an early, summing barrage of IPSCs and later intermittent IPSCs. Intraglomerular block of GABA_A receptors selectively and completely abolishes the early inhibition. Selective suppression of GABA release from GCs reduces late but not early IPSCs. Thus, intraglomerular circuits provide rapid inhibition of MCs.

Intraglomerular inhibition potently controls MC and ETC responses to ON input. When this inhibition is selectively blocked by restricted intraglomerular puffs of a GABA_A receptor antagonist, the MC spike response to ON stimulation increased ~6-fold, but the most pronounced effect was a nearly 10-fold increase in the duration of the spike response. In addition, glomerular GABA_A antagonists nearly doubled ON-evoked ETC spiking. Thus, intraglomerular inhibition exerts a strong brake upon sensory activation of MCs and ETCs. Because ETCs provide feedforward excitation of MCs, intraglomerular inhibition of ETCs should impact feedforward excitatory generation of LLDs in MCs. Indeed, intraglomerular block of GABA_A receptors significantly increased LLD currents, consistent with the generation and regulation of LLDs by intraglomerular circuits (Carlson et al. 2000; Schoppa and Westbrook 2001; Gire and Schoppa 2008; Urban and Sakmann, 2002). Thus, by both reducing ETC feedforward excitation and increasing postsynaptic inhibition of MCs, intraglomerular inhibition potently shapes the strength and temporal structure of glomerular output responses to sensory input.

Intraglomerular circuits also generate tonic inhibition of MCs. This derives from spontaneous bursting of ETCs, which monosynaptically excite two-thirds of all PG cells, causing tonic GABA release (Hayar et al. 2004; Shao et al. 2009). Spontaneous activity of MCs and other classes of tufted cells may also contribute excitatory drive PG cells to tonically release GABA. Tonic intraglomerular GABA release produces postsynaptic inhibition that reduces ON transmitter release by ~30% in slices (Shao et al. 2009) and intact animals (Pirez and Wachowiak 2008). Thus tonic presynaptic inhibition can regulate the gain of sensory input. Sustained intraglomerular GABA release also activates GABA_A receptors on MCs to cause tonic postsynaptic inhibition. This limits spontaneous spiking and may regulate the threshold for sensory-evoked spike generation. Together, tonic pre- and postsynaptic intraglomerular inhibition regulates the gain and threshold of the glomerular input-output function.

Two-thirds of PG cells are most efficiently activated by the ON→ET→PG circuit (Shao et al. 2009). The latency and synaptic jitter of ON-evoked intraglomerular IPSCs in MCs and ETCs is consistent with generation by this plurisynaptic circuit. Moreover, this circuit generates bursts of PG cell output consistent with the compound ON-evoked IPSCs observed in MCs and ETCs. A contribution from the ON→PG circuit cannot be ruled out based on latency data alone, however, and the predicted rapid feedforward IPSC from this circuit might superimpose with the ON-evoked EPSC and be difficult to identify with our approaches.

In addition to regulating magnitude and temporal structure of MC spiking, intraglomerular inhibition may suppress MC responses to weak sensory input. ETCs are activated by weaker ON inputs than MCs (Gire and Schoppa 2009; De Saint Jan et al. 2009; Najac et al. 2011; Gire et al. 2012). Weak sensory activation of ETCs would excite PG cells and increase GABA release above tonic levels. This should increase postsynaptic inhibition of MCs and reduce their sensitivity to weak sensory signals. Thus, intraglomerular circuits could provide fast, feedforward inhibition that could enhance contrast between glomeruli receiving weak and strong sensory signals, a mechanism that would not require spatially ordered chemotopic input to different glomeruli (Cleland and Sethupathy 2006; Cleland 2010).

The MC-GC-MC circuit mediates recurrent and lateral inhibition of MCs and deep tufted cells. There is evidence that GC inputs regularize MC spike timing (David et al. 2008; Giridhar et al. 2011; Schoppa 2006). MCs project heavily to the olfactory cortex, which sends strong feedback excitation to GCs (Balu et al. 2007; Strowbridge 2009). These feedback synapses support paired-pulse facilitation and spike-dependent plasticity in MCs (Balu et al. 2007; Gao and Strowbridge 2009). This cortical feedback might enhance the effectiveness of inhibition of MCs during repetitive activation when odors are sampled by sniffing or play a role in olfactory learning (Doucette et al. 2011).

Glomerulus input-output function. Each glomerulus receives signals from olfactory sensory neurons that express the same odorant receptor; different glomeruli receive input from olfactory sensory neurons sensitive to different odorants. Thus, odor quality is determined, in part at least, by a combinatorial code computed across spatial and temporal patterns of glomerular activity.

The glomerulus input-output function must ensure that sensory signals are processed such that downstream circuits can
decode information across glomeruli and across time. In mammals, odors are sampled by repetitive sniffing. MCs faithfully encode sensory signals across the dynamic range of active sniffing up to 10 Hz (Carey and Wachowiak, 2011; Smear et al. 2011). Interestingly, while the rise time of the MC spike response envelope to odor stimulation is longer than the rise time after synchronous electrical stimulation in slices, the decay times of spiking responses are surprisingly similar (~100 ms) in vivo and in vitro. In vivo, as sniff frequencies increase, MC response rise and decay times are shortened (Carey and Wachowiak 2011). This sharpens temporal response patterns to maintain fidelity with the time-varying input.

Intraglomerular excitatory and inhibitory circuits are well suited to regulate the temporal patterning of MC responses. Spontaneous activity in the ET→PG circuit generates tonic inhibition of MCs, limiting spike generation to background odorants. Weak sensory input should increase tonic postsynaptic inhibition of MCs and raise the threshold for spike initiation. Stronger sensory input produces monosynaptic excitation of MCs augmented by excitatory spike bursts from the ON→ET→MC circuit. This generates a LLD that overcomes tonic inhibition and drives the MC to threshold for action potentials. Excitation is rapidly and potently opposed by inhibition generated by intraglomerular circuits. The time interval between sensory-evoked excitation and intraglomerular inhibition creates a brief window for action potential generation in MCs. The relative strengths of excitation and inhibition, and the timing of these excitatory-inhibitory sequences, determine how MC spiking is gated. Thus, fast feedforward intraglomerular inhibition may be important for gating MC spike output, although more difficult odor discriminations involving longer time courses in vivo may be impacted by late IPSCs from GCs (Abraham et al. 2010).

When ON input frequency increases, as at higher sniffing rates, more ETCs are entrained (Hayar et al. 2004b; Wachowiak and Shipley 2006), increasing synchronous excitatory drive to MCs. This should reduce the MC response rise time. ETC entrainment also increases excitatory drive on the ET→PG→MC circuit, resulting in strong, summatting postsynaptic inhibition, which shortens the decay time of MC spike responses. Indeed, when we blocked intraglomerular inhibition, the spiking decay in MCs was ~10 times longer, indicating that intraglomerular inhibition normally strongly limits the duration of the MC spike response. Thus, the net effect of increased input frequency may be to preserve MC response patterns across changes in sniff frequency by reducing both response rise and decay times. Indeed, a recent in vivo study has shown that increased sniff frequencies were associated with decreased rise and decay times in MC spike responses (Carey and Wachowiak 2011). Such changes in the temporal structure of the MC response may impact processing of olfactory information by downstream olfactory circuits. Cury and Uchida (2010) reported that substantial odor encoding occurs in the first ~100–150 ms of the sniff cycle. The frequency-dependent response window generated by intraglomerular circuits may constrain MCs to encode odor signals early in each sniff cycle.

Although intraglomerular inhibition reduces LLD amplitude, the LLD persists for up to a second. However, spike responses do not summate across sniff cycles in vivo (Carey and Wachowiak 2011). Why don’t excitatory inputs summate with previous LLDs to increase MC responses to subsequent sniffs? One possibility is that MC spike output engages cortical feedback excitation of GCs to inhibit MCs and reduce their sensitivity until the next sniff. Cortical feedback inhibition proportional to MC output strength could operate conjointly with intraglomerular and recurrent GC inhibition to maintain optimal sensitivity to sensory input across changes in sniff frequency and odorant concentration.

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