Novel Cell Types and Circuits in the Mouse Main Olfactory Bulb

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NOVEL CELL TYPES AND CIRCUITS IN THE MOUSE MAIN OLFACTORY BULB

by

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The mammalian olfactory system is strikingly shallow. While peripheral input in other sensory systems is sequentially processed by brainstem, midbrain, and thalamic nuclei before reaching primary sensory and associational cortices, olfactory input is processed by only a single region of the brain – the main olfactory bulb – before reaching higher cortical areas. A tremendous amount of neural processing is thus compressed within the main olfactory bulb, making this region of the brain uniquely well suited for investigating fundamental principles of neural processing.

Currently, the identity and functional roles of multiple cell types and circuits within the main olfactory bulb remain almost entirely unknown, significantly limiting our understanding of olfaction. Herein, I describe a set of studies addressing this broad gap in knowledge. In Chapter 1, I introduce the known cellular and circuit components of the main olfactory bulb. In Chapter 2, I examine the complexity in biophysical cell-to-cell differences among mitral cells, a class of principal neurons in the main olfactory bulb, and quantify how this within-class diversity regulates mitral cell synchrony. In Chapter 3, I systematically explore synaptic and intrinsic biophysical properties to functionally establish mitral cells and tufted cells as two distinct classes of principal neurons in the main olfactory bulb. In Chapter 4, I reveal that disinhibitory circuitry mediated by a largely uncharacterized class of interneurons is widespread throughout the main olfactory bulb and critically involved in regulating the sensory-evoked activity of inhibitory granule cells. In Chapter 4 Appendix, I provide the first quantitative evidence for the morphological and functional subdivision of granule cells into two distinct classes that separately interact with mitral cells and tufted cells. In Chapter 5 and Chapter 5 Appendix, I molecularly identify a novel class of deep short-axon cells and show that this class of interneurons integrates centrifugal cholinergic input with broadly tuned sensory input and provides highly divergent synaptic output to dynamically regulate the balance of activity between mitral cells and tufted cells. Finally, in Chapters 6 and 7, I present general conclusions from these studies and provide a reappraisal of inhibitory circuitry within the main olfactory bulb.
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Preface

This document describes my main contributions to science while training under Nathaniel N. Urban, and is structured as follows. Chapter 1 is written to first provide a broad overview of the mammalian main olfactory system in general and the main olfactory bulb in particular, and second to briefly motivate the studies presented in Chapters 2, 3, 4, and 5. The conceptual models outlined in Chapter 1 are largely limited to the current consensus models of the field. In Chapters 2, 3, 4, and 5, I provide specific background, experimental methods, results, and discussion of multiple studies examining different aspects of neural processing in the mammalian main olfactory bulb. I present general conclusions from these studies in Chapter 6. Finally, in Chapter 7 I provide a reappraisal of the inhibitory circuits of the mammalian main olfactory bulb within the context of several recent findings in the field, including those described in Chapters 3, 4, and 5.

Throughout this dissertation, the focus is decidedly on mammalian olfaction. While findings from invertebrate and lower vertebrate systems (as well as other regions of the mammalian brain) have proven invaluable in framing the studies described herein, the novel cell types and circuits studied (in particular, see Chapters 3, 4, and 5) currently lack clear analogs in non-mammalian systems. Rather than seeking to generalize the results observed across phyla, I have thus instead explored in detail some of the many complexities specific to the mammalian olfactory system.

Note that modified versions of Chapters 2, 3, 4, and 4 Appendix have previously been published in Burton et al. (2012), Burton and Urban (2014), Burton and Urban (2015), and Geramita et al. (2016), respectively. In addition to these publications, modified versions of Chapter 5 and Chapter 5 Appendix are in preparation for submission. Other publications resulting from my graduate training under Nathaniel N. Urban but not explicitly discussed herein include: Arthur et al. (2013), Zhou et al. (2013), Tripathy et al. (2014), Tripathy et al. (2015), Zhou et al. (2015), and Yu et al. (2015).
1. Introduction

1.1. Investigating neural processing in the main olfactory bulb

Several key features make the mammalian olfactory system uniquely well suited for investigating fundamental properties of neural processing and local circuit interactions in the brain. First, olfaction is crucial to mammalian survival, providing information about predators, food, and conspecifics (for review, see Doty, 1986; Ache and Young, 2005). This ethological relevance is accompanied by a close link between neural activity in the main olfactory system and measurable behavioral outcomes, enabling basic findings about neural processing to provide direct and profound insight into the neural basis of behavior and cognition (for review, see Mainen, 2006; Martin and Ravel, 2014). Second, certain features of the mammalian olfactory system are conserved from lower vertebrates and closely parallel features in the analogous invertebrate structures (for review, see Dryer and Graziadei, 1994; Ache and Young, 2005; Wilson and Mainen, 2006; Kay and Stopfer, 2006; Wilson, 2013; Friedrich, 2013), likely identifying these features as evolutionarily robust strategies for olfactory processing. Third, odors constitute a high-dimensional and dynamic stimulus, with no clear variable along which to form a simple chemotopic map akin to the retinotopic, tonotopic, or somatotopic maps of the respective visual, auditory, and somatosensory systems (for review, see Wilson and Mainen, 2006; Murthy, 2011). Advances in understanding olfaction thus stand to provide significant insight into complex neural processing both within and beyond sensory perception. Finally, the mammalian olfactory system is strikingly shallow. While peripheral input in other sensory systems is sequentially processed by brainstem, midbrain, and thalamic nuclei before reaching primary sensory and associational cortices, olfactory input is processed by only a single region of the brain – the main olfactory bulb (MOB) – before reaching higher cortical areas (for review, see Wilson and Mainen, 2006). A tremendous amount of neural processing, such as that performed by other brain regions (for review, see Kay and Sherman, 2007; Gire et al., 2013), is thus compressed within the MOB, making the MOB in particular an excellent brain region in which to investigate fundamental principles of neural processing (for review, see Cleland, 2014).

1.2. Basic structure of the main olfactory bulb

1.2.1. The organization of odorant receptors

Sensory information enters the main olfactory system when odors bind to specific but overlapping sets of odorant receptors (ORs), which are expressed on the dendrites of olfactory sensory neurons (OSNs) in the main olfactory epithelium (MOE). Each mature OSN in the mouse expresses 1 of 1,000-1,200 ORs, and is therefore responsive to a distinct subset of odors. OSNs expressing the same OR are generally confined to one of a few broad dorsal-ventral zones within the MOE. Within each zone, however, OSNs are broadly distributed independent of OR identity (for review, see Mombaerts, 2004; Schoenfeld and Cleland, 2005; Mori and Sakano, 2011).

All OSNs expressing the same OR project axons to precisely converge within ~2 glomeruli on the surface of the ipsilateral MOB, forming a pair of OR-specific mirror-symmetric glomeruli within each MOB (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). OSN
projections from specific MOE zones generally converge onto corresponding dorsal-ventral domains across the MOB. However, similar to the random distribution of OR-specific OSNs within each MOE zone, no obvious relationship exists between OR identity and glomerular position within a MOB domain (for review, see Mombaerts, 2004; Schoenfeld and Cleland, 2005; Mori and Sakano, 2011). Likewise, while glomeruli that are activated by similar odors are loosely clustered within MOB domains, no such relationship exists at finer scales between neighboring glomeruli (Cleland and Sethupathy, 2006; Soucy et al., 2009; Ma et al., 2012). Thus, no precise chemotopical map exists within the main olfactory system (for review, see Murthy, 2011). Nevertheless, glomerular positions are remarkably well conserved between the two MOBs of each mouse brain, across different mice, and even partially across mice and rats (Soucy et al., 2009; Ma et al., 2012). Sensory input to the MOB thus represents an exquisitely organized labeled-line code with ~1,000 distinct sensory input channels in the mouse.

1.2.2. Intraglomerular and interglomerular organization
OR-specific glomeruli are located in the glomerular layer (GL) of the MOB and represent the primary odor coding modules of the main olfactory system (for review, see Wachowiak and Shipley, 2006; Cleland, 2010). Each glomerulus is a large microcircuit harboring a complex array of interconnections (Figure 1.1A). Within each glomerulus, OSN terminals release glutamate with high release probability (Murphy et al., 2004) to excite the apical dendritic tufts of mitral and tufted cells (M/TCs) (Najac et al., 2011; Gire et al., 2012), the principal neurons of the MOB. Each mature M/TC sends an apical dendrite to a single glomerulus (Malun and Brunjes, 1996; Lin et al., 2000; Blanchart et al., 2006), with a few tens of “homotypic” or “sister” M/TCs connected to each glomerulus (Royet et al., 1998; Sosulski et al., 2011; Ke et al., 2013). OSN terminals additionally provide input to numerous juxtaglomerular interneurons, the majority of which are GAD65+ GABAergic periglomerular cells (PGCs) (Parrish-Aungst et al., 2007). PGCs are compact, typically axonless interneurons that extend a small dendritic tuft within a single glomerulus (Pinching and Powell, 1971a; Hayar et al., 2004a; Kosaka and Kosaka, 2005; 2011; Shao et al., 2009; Kiyokage et al., 2010). PCCs release GABA into the glomerulus to inhibit both presynaptic OSN terminals (for review, see McGann, 2013) and postsynaptic M/TCs (Murphy et al., 2005; Hayar et al., 2005; Gire and Schoppa, 2009; Kiyokage et al., 2010; Shao et al., 2012), collectively shaping the amplitude and duration of sensory input to M/TCs.

MC somata are arrayed in the compact MC layer (MCL) deep to the GL and external plexiform layer (EPL), while TC somata reside throughout the EPL (for review, see Macrides et al., 1985). Each M/TC emits 2-5 lateral dendrites that extend up to ~1 mm radially within the EPL (Mori et al., 1983; Orona et al., 1984). Action potentials propagate actively down M/TC lateral dendrites (Margrie et al., 2001; Charpak et al., 2001; Xiong and Chen, 2002; Lowe, 2002; Christie and Westbrook, 2003; Debarbieux et al., 2003; Djurisic et al., 2004) and drive glutamate release onto axonless GABAergic granule cells (GCs) at reciprocal dendrodendritic synapses (Rall et al., 1966). At these synapses, GC excitation in turn drives GABA release back onto the M/TC lateral dendrite. Activation of a single M/TC can thus yield recurrent self-inhibition as well as lateral inhibition of other M/TCs synapsing onto the same GCs (for review, see Schoppa and Urban, 2003; Egger and Urban, 2006). M/TC axons leave the olfactory bulb via the lateral olfactory tract (LOT) and project to various cortical and subcortical targets (for review, see Mori and Sakano, 2011). The downstream targets of M/TCs in turn project centrifugal fibers back to the
MOB to primarily excite GCs (Mori and Takagi, 1978a; Nakashima et al., 1978; Balu et al., 2007; Markopoulos et al., 2012; Boyd et al., 2012).

Figure 1.1. New insights into sensory processing in the main olfactory bulb
A: Schematic of the MOB circuitry, focusing on the current consensus model of sensory processing. Cell colors correspond to OR and glomerular identity, with cells receiving excitatory sensory input from multiple glomerular microcircuits colored black. B: Physiological diversity in biophysical properties among neurons can support differential processing of sensory information (see Chapter 2). C: Intrinsic biophysical and extrinsic synaptic differences separate excitatory ETCs, TCs, and MCs into distinct cell classes capable of encoding complementary sensory information (see Chapter 3). Intrinsic and synaptic differences likewise separate inhibitory sGCs and dGCs into distinct cell classes (see Chapter 4 Appendix). D: The MOB contains a large diversity of inhibitory interneurons that significantly influence sensory processing. In particular, dSACs: (1) mediate feedforward inhibition onto GCs to regulate sensory-evoked GC recruitment (see Chapter 4), (2) mediate widespread long-lasting inhibition of TC apical dendrites (see Chapter 5), and (3) are a central component of the cholinergic modulation of MOB sensory processing (see Chapter 5 Appendix). Abbreviations: glomerular layer (GL); external plexiform layer (EPL); mitral cell layer (MCL); internal plexiform layer (IPL); granule cell layer (GCL); olfactory sensory neuron (OSN); superficial short-axon cell (sSAC); periglomerular cell (PGC); external tufted cell (ETC); tufted cell (TC); mitral cell (MC); EPL interneuron (EPL-IN); GL-projecting deep short-axon cell (GL-dSAC); EPL-projecting deep short-axon cell (EPL-dSAC); GCL-projecting dSAC (GCL-dSAC); superficial granule cell (sGC); deep granule cell (dGC);
intrabulbar projection (IBP); lateral olfactory tract (LOT); odorant receptor (OR); hyperpolarization-activated mixed cation current (I\text{h}); A-type potassium current (I\text{A}).

1.3. Current models of sensory processing in the main olfactory bulb

Multiple mechanisms have been proposed to explain how sensory information is encoded in odor-evoked patterns of M/TC firing, including: 1) a receptive field-based rate code, 2) slow timescale decorrelation, and 3) fast timescale oscillatory synchrony. Of note, these proposed mechanisms are not mutually exclusive, and considerable evidence supports the likelihood that each mechanism encodes at least some aspects of sensory information.

1.3.1. Receptive field-based rate codes

With the landmark discovery of the OR gene family (Buck and Axel, 1991) and the OR-specific identity of glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996), a receptive field-based rate code inspired by sensory processing in other sensory systems (e.g., Hubel and Wiesel, 1968) emerged as the first unifying theory of sensory processing in the MOB (Mori and Shepherd, 1994; Yokoi et al., 1995). In this theory, principal M/TCs are excited by odors that activate their cognate glomerulus and inhibited, via GC-mediated lateral inhibition, by chemically similar odors that activate surrounding glomeruli (i.e., “center-surround inhibition”). GC-mediated lateral inhibition thereby narrows the set of total possible odors that increases the firing rate of each M/TC, defining the “receptive field” or “excitatory molecular receptive range” of that M/TC. Consequently, M/TCs are more narrowly and specifically tuned than their presynaptic OSNs.

Supporting this theory, Yokoi et al. (1995) observed in a subset of M/TCs recorded in anesthetized rabbits that highly similar odors evoked M/TC excitation, while less similar odors evoked M/TC inhibition and chemically unrelated odors had no effect on M/TC firing rates. Moreover, application of GABA\text{A}R antagonists in rabbits (Yokoi et al., 1995) and rodents (Margrie et al., 2001; Tan et al., 2010) suppresses odor-evoked M/TC inhibition and broadens M/TC receptive fields, consistent with the hypothesis that GCs – which are more broadly tuned than M/TCs (Tan et al., 2010), narrow M/TC tuning. The receptive field-based rate code theory further predicts that at the level of glomeruli, excitation onto an M/TC will be narrow (reflecting the uniglomerular connection of single M/TC apical dendrites) while inhibition will be broad (reflecting the radial extension of multiple M/TC lateral dendrites). Supporting this prediction, M/TC depolarization correlates with glomerular activation in nearby focal regions of the GL (i.e., the cognate glomerulus of the M/TC) while glomerular activation in broad neighboring regions of the GL (i.e., neighboring glomeruli) correlates with M/TC hyperpolarization (Ezeh et al., 1993; Luo and Katz, 2001).

Several objections to a receptive field-based rate code have been raised, however (for review, see Wilson and Mainen, 2006). Foremost, the lack of precise chemotopy in the organization of glomeruli (Cleland and Sethupathy, 2006; Soucy et al., 2009; Ma et al., 2012) precludes narrowing of M/TC tuning by a classical center-surround organization of lateral inhibition. That is, neighboring glomeruli do not respond to chemically similar odors; therefore, lateral inhibition from neighboring glomeruli should not narrow the receptive field of M/TCs to a total set of chemically similar odors. However, this objection is somewhat obviated by the active propagation of action potentials along the unusually long M/TC lateral dendrites, which can
support functional GC-mediated lateral inhibition between M/TCs separated by several hundred micrometers (e.g., see Migliore and Shepherd, 2008). Therefore, the MOB can support functional center-surround inhibition (with respect to chemical odor similarity) via non-topographical mapping of interglomerular interactions.

Second, in contrast to the broad M/TC inhibition predicted by the receptive field-based rate code theory and observed with intracellular M/TC recordings (Ezeh et al., 1993; Luo and Katz, 2001), extracellular M/TC recordings have demonstrated surprisingly sparse inhibitory responses (Davison and Katz, 2007) and interglomerular interactions (Fantana et al., 2008). Such sparse inhibitory responses and interglomerular interactions suggest that GC-mediated lateral inhibition only minimally narrows M/TC receptive fields. Moreover, Fantana et al. (2008) observed only chance levels of correlation among the spectra of odors found to activate glomeruli correlated with the firing of any given M/TC. Importantly, however, this measure of tuning (i.e., odor response spectra correlation) neglects chemical similarity among different odors, and thus does not directly address the predictions originally raised by Mori and colleagues (Mori and Shepherd, 1994; Yokoi et al., 1995). Indeed, using a comprehensive 348-odor panel, Davison and Katz (2007) found greater-than-chance levels of chemical similarity among the odors influencing M/TC firing. Further, the sparseness of inhibitory responses and interglomerular interactions observed by Davison and Katz (2007) and Fantana et al. (2008) necessarily represent lower bounds, reflecting the bias of extracellular recordings to the strongest effects on firing. The actual density of interglomerular interactions influencing M/TC receptive fields therefore lies between the intracellular (Ezeh et al., 1993; Luo and Katz, 2001) and extracellular (Davison and Katz, 2007; Fantana et al., 2008) estimates, and thus remains compatible with the receptive field-based rate code theory.

Finally, only a subset of M/TCs exhibits clear odor-evoked increases or decreases in firing rate in anesthetized preparations, while extracellular recordings from M/TCs in awake preparations exhibit high spontaneous firing rates and complex changes in firing patterns that do not obviously abide by a rate code (for review, see Rinberg and Gelperin, 2006). Importantly, however, identification of neurons with extracellular recordings inherently requires nonzero spontaneous firing rates, and a recent investigation using whole-cell recordings (which lacks such spontaneous firing rate-dependence) observed similar odor-evoked M/TC responses across anesthetized and awake states (Kollo et al., 2014). Moreover, temporally aligning and/or “warping” M/TC responses by sniff phase reveals strong phasic increases in odor-evoked firing rates in awake preparations (Cury and Uchida, 2010; Shusterman et al., 2011), supporting the possibility of a sniff-based rate code. Importantly, such a code is immediately compatible with the extensive physiological and behavioral data identifying the sniff as a central computational unit of olfaction (for review, see Wachowiak, 2011).

In total, a modified receptive field-based rate code integrating non-topographical interglomerular interactions and sniff-based timing remains a viable mechanism by which the MOB may encode certain olfactory information (e.g., odor identity, chemical structure, etc.). Re-examination of how interglomerular interactions influence M/TC receptive fields (e.g., Fantana et al., 2008) with and without temporal warping by sniff phase in awake preparations (e.g., Shusterman et al., 2011) will thus be critical in future evaluations of the receptive field-based rate code. Systematic
analysis of interneuron connectivity supporting interglomerular interactions will likewise prove critical in evaluating a mechanistic basis of a receptive field-based rate code in the MOB.

1.3.2. Slow timescale decorrelation
Odors activate specific but overlapping combinations of glomeruli across the MOB (for review, see Wachowiak and Shipley, 2006), with no precise chemotopical mapping of odorants (Cleland and Sethupathy, 2006; Soucy et al., 2009; Ma et al., 2012). These patterns of glomerular activation in turn evoke firing responses in large and overlapping populations of M/TCs (Fletcher et al., 2009; Kato et al., 2012; Wachowiak et al., 2013; Blauvelt et al., 2013; Adam et al., 2014). Thus, across the entire MOB, sensory input initially yields highly correlated and therefore poorly discriminable patterns of combinatorial M/TC activity (Bathellier et al., 2008; Cury and Uchida, 2010; Kato et al., 2012; Gschwend et al., 2015). Downstream of sensory input to the MOB, however, M/TC firing is shaped in complex temporal patterns by local circuit interactions. In particular, activated M/TCs recruit numerous GCs with asynchronous and glomerulus-specific latencies (Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Urban and Sakmann, 2002; Kapoor and Urban, 2006) arising in part from the differential expression of slow, inactivating K⁺ channels in M/TCs and GCs (Schoppa and Westbrook, 1999; Christie and Westbrook, 2003; Balu et al., 2004; Kapoor and Urban, 2006; Fadool et al., 2011; Giridhar and Urban, 2012). Through GC-mediated recurrent and lateral inhibition, M/TC activity evolves across tens to hundreds of milliseconds in a stimulus-specific manner (Giridhar et al., 2011; Giridhar and Urban, 2012), leading to progressive decorrelation and enhanced discriminability of combinatorial M/TC activity patterns evoked by distinct odors (Bathellier et al., 2008; Cury and Uchida, 2010; Kato et al., 2012; Gschwend et al., 2015).

Supporting this slow timescale decorrelation theory of sensory encoding in the MOB, the degree of decorrelation in combinatorial M/TC activity significantly correlates with trial-by-trial and overall behavioral performance in difficult odor discrimination tasks, while total population firing rates (such as with a receptive field-based rate code) do not (Cury and Uchida, 2010; Gschwend et al., 2015). Further, decorrelation of combinatorial M/TC activity is significantly slower in anesthetized preparations in which GC activity is heavily suppressed (Kato et al., 2012; Cazakoff et al., 2014), consistent with the importance of GC recruitment in temporally shaping M/TC firing. Critically, Carleton and colleagues have also recently demonstrated that optogenetic activation (or chemogenetic inhibition) of GCs can enhance (suppress) decorrelation of M/TC firing and accelerate (decelerate) odor discrimination learning (Gschwend et al., 2015). This study thus excitingly suggests a causal link between GC-mediated inhibition, slow timescale combinatorial M/TC decorrelation, and behavioral odor discrimination. As a caveat, however, these manipulations of GC activity likewise yielded pronounced changes in spontaneous and odor-evoked M/TC firing rates (Gschwend et al., 2015), which may also have contributed (e.g., through a rate code) to the changes in behavioral performance. Further, other manipulations of GC activity have not yielded changes in odor discrimination learning (Abraham et al., 2010; Nunes and Kuner, 2015). While combinatorial M/TC activity thus dynamically evolves in a stimulus-specific manner, further investigations are needed to understand the underlying mechanism and behavioral relevance of this phenomenon to olfaction.
1.3.3. **Fast timescale oscillatory synchrony**

Odors evoke prominent beta (~15-40 Hz) and gamma (~40-80 Hz) frequency oscillations in MOB electroencephalogram and local field potential (LFP) recordings, suggesting that sensory input to the MOB evokes synchronous periodic firing across a large network of M/TCs (for review, see Kay, 2014; Martin and Ravel, 2014). Indeed, simultaneous LFP and extracellular recordings *in vivo* have confirmed that odor-evoked M/TC firing phase-locks to the LFP gamma cycle (Eeckman and Freeman, 1990; Kashiwadani et al., 1999; David et al., 2009; Lepousez and Lledo, 2013; Li et al., 2015). Interestingly, blocking bulbo-cortical transmission suppresses beta but not gamma oscillations in the MOB, identifying gamma oscillations as a sensory processing feature intrinsic to the MOB (Gray and Skinner, 1988; Neville and Haberly, 2003; Martin et al., 2004; 2006).

Within the MOB, fast timescale synchronous M/TC firing (i.e., on the order of milliseconds) emerges across distinct spatial scales, reflecting the OR-specific organization of the MOB (see above). Specifically, intraglomerular synchrony emerges between homotypic M/TCs predominantly via direct electrical coupling and indirect electrical coupling of AMPAR-mediated autoreceptor potentials between M/TC apical dendritic tufts (Urban and Sakmann, 2002; Schoppa and Westbrook, 2002; Christie et al., 2005; Christie and Westbrook, 2006; Pimentel and Margrie, 2008; Maher et al., 2009; Ma and Lowe, 2010; Gire et al., 2012). In turn, interglomerular synchrony emerges between heterotypic M/TCs via GC-mediated lateral inhibition (Kashiwadani et al., 1999; Nusser et al., 2001; Neville and Haberly, 2003; Friedman and Strowbridge, 2003; Lagier et al., 2004; 2007; Schoppa, 2006b; Giridhar et al., 2011; Lepousez and Lledo, 2013), though the exact dynamical mechanism remains debated (e.g., see Galán et al., 2006; Bathellier et al., 2006; Brea et al., 2009).

Of great interest, the distinct composition of M/TCs participating in intra- vs. interglomerular M/TC synchrony (i.e., homotypic vs. heterotypic) can subserve at least two key aspects of sensory coding: signal propagation and feature binding. Synchronization of homotypic M/TCs, which exhibit similar odor tuning (Tan et al., 2010; Dhwale et al., 2010; Kikuta et al., 2013), will provide a degree of redundancy to the activity propagated to downstream brain regions, ensuring faithful propagation of the information encoded by the activation of an OR-specific glomerulus (Giridhar et al., 2011). In parallel, synchronization of heterotypic M/TCs with complementary odor tuning may act to bind together distinct odor features into single percepts (Kashiwadani et al., 1999). Fast timescale synchrony across homotypic or heterotypic M/TCs (or both) may also signify expectation or odor valence in olfactory-guided behavioral tasks (Doucette et al., 2011).

While the specific sensory information encoded by M/TC synchrony and phase-locking within network rhythms thus remains to be determined, perturbation of gamma frequency oscillations and M/TC synchrony through either genetic (Nusser et al., 2001) or pharmacological manipulations (Lepousez and Lledo, 2013) significantly alters olfactory-guided behavior. In turn, network oscillations in the MOB are strongly modulated by both learning and task difficulty during olfactory-guided behavior (for review, see Martin and Ravel, 2014). These findings thus strongly argue for a critical role of fast timescale M/TC synchrony and network oscillations in sensory processing in the olfactory system. Further investigation into how changes in network oscillations and M/TC synchrony influence activity in downstream brain regions and overall
behavior will be pivotal in defining the sensory information encoded by the specific temporal patterning of M/TC activity. Toward this end, multiple studies have recently demonstrated that mice can discriminate between the differential temporal activation of OSNs or M/TCs with a precision on the timescale of gamma oscillations (i.e., 10-20 ms) (Smear et al., 2011), even from a single glomerulus (Smear et al., 2013), and independent of sniff phase (Rebello et al., 2014; Li et al., 2014). Encoding of sensory information in gamma frequency oscillations is thus also compatible with the encoding of potentially complementary sensory information within sniff-coupled theta frequency oscillations (e.g., see Schaefer and Margrie, 2007).

1.4. New insights into sensory processing in the main olfactory bulb

1.4.1. Physiological cell-to-cell diversity

Biophysical diversity is widespread across the mammalian brain, both between neuron classes (Tripathy et al., 2014; 2015) and within neurons of the same class (Figure 1.1B; Padmanabhan and Urban, 2010; 2014). Such biophysical diversity can emerge from multiple factors, including differences in morphology, ion channel expression, subcellular ion channel distribution, and neuromodulation. Several examples and general principles of how these factors contribute to biophysical neuronal diversity are reviewed below.

In his seminal work, Ramón y Cajal (1911) elegantly illustrated the striking, pervasive morphological diversity between all neurons throughout the brain, and inferring a structure-function relationship, was able to classify the vast majority of functional neuron classes. The existence of morphological diversity between neurons of different classes, and even between neurons of the same class, was thus soundly established more than a century ago. Later pioneering theoretical work from Rall offered the first principles by which differences in morphology could influence neuronal function (e.g., Rall, 1959). However, it was not until the wide-scale application of patch-clamp recording techniques that the impact of morphological diversity on neuron function was directly demonstrated.

Using multi-compartmental simulations guided by somatodendritic patch-clamp recordings, early modeling efforts demonstrated that highly diverse patterns of action potential firing and propagation could emerge from neurons with identical ion channel densities but distinct somatodendritic morphologies, including stellate, pyramidal, Purkinje, and substantia nigra dopaminergic cell morphologies (Mainen and Sejnowski, 1996; Vetter et al., 2001). In particular, relatively simple dendritic arborizations foster highly regular firing and efficient dendritic propagation of action potentials, while more elaborate arborizations support action potential bursting and limit dendritic propagation (for review, see Waters et al., 2005). The ability of between-class morphological diversity to regulate neuron function was also recently extended to more physiologically realistic models built on the stochastic gating of ion channels (Cannon et al., 2010).

Morphological diversity also contributes to functional differences between neurons of the same functional class. In a combined experimental and simulation study using dual somatodendritic recordings and multi-compartmental simulations, Schaefer et al. (2003) provided compelling evidence that even minor within-class morphological diversity, independent of changes in ion channel properties and distributions, can significantly regulate neuronal function. Specifically,
the position of one or a few oblique dendritic branches along the apical dendrite of neocortical layer 5 pyramidal cells proved sufficient to control the ability of back-propagating axosomatic action potentials to facilitate dendritic regenerative Ca\textsuperscript{2+} spikes, a phenomenon critical to sensory perception and cognitive processing (for review, see Larkum, 2013). Similar diversity in axosomatic and active dendritic coupling arising from morphological diversity has also been experimentally observed in hippocampal CA1 pyramidal cells (Golding et al., 2001), even at the level of cellular differences in dendritic branch point morphology (Ferrante et al., 2013), and may underlie the recent discovery of two morphologically and functionally distinct subtypes of CA1 pyramidal cells (Graves et al., 2012).

Neuronal activity emerges from the expression of unique combinations of ion channels that support not only a negative resting membrane potential, but also the ability to dynamically change membrane potential through regenerative action potentials (for review, see Hille, 2001). There exists a tremendous diversity of ion channels, including 9 α- and 4 auxiliary β-subunits of the voltage-gated Na\textsuperscript{+} channel, more than 10 voltage-gated Ca\textsuperscript{2+} channel subunits, and more than 100 subunits within the K\textsuperscript{+} channel superfamily (for review, see Hofmann et al., 1994; Coetzee et al., 1999; Catterall et al., 2005). The differential expression of a subset of these ion channels within each neuron thus provides a mechanism for generating both between- and within-class neuronal diversity in a wide array of biophysical properties, including resting membrane potential, action potential shape and firing pattern, and axosomatic and active dendritic coupling.

One example of an ion channel family contributing to both between- and within-class biophysical neuronal diversity is the hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels (Figure 1.2), which conduct a hyperpolarization-activated mixed cation current (\(I_h\)) that drives a membrane potential “sag” in response to hyperpolarization (for review, see Robinson and Siegelbaum, 2003). \(I_h\) can play a critical role in several neuronal functions, such as regulating the spatiotemporal summation of synaptic input (for review, see Magee, 2000) and driving rhythmic subthreshold oscillations (e.g., Dickson et al., 2000) and bursting (e.g., Liu and Shipley, 2008b). Yet not all neurons exhibit significant \(I_h\). For example, hippocampal CA1 pyramidal neurons and basket cells, entorhinal cortical stellate cells, neocortical layer 5 and 6 pyramidal cells, MOB MCs, cerebellar Purkinje cells, and dopaminergic substantia nigra neurons all exhibit significant \(I_h\) and sag, while hippocampal granule cells, piriform cortical pyramidal cells, neocortical layer 2/3 pyramidal cells, and GABAergic substantia nigra neurons do not (e.g., Spruston and Johnston, 1992; Stuart and Häusser, 1994; Häusser et al., 1995; Dickson et al., 2000; Aponte et al., 2006; Larkum et al., 2007; Bathellier et al., 2009; Ledergerber and Larkum, 2010; Angelo and Margrie, 2011). Thus, the presence or absence of \(I_h\) and membrane potential sag at the whole-cell level strongly contributes to the diversity of neuron classes across the brain (Figure 1.2A,B).
Figure 1.2. Ion channel expression and subcellular distribution drives between- and within-class neuronal diversity
A: Schematized membrane potential response to hyperpolarizing somatic step current injection for a piriform cortical pyramidal cell, cerebellar Purkinje cell, and neocortical layer 5 pyramidal cell. Morphologies reproduced from Bathellier et al. (2009), Roth and Häusser (2001), and Oswald et al. (2013), respectively. B: Plot of subcellular $I_h$ current density, aligned such that y-axis corresponds to morphologies in A. C,D: Heterotypic MCs exhibit greater sag diversity than homotypic MCs (C), matching immunohistochemical differences in glomerular HCN2 expression (D); reproduced from Angelo et al., 2012.

Cell-attached somatodendritic recordings have also revealed distinctions in subcellular localizations of $I_h$ between neuron classes (Figure 1.2A,B; for review, see Nusser, 2009). Magee (1998) demonstrated a gradient of increasing $I_h$ magnitudes from soma to distal apical dendrites in hippocampal CA1 pyramidal cells that was likewise observed in neocortical layer 5 pyramidal cells (Williams and Stuart, 2000; Berger et al., 2001). Other, non-pyramidal excitatory projection neurons, such as MOB MCs, also exhibit strong dendritic and weak somatic $I_h$ (Angelo and Margrie, 2011). In contrast, however, cerebellar Purkinje cells exhibit uniform $I_h$ currents.
throughout their somatodendritic axis (Stuart and Häusser, 1994; Angelo et al., 2007), while cerebellar basket cells express strong \( I_h \) in both soma and axon termini (Southan et al., 2000).

Differences in cellular and subcellular \( I_h \) also contributes to within-class biophysical diversity. Neocortical layer 5 pyramidal cells with corticospinal, corticothalamic, corticostriatal, or corticocortical axonal projections exhibit distinct sag amplitudes (Oswald et al., 2013). Likewise, CA1 pyramidal cells positioned within the dorsal or ventral hippocampus express distinct \( I_h \) amplitudes and voltage-dependencies (Dougherty et al., 2013), while entorhinal cortical stellate cells similarly exhibit a dorso-ventral gradient of \( I_h \) kinetics (Giocomo and Hasselmo, 2008). This anatomically patterned diversity plays a critical role in the proper establishment of place and grid fields that are critical to effective spatial navigation (Garden et al., 2008; Giocomo et al., 2007; 2011). However, not all within-class diversity exhibits such clear anatomical patterning, even among CA1 pyramidal cells (Graves et al., 2012). In particular, changes in activity at the network or even individual synaptic level can modulate \( I_h \) at the whole-cell level in CA1 pyramidal cells (van Welie et al., 2004; Fan et al., 2005). Such activity-dependent generation of within-class diversity is clearly demonstrated by MOB MCs. The strong diversity in \( I_h \) and sag across MCs contributes to dramatic differences in excitability and action potential firing patterns (Angelo and Margrie, 2011) and is at least partially generated by the history of sensory input (Angelo et al., 2012), leading to a mosaic pattern of \( I_h \)-dependent within-class diversity reflecting the unique circuit architecture of the main olfactory system (Figure 1.2C,D).

Morphological and ion channel properties diversify neurons across many scales, and thereby provide the biological basis for both between- and within-class neuronal diversity. How this diversity translates into differences in neuronal function at the cellular, network, and systems level is often unintuitive, however. Indeed, individual ion channel currents typically contribute to multiple biophysical properties in each neuron, such as input resistance, resting membrane potential, and action potential threshold (e.g., Günay et al., 2008), and further regulate action potential waveforms in complex ways (for review, see Bean, 2007). Thus, to understand the functional impact of diversity at the cellular, network, and systems level, it is often necessary to examine more composite features than, for example, single ion channel current properties.

In particular, the spike-triggered average (STA) represents one such composite feature capable of intuitively relating several aspects of biophysical diversity to the stimulus coding properties of a neuron (Figure 1.3). The STA specifically describes the optimal current waveform triggering an action potential (Bryant and Segundo, 1976; Forger et al., 2011; Mainen and Sejnowski, 1995) and thereby reflects the intrinsic dynamical states of the neuron and its various ion channels that are relevant to action potential firing (e.g., Fricker and Miles, 2000; Mease et al., 2013; Slee et al., 2005; Svirskis et al., 2002). Moreover, the STA provides a tractable, intuitive measure of the collective integrative and resonant properties of the neuron (Das and Narayanan, 2014; Hong et al., 2012; Ratté et al., 2013) and its ability to encode information (Padmanabhan and Urban, 2010). Importantly, the STA of a periodically firing (i.e., oscillating) neuron can also be directly related to its phase-response curve (PRC) (Ermentrout et al., 2007; Torben-Nielsen et al., 2010), which describes the response (phase advance or delay) of the neuron to transient inputs (for review, see Smeal et al., 2010). Using this relationship, we have measured STA and PRC diversity across MCs to quantitatively examine how physiological biophysical diversity influences the ability of correlated input to synchronize oscillating neurons (see Chapter 2).
Biophysical diversity in numerous morphological and ion channel properties systematically shifts the collective integrative and resonant components of the STA.

1.4.2. Functional differentiation of mitral, tufted, and external tufted cells
More than a century ago, Ramón y Cajal (1911) divided the excitatory neurons of the MOB into two distinct classes – MCs and TCs – on the basis of their respective somatic depth in the MCL and throughout the EPL. Reinforcing this division, quantitative examination of M/TC lateral dendrite and axon collateral distributions within the MOB has revealed that most MCs (i.e., “type I MCs”) extend lateral dendrites and axon collaterals within the deep EPL and GCL, respectively, while TCs extend lateral dendrites and axon collaterals within the superficial EPL and GCL, respectively (Mori et al., 1983; Kishi et al., 1984; Orona et al., 1984). These complementary lateral dendritic patterns strikingly match the putative division of deep (i.e., “type 2”) GC and superficial (i.e., “type 3”) GC apical dendritic arborizations within the deep and superficial EPL, respectively (Orona et al., 1983; Mori et al., 1983). TCs have additionally been further subdivided into external or superficial TCs (ETCs), middle TCs, and deep or internal tufted cells, based on: 1) their respective somatic depth in the EPL/GL border, superficial EPL, and deep EPL; 2) the lack of lateral dendrites in the majority of ETCs; and 3) the (likely) exclusive intrabulbar axonal projections of ETCs, identifying ETCs as interneurons (Figure 1.1C; Ramón y Cajal, 1911; Macrides and Schneider, 1982; Orona et al., 1984; Schoenfeld et al., 1985; Liu and Shipley, 1994; for review, see Macrides et al., 1985). Beyond the MOB, MCs and TCs (i.e., deep and middle TCs) also exhibit complementary, non-overlapping axonal projections to downstream cortical and subcortical regions (Nagayama et al., 2010; Igarashi et al., 2012). Despite these extensive anatomical and morphological differences, however, principal MC and TCs and interneuron ETCs remain widely viewed as functionally equivalent cells within the MOB circuit (Figure 1.1A), likely in part to maintain strict parallelism with the apparent single class of principal neurons in the lower vertebrate and invertebrate olfactory systems.
Recent functional investigations have begun to challenge this prevailing view. In particular, the Shipley laboratory and others have demonstrated striking functional differences between ETCs and M/TCs. Specifically, ETCs exhibit rhythmic intrinsic bursting at theta frequencies (Hayar et al., 2004a, b; 2005; Antal et al., 2006; Liu and Shipley, 2008b), while M/TCs exhibit a range of regular tonic firing to irregular firing of action potential clusters interspersed with subthreshold membrane potential oscillations (Chen and Shepherd, 1997; Desmaisons et al., 1999; Friedman and Strowbridge, 2000; Balu et al., 2004; Schaefer et al., 2006; Padmanabhan and Urban, 2010; Angelo and Margrie, 2011; Fadool et al., 2011). Recurrent excitation specifically between homotypic ETCs (Hayar et al., 2004b; 2005) has further been shown to play a central role in activating other neurons within the glomerular microcircuit. In particular, OSN input to a glomerulus triggers rapid nonlinear ETC excitation (Liu and Shipley, 2008a), leading to a disynaptic ETC-mediated burst of excitatory synaptic input to most PGCs and superficial short-axon cells (sSACs) (Hayar et al., 2004a; Murphy et al., 2005; Shao et al., 2009; Kiyokage et al., 2010) and a regenerative, all-or-none ETC-mediated long-lasting depolarization (LLD) in M/TCs (Carlson et al., 2000; De Saint Jan and Westbrook, 2007; Gire and Schoppa, 2009; De Saint Jan et al., 2009; Najac et al., 2011; Gire et al., 2012). The intrinsic bursting of ETCs can further be entrained by rhythmic OSN input at physiological sniff rhythms (Hayar et al., 2004b), attractively suggesting that the ETC-mediated LLD mechanistically underlies the sniff-locked subthreshold depolarization observed in M/TCs in vivo (Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Phillips et al., 2012; Fukunaga et al., 2012; 2014), though this remains untested. In total, overwhelming evidence has now accumulated demonstrating that ETCs are anatomically, morphologically, functionally, and even neurochemically (Liu and Shipley, 1994; Tobin et al., 2010; Tatti et al., 2014) distinct from M/TCs.

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<th>Table 1.1. Differences in odor-evoked MC vs. TC activity</th>
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Numerous functional differences between odor-evoked MC and TC activity in vivo (Table 1.1) further suggest that MCs and TCs may represent two distinct cell classes capable of encoding complementary sensory information. These differences at least partially arise from stronger OSN input and weaker lateral inhibitory input to TCs than MCs (Ezeh et al., 1993; Christie et al., 2001; Gire et al., 2012). Whether differences in synaptic input are sufficient to explain the differences in odor-evoked MC vs. TC activity in vivo, and whether MCs and TCs also differ in
their intrinsic biophysical properties to indeed form distinct cell classes, is examined below (see Chapter 3). In addition, the putative subdivision of GCs and their corresponding MC vs. TC interactions are also examined below (see Chapter 4 Appendix).

1.4.3. Novel inhibitory interneuron circuitry
Inhibitory GABAergic signaling critically regulates several aspects of sensory processing in the MOB. Current models of olfaction focus almost exclusively on the role of GCs and PGCs in mediating GABAergic signaling in the MOB (Figure 1.1A). However, in addition to PGCs and GCs, the MOB contains a large diversity of other interneuron classes, including combined dopaminergic/GABAergic sSACs, GABAergic EPL-INs, and GABAergic dSACs (Figure 1.1D; for review, see Nagayama et al., 2014). While comparatively less is known about sSACs, EPL-INs, and dSACs, multiple recent findings suggest that each of these cell classes is critically involved in MOB sensory processing, motivating a re-evaluation of inhibition in the MOB (see Chapter 7).

Of particular interest, dSACs comprise a large population of MOB interneurons (Price and Powell, 1970b), numbering ~13,500 per rat MOB or three to four principal neurons per dSAC (Eyre et al., 2009), comparable with the principal neuron/interneuron ratio in the neocortex (Markram et al., 2004). Unlike PGCs and GCs, dSACs form extensive axonal arbors, and can be subdivided into GL-projecting, EPL-projecting, and GCL-projecting dSACs (GL-, EPL-, and GCL-dSACs, respectively) (Eyre et al., 2008). Critically, these extensive axonal arbors support a high degree of dSAC connectivity with other MOB interneurons (Eyre et al., 2009; Miyamichi et al., 2013), including adult-born neurons (Arenkiel et al., 2011; Deshpande et al., 2013), suggesting that dSACs may powerfully regulate local circuits and plasticity within the MOB. Indeed, in a pioneering functional examination of dSACs, Pressler and Strowbridge (2006) found that a subset of predominantly EPL-projecting dSACs (i.e., “Blanes cells”) are capable of suppressing GC firing via monosynaptic inhibition, introducing the first known disinhibitory circuit within the MOB. How prevalent such dSAC-mediated GC inhibition is, when dSAC-mediated GC inhibition occurs with respect to sensory activation of the MOB, and how dSAC-mediated GC inhibition influences sensory-evoked GC activity is examined in detail below (see Chapter 4).

Owing to the limitations of Golgi staining, GL-dSACs were remarkably first identified in 2008 (Eyre et al., 2008). Of outstanding interesting, each GL-dSAC innervates multiple neighboring glomeruli, and is thus poised to significantly regulate sensory-evoked MOB activity (Eyre et al., 2008). Moreover, while lateral and superficial-to-deep circuits dominate the propagation of sensory-evoked activity through the MOB circuit (Figure 1.1A), GL-dSACs are uniquely positioned within the IPL but provide output to the GL, and thus form a novel deep-to-superficial circuit (Eyre et al., 2008). Further, the IPL is densely innervated by ETC and TC axon collaterals (Macrides and Schneider, 1982; Orona et al., 1984; Kishi et al., 1984; Schoenfeld et al., 1985; Liu and Shipley, 1994; for review, see Macrides et al., 1985), glutamatergic and GABAergic centrifugal feedback (Boyd et al., 2012; Markopoulos et al., 2012; Nunez-Parra et al., 2013), and neuromodulatory input (Linster and Fontanini, 2014), making GL-dSACs a likely hub for coordinating widespread MOB activity across distinct brain states. While GL-dSACs are thus likely of critical importance to sensory processing in the MOB, the current lack of known selective molecular markers for any subclass of dSACs (Eyre et al., 2009) has strongly impeded
functional investigation of GL-dSACs. In Chapter 5 below, we identify nicotinic acetylcholine receptor subunit α2 (*chrna2*) as the first selective marker of GL-dSACs, and use transgenic Chrna2-Cre mice to systematically investigate the structure, intrinsic physiology, sensory recruitment, neuromodulation, synaptic output, and functional role of GL-dSACs in the MOB circuit. In addition, using targeted expression of channelrhodopsin in basal forebrain cholinergic neurons, we reveal pronounced nicotinic postsynaptic currents selectively in GL-dSACs following endogenous acetylcholine release, confirming that GL-dSACs are a central component in the neuromodulatory control of MOB activity (see Chapter 5 Appendix).
2. Intrinsic heterogeneity in oscillatory dynamics limits correlation-induced neural synchronization

2.1. Abstract

Synchronous neural oscillations are found throughout the brain and are thought to contribute to neural coding and the propagation of activity. Several proposed mechanisms of synchronization have gained support through combined theoretical and experimental investigation, including mechanisms based on coupling and correlated input. Here, we ask how correlation-induced synchrony is affected by physiological heterogeneity across neurons. To address this question, we examined cell-to-cell differences in phase-response curves (PRCs), which characterize the response of periodically firing neurons to weak perturbations. Using acute slice electrophysiology, we measured PRCs across a single class of principal neurons capable of sensory-evoked oscillations in vivo: the olfactory bulb mitral cells (MCs). Periodically firing MCs displayed a broad range of PRCs, each of which was well fit by a simple three-parameter model. MCs also displayed differences in firing rate-current relationships and in preferred firing rate ranges. Both the observed PRC heterogeneity and moderate firing rate differences (~10 Hz) separately reduced the maximum correlation-induced synchrony between MCs by up to 25–30%. Simulations further demonstrated that these components of heterogeneity alone were sufficient to account for the difference in synchronization among heterogeneous vs. homogeneous populations in vitro. Within this simulation framework, independent modulation of specific PRC features additionally revealed which aspects of PRC heterogeneity most strongly impact correlation-induced synchronization. Finally, we demonstrated good agreement of novel mathematical theory with our experimental and simulation results, providing a theoretical basis for the influence of heterogeneity on correlation-induced neural synchronization.

2.2. Introduction

Synchronized oscillatory firing is a prominent feature of neuronal activity (Buzsáki and Draguhn, 2004). Oscillatory synchrony can be task and state dependent (Salinas and Sejnowski, 2001), is believed to critically contribute to neural coding (Panzeri et al., 2010), and is altered in several disorders (Uhlhaas and Singer, 2006). Considerable progress has been made in elucidating mechanisms of oscillatory synchronization, particularly through combined theoretical and experimental approaches (Wang, 2010). Direct coupling of inhibitory neurons or mixed populations of excitatory and inhibitory neurons by electrical and chemical synapses can synchronize oscillating neurons (Traub et al., 2004), and physiological heterogeneity can destabilize such coupling-induced synchronization (Kopell and Ermentrout, 2002). Alternatively, fluctuating correlated input, such as that from common synaptic partners, can synchronize coupled or uncoupled oscillating neurons (Galán et al., 2006). How physiological heterogeneity in biophysical properties and firing rates affects correlation-induced synchronization is currently unknown.

The stereotyped circuitry and sensory-evoked oscillations of the rodent olfactory bulb (OB) affords an excellent model for studying the impact of heterogeneity on oscillatory synchronization (Kay et al., 2009; Padmanabhan and Urban, 2010). Sensory-evoked excitation
and asynchronous lateral inhibition drive mitral cells (MCs), the OB principal neurons, with correlated fluctuating input. Oscillating MCs are thus subject to both inhibitory coupling and noisy correlated input (Friedman and Strowbridge, 2003; Galán et al., 2006; Lagier et al., 2004; Schoppa, 2006b). Moreover, intrinsic diversity within MCs, as measured through spike-triggered average (STA) heterogeneity, can significantly impact MC spike train correlations and information content (Padmanabhan and Urban, 2010).

The degree to which oscillating neurons synchronize depends on their response to transient inputs, as captured by the phase-response curve (PRC) (Smeal et al., 2010). The PRC describes the response (phase advance or delay) of an oscillator to small transient inputs as a function of the phase at which the input arrives. Neurons in which the phase of spiking is always advanced by positive inputs have type I PRCs. Neurons in which positive inputs can either advance or delay spike phase have type II PRCs (Hansel et al., 1995). Previously, we have shown that homogeneous neurons with identical type II PRCs synchronize more strongly when driven by correlated inputs than do neurons with type I PRCs (Abouzeid and Ermentrout, 2009; Galán et al., 2007; Marella and Ermentrout, 2008). We hypothesize that, in turn, physiological levels of PRC heterogeneity significantly limit correlation-induced synchronization.

In this article, we present new data and analysis quantifying the influence of physiological heterogeneity on correlation-induced synchrony. In the first half of our study, we quantify the intrinsic biophysical diversity within oscillating MCs in vitro using acute slice electrophysiology. In the second half, we analyze how this observed heterogeneity, as well as systematic PRC heterogeneity in general, influences correlation-induced synchrony. We find that MCs firing in a roughly periodic manner (similar to odor-evoked oscillations observed in vivo) exhibit a broad range of type II PRCs that are surprisingly well characterized by a generalized three-parameter model. MCs also vary in their firing rate-current (FI) relationships and evoked periodic firing rates. Intrinsic biophysical diversity within this single class of principal neurons thus manifests as substantial and largely independent PRC and firing rate heterogeneity. Using within-cell comparisons to define the maximum synchrony possible among homogeneous MCs, we find that PRC differences between heterogeneous MCs reduce output synchrony by up to 30%. This is equivalent to the reduction in output correlation caused by reducing input correlation by 0.1. In turn, moderate firing rate differences (~10 Hz) impose up to a comparable 25% reduction in maximum output synchrony. Combined, PRC and firing rate heterogeneity reduce maximum output synchrony by up to 40%, equivalent to reducing input correlation by as much as 0.2. Simulations further demonstrate that these two factors (physiological levels of PRC and firing rate heterogeneity) completely account for the difference in correlation-induced synchrony among heterogeneous vs. homogeneous populations in vitro. Independent modulation of PRC features reveals that correlation-induced synchrony among neurons with small-amplitude type II PRCs is more susceptible to PRC heterogeneity than synchrony among neurons with large-amplitude type II PRCs. Finally, we extend our previous analytical theory describing correlation-induced synchronization of uncoupled oscillators (Marella and Ermentrout, 2008) to account for intrinsic oscillator heterogeneities. Predictions made with this generalized formulation closely match our experimental results, providing a theoretical context for how intrinsic diversity impacts correlation-induced synchronization of uncoupled oscillators.
2.3. Experimental procedures

2.3.1. Slice preparation
Coronal OB slices (310–320 μm thick) were prepared from postnatal day 12–18 mice of both sexes, as described previously (Giridhar et al., 2011). Briefly, C57BL/6 mice were anesthetized with isoflurane and decapitated into ice-cold oxygenated Ringer’s solution containing (in mM) 125 NaCl, 25 glucose, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, and 2.5 CaCl₂. OBs were isolated, and slices were obtained using a vibratome (VT1200S; Leica). Slices were removed to 37°C oxygenated Ringer solution for ~15 min before recovering at room temperature for at least 30 min before electrophysiological recording. All experiments were completed in compliance with the guidelines established by the Institutional Animal Care and Use Committee of Carnegie Mellon University.

2.3.2. Electrophysiology
Slices were continuously superfused with oxygenated Ringer’s solution warmed to 37°C. MCs were identified by morphology and laminar position using infrared differential interference contrast (IR-DIC) microscopy. Unpaired whole cell current-clamp recordings were made from individual MCs using electrodes filled with (in mM) 120 K-gluconate, 2 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 Mg-ATP, 0.3 Na₃GTP, and 1 EGTA. Synaptic transmission was not blocked, based on our previous observation that intrinsic MC biophysical diversity, as measured by 1) STA heterogeneity, 2) firing response across MCs to an identical input current waveform, and 3) heterogeneity in FI relationships, is independent of intact synaptic transmission in unpaired recordings (Padmanabhan and Urban, 2010). The insensitivity of STAs to synaptic blockade in particular is critical to our current results, because we use the STA to calculate the PRC for each oscillating MC (see below). Others have also noted that ongoing synaptic transmission in acute slice preparations does not strongly impact the accuracy of PRC estimation (Netoff et al., 2012). Data were low-pass filtered at 4 kHz and digitized at 10 kHz using MultiClamp 700A and 700B amplifiers (Molecular Devices) and an ITC-18 acquisition board (Instrutech) controlled by custom software written in IGOR Pro (Wavemetrics).

2.3.3. Stimulus generation
To study synchronization of oscillating neurons, we drove MCs to fire in a roughly periodic manner by injecting constant step currents (150–700 pA). These step currents were overlaid with frozen colored noise (σ = 15–20 pA) to 1) more realistically mimic physiological synaptic input; 2) estimate the STA, a biophysical metric unique to each neuron (Padmanabhan and Urban, 2010) and directly related to the PRC for interspike interval (ISI) coefficient of variations (CV_ISI) <0.4 (Ermentrout et al., 2007); and 3) examine the ability of small-amplitude, correlated fluctuating input to synchronize spiking (i.e., correlation-induced, “noise-induced,” or “stochastic” synchrony). To study correlation-induced synchrony, pairs of white noise inputs, \( \xi_{1,2} \), with varying levels of correlation were produced by mixing common and independent white noise sources via \( \xi_j \ t = C_{in} \xi_C \ t + 1 - C_{in} \eta_j \ t \), where \( \xi_C \ t \) is a source of common noise and \( \eta_{1,2} \) are two sources of independent noise. \( C_{in} \) governs the correlation between the resulting pair of noise inputs, with \( C_{in} = 0 \) corresponding to no input correlation and \( C_{in} = 1 \) corresponding to perfect input correlation. \( \xi_{1,2} \ t \) were then normalized by \( 1 - 2C_{in} + 2C_{in}^2 \) to maintain equal spectral properties and convolved with an alpha function:
\[\alpha t = t \tau \exp(-t \tau) \quad \text{with} \quad \tau = 3 \text{ ms}.\]

Constant step current injections were then overlaid with these correlated colored noise inputs.

2.3.4. PRC estimation

To estimate the PRC for each in vitro MC, we took advantage of the direct relationship between the STA and PRC of a neuron firing in a roughly periodic manner (\(CV_{\text{ISI}} < 0.4\)) (Ermentrout et al., 2007; Torben-Nielsen et al., 2010). STAs were determined first, as follows. Each MC was injected with a series of noisy step current injections (see above), each 6 s in duration with 10 s separating each stimulus presentation. Binary strings of spike times were extracted from each resulting voltage trace. The initial 1 s of each trace was discarded to minimize effects of spike-frequency adaptation, and the remaining spikes were used to calculate \(CV_{\text{ISI}}\). Under these conditions and in response to the described inputs, \(\sim 80\%\) of MCs exhibited noisy periodic firing (\(CV_{\text{ISI}} < 0.4\)) and were used for PRC and synchrony analyses. The STA of each MC firing in this noisy periodic regime was calculated as the mean injected current (with step current subtracted) preceding each spike by the natural period of the cell, defined as the mean ISI. STAs were calculated over 805–4,090 spikes (\(\mu = 1,951\) spikes; \(\sigma = 690\) spikes), which well exceeds the few hundred spikes necessary for accurate PRC estimation using the STA method (Torben-Nielsen et al., 2010). To confirm that a sufficient number of spikes were used in each STA extraction, we compared the final STA with “trial-subsampled” STAs extracted from randomly sampling half of the total number of spikes (Padmanabhan and Urban, 2010). To confirm consistency of the extracted STA across the duration of the recording, we compared the final STA with “time-subsampled” STAs extracted from the first and second halves of all spikes (Padmanabhan and Urban, 2010). All final STAs closely agreed with their respective trial- and time-subsampled STAs (\(R^2 > 0.95\) for all cells), confirming accurate and consistent STA extraction.

The PRC of each oscillating MC was then derived from the STA using

\[
PRC \theta = -2\pi T \sigma^2 R_{in}^{-1} \int_0^T STA(t) \, dt
\]

where \(T\) is the natural period (mean ISI), \(\sigma\) is the standard deviation of the injected current fluctuations, and \(R_{in}\) is the input resistance (Ermentrout et al., 2007). Time \(t\) is mapped into phase \(\theta\) in terms of radians by the conversion factor \(2\pi T\) to reflect the periodic nature of activity. While experimentally accessible and robust to various sources of noise (Torben-Nielsen et al., 2010), this method of PRC estimation can yield estimates of the PRC with large divergences from 0 at \(\theta = 2\pi\) and discontinuities at \(\theta = 0,2\pi\). These deviations from theoretical PRC predictions arise largely from uncertainty in defining the natural period of a noisy oscillator (Ermentrout et al., 2007). Experimentally, such uncertainty can arise from: 1) imperfect estimation of the period in a noisy periodically firing cell, 2) variance in spike widths within and between cells, and 3) variance in spike-threshold within and between cells. To account for this uncertainty and minimize deviations of our estimated PRCs from theoretical predictions, we allowed the duration and exact timing of the STA to vary by 5% of the mean ISI for each MC. We then used whichever STA and corresponding period that yielded a PRC closest to 0 at \(\theta = 2\pi\). This method led to negligible changes in estimated STAs but yielded estimated PRCs that more closely matched theoretical PRC predictions. We also note that the above PRC estimation method yields a deterministic PRC for each in vitro MC; phase-dependent PRC variability was not examined but should not significantly influence the accuracy of our PRC.
estimate (Ermentrout et al., 2011). To systematically evaluate differences in PRCs across neurons, each PRC was fit to the generalized phenomenological model

\[ PRC \theta = A \sin B - \sin B + \theta e^{C \theta - 2\pi} \]  

(2)

using a mean-squared error minimization algorithm. A sinusoidal component provides the general form of the model, with the \( B \) parameter governing the node or zero point of the curve. An amplitude component with parameter \( A \) describes the reactivity of the cell to phasic inputs, with higher values of \( A \) yielding greater advances and delays in spike timing for a given input. Finally, an exponential component governs the balance between phase advance and delay regimes, with high values of parameter \( C \) corresponding to little or no phase delays (i.e., closer to type I PRCs). This model provided highly accurate fits to all PRCs recorded \textit{in vitro} (\( R^2 = 0.9979 \pm 0.0013 \), mean ± SD), facilitating quantitative analysis of PRC heterogeneity. More traditional two-parameter sinusoidal models, e.g., \( PRC \theta = A \sin B - \sin B + \theta \), provided less accurate fits (\( R^2 = 0.8123 \pm 0.1009 \)) and were not considered further.

2.3.5. Synchrony analysis

To examine correlation-induced synchrony, MCs were injected with six pairs of noisy inputs, with correlation between inputs of a pair ranging from \( C_{in} = 0 \) to \( C_{in} = 1 \) in steps of 0.2. Pairs of inputs were presented to each individually recorded cell once in a pseudorandom order. Resulting binarized spike trains were convolved with a square-wave pulse of 8 ms to account for spike time jitter and differences in spike thresholds (Galán et al., 2008). Convolved spike trains evoked by each input of a correlated pair were then compared within the same cell or between different cells to analyze correlation-induced synchrony within homogeneous or heterogeneous MC populations, respectively. Synchrony was measured using magnitude squared coherence, which normalizes the cross-spectral density between two spike trains by the autospectral density of each individual spike train, yielding a measure of spectral power dependent only on synchrony between spike trains. Cross power spectra were additionally calculated to help visualize synchronization of oscillations. Coherence and power spectra were calculated using the Welch method with a total signal length of 50,000 samples per trace (i.e., 5 s at a sampling rate of 10 kHz), a window size of 1,024 samples, and 50% window overlap. These windowing parameters facilitated clear comparison of output synchrony across conditions while providing sufficient spectral resolution of the target envelope (15–75 Hz). Final results were invariant to square-wave width used in spike train convolution and window size used in spectral analyses (see Figure 2.7; Galán et al., 2008). Statistical comparisons made are described in Results. For direct comparison with theoretical predictions, phase difference densities were derived from \textit{in vitro} spike trains by linearly interpolating MC phase across each ISI.

2.3.6. Simulations

Oscillating MCs recorded \textit{in vitro} were modeled as simple noisy phase \( \theta \) oscillators by

\[ d\theta_j = \omega_j + \Delta_j \theta \left[ I(t) + \eta \right], \]

where \( \omega_j = 2\pi/T_j \) is the natural frequency of neuron \( j \) calculated using the experimentally derived mean ISI (\( T_j \)), and \( \Delta_j \) is the experimentally derived PRC of neuron \( j \) (Galán et al., 2008). \( \theta_j \) ranges from 0 to 2\( \pi \) for each model neuron \( j \); we take \( \theta_j = 0 \) to be the time of a spike. Model neurons were driven by shared synaptic input, \( I(t) \), identical to \textit{in vitro} neurons (see above) except that the amplitude was uniformly reduced and balanced with independent white noise \( \eta \) to evoke equivalent “noisy periodic” firing rates and ISI distributions.
as observed in vitro. As for in vitro neurons, \( C_{in} \) characterizes the degree of correlation between pairs of frozen colored noise inputs, \( I(t) \). Coherence of output spike trains of model neurons was analyzed as for in vitro neurons (see above). Independent PRC and firing rate manipulations were performed as described in Results.

2.3.7. Mathematical theory

In this section, we extend previous theory that we have developed to study correlation-induced synchronization of uncoupled neural oscillators. We start with a set of phase models, similar to those above, which arise when the inputs to the oscillators are small. The general model takes the form

\[
d\theta_j \ dt = \omega_j + \sigma \xi_j(t) \Delta_j \theta_j,
\]

where \( j = 1,2 \), representing two neural oscillators, and \( \xi_j(t) \) is the broadband input of magnitude \( \sigma \) given to the oscillator. \( \theta_j, \omega_j, \) and \( \Delta_j \) are as defined above. In our prior work (Marella and Ermentrout, 2008), we derived equations for the phase difference, \( \phi = \theta_2 - \theta_1 \), between two identical oscillators (\( \omega_1 = \omega_2 \) and \( \Delta_1 = \Delta_2 \)) that received a mixture of correlated and uncorrelated white noise \( \xi_j(t) \) where \( c \) is the correlation, \( \eta_{1,2} \) are independent noise sources, and \( \xi_C \) is the common noise. The result of this calculation is an explicit formula for the density of the phase difference, \( P_s \phi \). This density function can be directly related to the more familiar quantity of spike time cross-correlation, \( C_{ij} \), by

\[
C_{ij} \tau = P_s \phi = 1 - 2 \pi \delta(\tau) \tag{4}
\]

(for details, see Appendix). Thus, if the noise to the oscillators is uncorrelated, then \( P_s \phi = 1 \) and the cross-correlation is zero. We now report that this formula for the phase difference density can be generalized to take into account other kinds of heterogeneity besides differences in sources of noise, such as heterogeneity in PRC dynamics. Given weak noise (small \( \sigma \)) and small heterogeneities, we obtain the following expression for \( P_s \phi \):

\[
0 = -d \phi \beta P_s \phi + \sigma^2 2 d^2 \phi^2 \alpha_1 + \alpha_2 - 2ch \phi P_s \phi \tag{5}
\]

where \( \beta \) arises due to heterogeneity in the frequencies of the oscillators, \( \alpha_j \) is the average of the squared amplitude of the PRC, and the factor \( ch \phi \) provides a sense of how much correlated noise synchronizes the two oscillators (for details, see Appendix). Heterogeneity in the natural frequencies has a large effect on the ability of the noisy oscillators to synchronize. If \( \sigma^2 = \beta \) (i.e., there is a modest degree of heterogeneity in the frequencies of the 2 oscillators), then \( P_s \phi \approx 1 \) and \( P_s \phi \approx 2 \pi \delta(\tau) \), and \( P_s \phi \) is nearly flat. If \( \beta = 0 \) (i.e., no heterogeneity in the frequencies of the 2 oscillators), then \( P_s \phi = N \alpha_1 + \alpha_2 - 2ch \phi \), where \( N \) is a normalization constant so that the integral of \( P_s \phi \) is 1. A closed-form solution for Eq. 5 is possible but not terribly useful, involving a number of integrals that must be evaluated numerically. We thus instead solve Eq. 5 numerically, using the periodic boundary condition, \( P_s \phi = P_s \phi + 2\pi \), and the normalization requirement, \( \int_0^{2\pi} P_s \phi d\phi = 1 \).
As an illustration of this method applied to experimental data (where oscillators are indeed noisy and heterogeneous), we modeled two oscillators (*Eq. 3*) with $\omega_{1,2} = 1$, using the function form of the PRC (*Eq. 2*) with values derived from two randomly chosen *in vitro* MCs oscillating at roughly the same periodic rate:

$$PRC_1 \theta_1 = \Delta_1 \theta_1 = 0.248 \sin 0.103 - \sin 0.103 + \theta_1 \ e^{0.232 \theta_1 - 2\pi}$$

$$PRC_2 \theta_2 = \Delta_2 \theta_2 = 0.412 \sin 0.634 - \sin 0.634 + \theta_2 \ e^{0.205 \theta_2 - 2\pi}$$

We then applied identical noise to both oscillators ($c = 1$) with $\sigma = 0.25$. We integrated the phase models using the Euler method with a step size of 0.05, throwing out the first 10,000 time units and computing 390,000 subsequent time units. We constructed a histogram of $\phi = \theta_2 - \theta_1$ between $-\pi, \pi$ with 100 bins. We also created a “spike train”, $s_j \ t = \exp -400 \ 1 - \cos \theta_j \ t /0.125371$, scaled to be compatible with the binning of $\phi$ into 100 bins, and computed the cross-correlation of $s_1, s_2$. Finally, we solved *Eq. 5* with $\beta = 0$ and $c = 1$ to get $P_s \phi$. For these noisy oscillators with distinct PRCs (Figure 2.1A), we note that the numerical phase difference density and spike time correlogram are nearly indistinguishable and that the theoretical density predicted from *Eq. 5* also provides a close match (Figure 2.1B).

![Figure 2.1](image)

**Figure 2.1. Predicted impact of heterogeneity on correlation-induced synchronization of uncoupled oscillators**

A: Sample phase-response curves (PRCs; $\Delta_1, \Delta_2$) estimated from 2 *in vitro* mitral cells (MCs). B: Numerical (green) and theoretical (blue) phase difference densities $P_s(\phi)$ (normalized to an integral of 1) of 2 sample oscillators with PRCs shown in A, given perfectly correlated input. Note the close correspondence between the phase difference density and scaled estimate of the spike time correlogram (red). C: Phase difference density of the 2 sample oscillators given perfectly correlated input across different values of $\beta$ (the degree of heterogeneity in the natural frequencies of the 2 oscillators; color index identical to that in D). D: Increasing values of $\beta$ reduce correlation-induced synchrony, measured here as the sharpness in the phase difference density of the 2 sample oscillators, across all levels of input correlation, $c$. OP, order parameter.
This result is consistent across all other oscillator pairs examined. We cannot expect a perfect fit by the theory as the equations are strictly valid only as $\sigma \to 0$. Nevertheless, the theoretical phase difference density still provides an excellent prediction, thereby relating the phase difference density from dynamical theory to spike time cross-correlation. Moreover, this example demonstrates that we have successfully generalized our previous work (Marella and Ermentrout, 2008) to the completely heterogeneous case. We additionally note that, for equal firing rates, the noisy oscillators achieve a large degree of synchrony (Figure 2.1B) despite distinct PRC dynamics (Figure 2.1A), although with a noticeable phase shift from $\phi = 0$.

Next, we fixed $\sigma = 0.25$ while varying $\beta$, the difference in natural frequencies, and examined how the shape of the invariant density changed. To characterize the degree of resulting synchrony, we introduce an order parameter: $OP \equiv \frac{S^2 + C^2}{S_0^2}$, where $S = \int_0^{2\pi} \sin x \ P \ x \ dx$ and $C = \int_0^{2\pi} \cos x \ P \ x \ dx$. When $OP = 0$, the density is flat, and when $OP = 1$, the density has a sharp peak. Thus $OP$ characterizes the degree of synchrony. As stated above, for perfect input correlation ($c = 1$), intrinsic heterogeneity in the PRC dynamics of the two sample oscillators results in a phase shift in the peak of the density $P_\phi$. In this example, small increases in $\beta$ marginally flattened $P_\phi$ but surprisingly compensated for the phase shift from $\phi = 0$ (Figure 2.1C). It is thus possible for combined firing rate and PRC heterogeneities to yield a greater proportion of synchronized spikes and thereby encode stimulus-specific information (Friedrich et al., 2004). Moreover, this example demonstrates that firing rate and PRC heterogeneities do not exclusively yield reduced synchronization and further suggests that stochastic synchronization of neural oscillators may tolerate a degree of heterogeneity in oscillatory dynamics. In general, however, increasing values of $\beta$ flatten $P_\phi$ and thus reduce OP across all levels of input correlation (Figure 2.1D).

2.4. Results

2.4.1. Theoretical analysis of PRC heterogeneity

On the basis of the novel theory described above, we predicted the influence of PRC heterogeneity on stochastic synchronization of neural oscillators by using PRCs measured from two in vitro MCs (Figure 2.1; see Experimental procedures). The theory predicted with good accuracy the reduction in synchrony (as measured by the density of phase differences) observed for a given difference in PRCs and intrinsic firing rates. Specifically, we found that as firing rates of two heterogeneous neural oscillators become increasingly different, the phase difference density flattens (Figure 2.1C,D), corresponding to a decorrelation of the neurons’ firing phases. Moreover, as intrinsic firing rates diverge, the peak of the phase difference density shifts (Figure 2.1C), indicating that pairs of heterogeneous neurons with different intrinsic firing rates will fire with a reliable temporal offset or lag. For any given difference in firing rates, reducing the level of input correlation leads to an approximately linear reduction of output correlation (Figure 2.1D).

2.4.2. In vitro PRC heterogeneity

To investigate the impact intrinsic biophysical diversity has on synchronization of neural oscillations in vitro, we recorded membrane voltages from individual MCs in coronal slices of mouse main OB by using whole cell current-clamp electrophysiology. Each MC was driven by a
step current (150–700 pA) added to low-amplitude (σ = 15–20 pA) colored noise to evoke noisy periodic firing (CV_{ISI} < 0.4; Figure 2.2A) across a range of physiologically relevant rates (15–75 Hz). Critically, this low-CV_{ISI} regime provides a good approximation of periods during which odors evoke periodic firing in MCs and the homologous projection neurons (PNs) of the invertebrate antennal lobe (AL) in vivo (for example, see Cang and Isaacson, 2003; Friedrich et al., 2004; Ito et al., 2009; Kashiwadani et al., 1999; Laurent et al., 1996; Margrie and Schaefer, 2003; although see Discussion). For each oscillating MC, we calculated a STA over the natural period, defined as the cell’s mean ISI (Figure 2.2B). Oscillating MCs exhibited a broad range of STAs (Figure 2.2C), similar to the diversity measured previously in less periodically firing MCs (Padmanabhan and Urban, 2010).

The STA of a neuron firing in a roughly periodic manner (CV_{ISI} < 0.4) can be used to estimate the PRC of the neuron (Ermentrout et al., 2007; Torben-Nielsen et al., 2010). Thus, to translate the biophysical diversity captured by the STA heterogeneity into terms consistent with analyses of oscillatory synchrony, we estimated PRCs for all recorded neurons from their STAs (Figure 2.2B). Oscillating MCs displayed largely type II PRC dynamics (Figure 2.2D) consistent with our previous findings (Ermentrout et al., 2007; Galán et al., 2005). The predominance of type II PRC dynamics in MCs, along with our previous demonstration that stochastic synchronization is enhanced in neurons with type II PRCs (Abouzeid and Ermentrout, 2009; Galán et al., 2007; Marella and Ermentrout, 2008) agrees well with the observation that odorant stimulation can evoke synchronized oscillatory activity in the rodent OB (Kay et al., 2009). Within the class of type II PRC dynamics, however, MCs exhibited considerable diversity in PRC shape (Figure 2.2D). To classify in vitro MC PRC heterogeneity, we fit a generalized three-parameter model (Eq. 2) to each of the estimated PRCs (Figure 2.2B; see Experimental procedures). The proposed PRC model provided an excellent fit to PRCs recorded in vitro ($R^2 = 0.99 \pm 0.02$, μ ± σ). Projection of parameterized PRCs as single points into three-dimensional parameter space (Figure 2.2E) illustrates the intrinsic diversity and density of MC PRCs recorded in vitro.

Examination of 43 in vitro MC PRCs demonstrated considerable diversity in all model parameters (Figure 2.3A–C). Moreover, the parameter values obtained from the fits of these PRCs exhibited significant but moderate interdependence, with $R^2$ values ranging from 0.33 to 0.49 (Figure 2.3D–F). This moderate level of parameter interdependence suggests that the proposed three-parameter PRC model is not unnecessarily complex and further supports the conclusion that PRC heterogeneity is an intrinsic property of in vitro MCs that cannot be attributed to variation in any single parameter.
Figure 2.2. MC PRC diversity

A: Example “periodic” (A1, top) and “noisy periodic” (A2, top) firing evoked in the same MC by a 250-pA step current (A1, bottom) or by colored noise ($\sigma = 15$ pA) added to a 250-pA step current (A2, bottom). B: Example spike-triggered average (STA; primary axis, black) and corresponding PRC (secondary axis, red) of the cell shown in A. Invariance across mean (black), time-subsampled (light gray), and trial-subsampled (dark gray) STAs was used to confirm accurate and consistent STA extraction. Shown in dark red is the fit of the in vitro PRC to the model: $\text{PRC}(\theta) = A[\sin(B) - \sin(B + \theta)]\exp[C(\theta - 2\pi)]$, where parameter $A$ governs the model PRC amplitude, parameter $B$ governs the node of the sinusoidal base of the PRC model, and parameter $C$ governs the balance between negative (phase delay) and positive (phase advance) regions of the PRC model. C,D: STAs (C) and PRCs (D) for a population of MCs ($n = 43$ cells; 16 animals), indexed by color. E: Projection of parameterized PRCs as single points into 3-dimensional parameter space. Color index is same as in C,D.
2.4.3. Firing rate-dependent modulation of PRC dynamics

Changes in periodic firing rates can modulate PRC type and PRC shape within a single dynamical type (Fink et al., 2011; Gutkin et al., 2005; Schultheiss et al., 2010; Stiefel et al., 2008; 2009). Thus we next asked, to what extent is the observed MC PRC diversity a product of firing rate-dependent modulation? To address this question, we first characterized the range of noisy periodic firing rates evoked in the population of recorded MCs by step current injections overlaid with low-amplitude colored noise. For any given magnitude of step current, the population of MCs exhibited a broad range of resulting firing rates (Figure 2.4A). Indeed, no significant relationship was found between step current magnitude and evoked noisy periodic firing rate across the MC population ($P = 0.18$, linear regression). This heterogeneity in evoked firing rates was consistent with the substantial diversity observed within MC FI relationships (Figure 2.4B; Padmanabhan and Urban, 2010), and could not be directly attributed to differences in input resistance ($P = 0.53$, linear regression). In vitro MC heterogeneity is thus evident not only in the cells’ PRCs but also in differences in preferred firing rate ranges.
Figure 2.4. Firing rate dependence of PRC diversity across the MC population

A: Noisy periodic firing rate plotted against the corresponding step current injection for each of the 43 MCs recorded. Each point corresponds to a single MC. No significant relationship existed
To assess how the heterogeneity in evoked noisy periodic firing rates contributes to the heterogeneity in calculated PRCs, we next quantified the difference in parameterized PRC components between MCs firing at different periodic rates. Specifically, we calculated the mean absolute difference in PRC model amplitude, sinusoidal node, and exponential component parameters between MCs oscillating with firing rate differences of 0–5, 5–10, 10–15, and 15–20 Hz (Figure 2.4C–E). Surprisingly, across the MC population, none of the parameterized PRC components exhibited significant firing rate dependence (Figure 2.4E). Differences in individual parameterized PRC components are thus largely independent of firing rate differences between MCs.

Next, we visualized full PRC models and clustering of PRCs in three-dimensional parameter space on the basis of firing rate. Consistent with the above analysis, the set of parameterized PRCs exhibited considerable heterogeneity, even from MCs firing at identical rates (Figure 2.4F1, F2). To provide an additional objective measure for how PRC and firing rate heterogeneities relate, we performed multiple linear regression between parameterized PRCs and recorded periodic firing rates (Figure 2.4F3). A significant ($P = 0.013$, multiple linear regression) but weak ($R^2 = 0.24$) relationship was found between the recorded firing rate and the rate predicted by a linear combination of all three PRC parameters, with the sinusoidal node ($P = 0.027$) and exponential ($P = 0.0020$) component parameters providing significant predictive power. In other words, 24% of the heterogeneity in recorded PRCs could be attributed to the heterogeneity in evoked periodic firing rates. Thus PRC and firing rate heterogeneity are largely independent across the population of recorded MCs.

We additionally considered to what extent variance in spiking periodicity and passive membrane properties related to the recorded PRC heterogeneity. As expected, greater periodicity (i.e., lower CV_{ISI}) was associated with lower amplitude PRCs (Figure 2.4G1). In other words, MCs exhibiting reduced responsiveness to the aperiodic noisy current injection demonstrated behavior closer to that of a pure oscillator ($CV_{ISI} = 0$). The reduced amplitudes of PRCs in MCs exhibiting greater periodicity reflected both lower amplitude and exponential component parameters (Figure 2.4G2). In total, heterogeneity across the set of parameterized PRCs was significantly ($P = 0.013$, multiple linear regression) related to the recorded firing rate. 


= 5.8 × 10^{-4}, multiple linear regression; \( R^2 = 0.36 \) related to the observed variance in recorded CV_{ISI} (Figure 2.4G3), with the exponential component parameter providing significant predictive power (\( P = 0.014 \)). In contrast to heterogeneity in firing rates, PRC heterogeneity exhibited a relatively stronger (\( R^2 = 0.49 \)) and significant (\( P = 7.7 \times 10^{-6} \), multiple linear regression) relationship to variance in recorded input resistance across the MC population (data not shown), as expected from their explicit relationship (Eq. 1). Variance in recorded membrane time constant was only weakly related to the observed heterogeneity in PRCs (\( P = 0.045 \), multiple linear regression; \( R^2 = 0.19 \)), whereas no relationship existed between PRC heterogeneity and the magnitude of step current injection used to evoke periodic firing (Figure 2.4H1–H3; \( P = 0.11 \), multiple linear regression), consistent with intrinsic biophysical properties underlying PRC (and firing rate) heterogeneity.

To explore PRC modulation by firing rate changes further, we drove a subset of MCs at multiple firing rates and quantified the sensitivity of the parameterized PRC within each MC across different firing rates. Using this within-cell approach, we observed a small shift toward lower exponential component parameters with increased firing rates (Figure 2.5A,B). This firing rate dependence generally acted to increase type II PRC dynamics by decreasing negative (phase delay) PRC values (Figure 2.5C). The net effect of within-cell firing rate-dependent modulation of MC PRCs proved quite small over a large range of firing rates (15–75 Hz), however (Figure 2.5C), and thus cannot account for the much larger degree of total MC PRC heterogeneity. Intrinsic biophysical diversity within in vitro MCs thus gives rise to substantial and largely independent PRC and firing rate heterogeneity, which we collectively refer to as heterogeneity in “oscillatory dynamics.”

![Figure 2.5. Quantification of MC PRC modulation by firing rate](image)

**Figure 2.5. Quantification of MC PRC modulation by firing rate**

A: Trajectory of MC PRCs (\( n = 13 \) cells; 6 animals) through parameter space across multiple firing rates (\( \Delta FR \) mean = 16.9 Hz; range = 5.5–44.9 Hz). Note the minimal change in parameterized PRC components, in agreement with the above results (Figure 2.4C-F). B: Quantification of PRC parameter change per 1-Hz increase in firing rate. **\( P < 0.01 \); Student’s t-
test. C: Evolution of the mean parameterized MC PRC across firing rates according to the firing rate dependence of the exponential component calculated in B. As firing rate increases, changes in C yield greater type II PRC dynamics.

2.4.4. Effect of heterogeneity in oscillatory dynamics on correlation-induced synchronization in vitro

Our data thus far establish a considerable degree of intrinsic biophysical diversity within oscillating MCs, as measured by STA and PRC heterogeneity and reflected in heterogeneous FI relationships. We next sought to investigate the functional consequences of this cellular diversity. In particular, how does intrinsic biophysical diversity affect correlation-induced synchronization? To address this question, we drove a subset of 27 MCs with pairs of low-amplitude noisy correlated input (Figure 2.6), mimicking varying degrees of shared glomerular and granule cell synaptic input, and calculated the resulting correlation-induced coherence between evoked spike trains as a measure of synchronization. To eliminate possible contributions of coupling-induced synchronization via lateral synaptic inhibition to our measures of synchrony, we recorded from individual MCs in different slices. This in vitro approach, similar to that used by Galán et al. (2006) and Markowitz et al. (2008), allowed us to then simulate uncoupled pairs of real MCs receiving correlated input and to remove the potential influence of local circuitry on correlation. To specifically isolate the effect of intrinsic MC diversity on the resulting correlation-induced synchrony, we first measured the coherence between spike trains evoked within the same MC by correlated input (thus simulating a “homogeneous” pair of MCs) and then compared this with the coherence measured between spike trains evoked in two different MCs by correlated input (thus simulating a “heterogeneous” pair of MCs) (Figure 2.6). Importantly, the weak fluctuations used (σ = 15 pA) do not reliably drive spike timing (Galán et al., 2008), allowing us to examine spike correlation in the oscillatory regime and differentiating our experiments from those focused specifically on noise-induced spike precision and reliability (Mainen and Sejnowski, 1995). Critically, differences in periodic firing rates can significantly affect stochastic synchrony (Markowitz et al., 2008; and see below). Thus, to distinguish effects of biophysical diversity (as measured by PRC heterogeneity) from effects of firing rate differences, we first focused on correlation-induced spike time coherence between MCs firing at highly similar (|ΔFRij| ≤ 5 Hz) periodic rates.

Figure 2.6. Strategy for investigating the impact of intrinsic heterogeneity on correlation-induced synchronization

Pairs of aperiodic colored noise inputs with identical spectral properties and varying levels of correlation (defined by Cm) were generated by blending uncorrelated white noise processes convolved with an alpha function (τ = 3 ms). Correlated noisy inputs were then injected with step
currents into in vitro MCs to evoke noisy periodic firing within 15–75 Hz. Stochastic synchronization of homogeneous neurons was examined by comparing spike trains evoked within the same MC by sequentially injected correlated inputs. Stochastic synchronization of heterogeneous neurons was examined by comparing spike trains evoked across different MCs by correlated inputs. Sample voltage traces shown are from unpaired recordings of 2 in vitro MCs driven by the pair of correlated noisy inputs shown at left (time rescaled) added to step current injections.

Foremost, we observed a clear effect of input correlation on spike time cross power spectra and coherence in both homogeneous (Figure 2.7A,B) and heterogeneous (Figure 2.7C,D) MC populations, recapitulating the phenomenon of correlation-induced synchronization (Galán et al., 2006). This was further evident in mean spike time correlogram peaks, once time was rescaled to the natural period of oscillations (Figure 2.7A,C insets). To quantitatively compare the level of correlation-induced synchronization in homogenous vs. heterogeneous (|ΔFRij| ≤ 5 Hz) MC populations, we integrated the spike time coherence from 15–75 Hz for each level of input correlation considered. With the use of this measure, the extent of synchronization was significantly greater in homogeneous than in heterogeneous MC populations (Figure 2.7E1), with cell heterogeneity imposing up to a 30% reduction in maximum output coherence (Figure 2.7E2). Intrinsic biophysical diversity within MCs oscillating at similar rates thus significantly limits (but does not abolish) stochastic synchronization.

Markowitz et al. (2008) previously demonstrated that small firing rate differences can have a strong effect on output synchrony of pyramidal neuron pairs driven by perfectly correlated input. To what extent this firing rate effect extends across a population of neurons, depends on the level of input correlation, or relates to physiological neuronal heterogeneity remains unknown, however. Thus we next quantified the effect of firing rate divergence on stochastic synchrony. To do so, we compared the spike time coherence between oscillating MCs with firing rate differences of 0–5, 5–10, 10–15, or 15–20 Hz throughout the range of physiological beta/gamma frequencies. As expected, increasing firing rate divergence significantly reduced the extent of stochastic synchronization observed (Figure 2.7F1), with each 10-Hz increase in firing rate difference imposing up to a 25% reduction in maximum output coherence (Figure 2.7F2). This effect could not be attributed to any systematic shift in biophysical diversity, since PRC differences were consistent across all MC comparisons (Figure 2.7G). Moreover, firing rate differences within homogeneous MC populations also significantly reduced the extent of correlation-induced synchronization (Figure 2.7H) despite low firing rate-dependent PRC modulation (Figure 2.5). Thus, heterogeneity in oscillatory dynamics, comprising both PRC and firing rate heterogeneity, plays a key role in governing stochastic synchronization. Specifically, physiological PRC heterogeneity and low-to-moderate firing rate differences each separately reduce the maximum synchrony possible by up to 25–30%. Combined, these two sources of heterogeneity impose up to a 40% reduction in maximum output synchrony, consistent with a small but fraction of PRC heterogeneity (and its impact on output synchrony) arising from firing rate differences (Figure 2.5). We additionally note that, surprisingly, biophysically diverse MCs oscillating at rates as different as 20 Hz still supported a measure of correlation-induced synchronization (Figure 2.7F1). Our results thus establish stochastic synchronization as a robust phenomenon of real neurons in vitro.
Figure 2.7. Intrinsic MC diversity and firing rate differences limit correlation-induced synchronization

A–D: Mean within-cell (A,B; n = 27 comparisons) and between-cell (C,D; n = 85 comparisons, $|\Delta FR_{ij}| \leq 5$ Hz) spike time cross power spectra (A,C) and correlation-induced spike time coherence (B,D) as a function of MC firing rates in vitro (thin traces, ± SE). Insets: mean spike time correlogram with time rescaled to the period of each oscillating MC pair. For comparisons between cells with low firing rate differences (C), $T$ is the mean period of the 2 oscillating cells. In A1,C1, spike trains are convolved with an 8-ms square-wave pulse before cross power spectra is calculated. In A2,C2, the square-wave pulse is reduced to 2 ms. Note that while absolute magnitudes change, the observed impact of input correlation and heterogeneity on output coherence is essentially invariant to the convolution pulse width. E1: The correlation-induced spike time coherence integrated from 15–75 Hz is greater in homogeneous populations (within-cell comparisons; homo., solid line) than in heterogeneous populations (between-cell comparisons; $|\Delta FR_{ij}| \leq 5$ Hz; hetero., dashed line) ($P = 1.6 \times 10^{-4}$, 2-way ANOVA), reflecting the
impact of MC diversity on stochastic synchronization. This impact is plotted in E2 as the percent decrease in integrated coherence from the level achieved with perfect input correlation. F1: High firing rate differences between MCs (|ΔFR|: (0,5) Hz, n = 85; |ΔFR|: (5,10) Hz, n = 73; |ΔFR|: (10,15) Hz, n = 59; |ΔFR|: (15,20) Hz, n = 53) also significantly limit stochastic synchronization (P = 2.3 × 10⁻⁵, 2-way ANOVA). |ΔFR|: (0,5) Hz > |ΔFR|: (10,15) Hz, |ΔFR|: (15,20) Hz; |ΔFR|: (5,10) Hz > |ΔFR|: (15,20) Hz; |ΔFR|: (10,15) Hz > |ΔFR|: (15,20) Hz, Tukey’s test). Color index is same as in G. The impact of low (0–10 Hz) and high (10–20 Hz) firing rate differences on stochastic synchrony is plotted in F2 as in E2. Note that the impact of cell heterogeneity on output coherence closely matches the impact of moderate firing rate heterogeneity. G: Mean absolute PRC differences (|ΔPRC|; thin lines, ± SE) across the 4 levels of firing rate differences considered in F1. H: Firing rate differences and rate-dependent PRC modulation shown in Figure 2.5 similarly reduce stochastic synchrony in within-cell comparisons [equal firing rates: n = 27 comparisons, solid line; unequal firing rates: n = 16 comparisons (described in Figure 2.5), dashed line; P = 3.2 × 10⁻³, 2-way ANOVA].

2.4.5. Isolating the effect of PRC heterogeneity and firing rate differences on correlation-induced synchronization

We next sought to determine how completely PRC heterogeneity accounted for the reduction in stochastic synchrony observed between neurons oscillating at highly similar rates. That is, we sought to test whether aspects of neuronal dynamics not captured by the PRC contributed significantly to the reduced synchrony observed between vs. within MCs. We thus simulated MC responses using simple models in which all of the neuronal dynamics are derived from the PRC. Specifically, for each in vitro MC, we constructed a noisy phase oscillator using the MC’s recorded oscillatory frequency and parameterized PRC (see Experimental procedures). We then performed analyses of synchrony equivalent to those performed on the spike trains from the real neurons above. Within this highly reduced framework, we observed levels of spike time cross power spectra and correlation-induced spike time coherence in homogeneous (Figure 2.8A,B) and heterogeneous (Figure 2.8C,D) oscillator populations comparable to those in the corresponding in vitro populations (Figure 2.7A–D). Critically, the PRC heterogeneity measured in vitro proved sufficient in these simulations to account for at least the reduction in synchrony observed among heterogeneous MCs in vitro (compare Figure 2.8E with Figure 2.7E1). In other words, differences in firing rates were not required to explain the experimentally observed reduction in synchrony. Likewise, small to moderate differences in oscillatory frequencies yielded at least the same reduction in maximum output coherence among noisy phase oscillators (Figure 2.8F1) as among in vitro MCs (Figure 2.7F). Our simulations thus confirmed that the range of PRC heterogeneity and firing rate differences observed in vitro alone are sufficient to account for the observed changes in stochastic synchronization. Reducing PRC differences to zero significantly enhanced synchronization within heterogeneous populations of simulated oscillators with low firing rate differences (0–5 Hz) to levels near those observed within homogeneous populations (Figure 2.8E). In heterogeneous populations with high firing rate differences (15–20 Hz), however, reducing PRC differences to zero had no significant effect on synchronization of the simulated oscillators (Figure 2.8G). Our simulations thus further revealed that 1) both PRC heterogeneity and firing rate differences limit stochastic synchronization given low to moderate firing rate differences, and 2) high firing rate differences have a dominant effect on stochastic synchronization.
Figure 2.8. PRC heterogeneity and firing rate differences independently limit stochastic synchronization of model neurons

A–D: Mean within-cell (A,B; n = 27 comparisons) and between-cell (C,D; n = 91 comparisons, |ΔFRij| ≤ 5 Hz) spike time cross power spectra (A,C) and correlation-induced spike time coherence (B,D) as a function of firing rate in noisy phase oscillator models with frequencies, PRCs, and CV ISI distributions replicating in vitro findings (thin traces, ± SE). For these spectral analyses, spike trains are convolved with an 8-ms square-wave pulse before cross power spectra are calculated. Inset: mean spike time correlogram with time rescaled to the period of each model neuron pair. For comparisons between model neurons with low firing rate differences (C), $T$ is the mean period of the 2 model neurons. E: The correlation-induced spike time coherence integrated from 15–75 Hz is greater in homogeneous populations (within-cell comparisons; solid black line) than in heterogeneous populations (between-cell comparisons, |ΔFRij| ≤ 5 Hz; black
dashed line), as observed in vitro (Figure 2.7E). Reducing all between-cell PRC (=PRC; light gray line) or firing rate (=FR; dark gray line) differences to zero significantly increased coherence, confirming that both PRC heterogeneity and small firing rate differences (0–5 Hz) impact stochastic synchronization \(P = 1.8 \times 10^{-15},\) 2-way ANOVA, within-cell > =PRC, =FR > between-cell, \(|\Delta FR_{ij}| \leq 5\) Hz, Tukey’s test). \textbf{F1}: High firing rate differences between noisy phase oscillators \(|\Delta FR_{ij}|\): (0,5] Hz, \(n = 91\); \(|\Delta FR_{ij}|\): (5,10] Hz, \(n = 74\); \(|\Delta FR_{ij}|\): (10,15] Hz, \(n = 59\); \(|\Delta FR_{ij}|\): (15,20] Hz, \(n = 61\) also significantly limit correlation-induced spike time coherence \(P = 2.1 \times 10^{-42}\), \(|\Delta FR_{ij}|\): (0,5] Hz > \(|\Delta FR_{ij}|\): (5,10] Hz, \(|\Delta FR_{ij}|\): (10,15] Hz, \(|\Delta FR_{ij}|\): (15,20] Hz, Tukey’s test). \textbf{F2}: Mean absolute PRC differences (thin lines, ± SE) across the 4 levels of firing rate differences considered in \textbf{F1}. \textbf{G}: Reducing all between-cell PRC differences to zero (=PRC; light gray line) does not significantly increase spike time coherence between cells with high firing rate differences (15–20 Hz). In contrast, reducing all between-cell firing rate differences to zero (=FR; dark gray line) significantly raises coherence to levels near those observed in homogeneous populations \(P = 3.2 \times 10^{-75},\) 2-way ANOVA, within-cell > =PRC, =FR, between-cell, \(|\Delta FR_{ij}|\): (15,20] Hz; within-cell > =FR > =PRC, \(|\Delta FR_{ij}|\): (15,20] Hz, Tukey’s test). 

The effect of biophysical diversity on stochastic synchrony is illustrated in an example in which we examined the response of a random subset of in vitro MCs with heterogeneous PRCs (Figure 2.9A) firing at highly similar rates to a series of highly correlated inputs \((C_{in} = 0.8)\). Spike time raster plots (Figure 2.9B) show that each MC exhibited clear noisy periodic activity on each sweep as well as a considerable degree of sweep-to-sweep synchrony induced by the small-amplitude, highly correlated input (Figure 2.9C). Moreover, the subset of MCs also exhibited clear epochs of between-cell synchrony, often with different MC pairs aligning at different times, producing a pattern of phasic synchronization and yielding greater within-cell than between-cell oscillatory synchrony across the entire MC subset at any given instance. This effect was particularly evident in peristimulus time histograms (Figure 2.9D, top), where firing probability within a single cell exhibited sharper peaks and deeper interspike valleys than the firing probability across the entire subset (reflected in the absolute probability difference calculated in Figure 2.9D, bottom). Similar investigation in our modeling framework with the use of a large population of noisy phase oscillators yielded equivalent results (Figure 2.9E–H).
Figure 2.9. Visualizing the impact of PRC heterogeneity on stochastic synchronization

A: PRCs estimated for a small random subset (n = 5) of *in vitro* MCs firing at similar rates (μFR = 35.7 Hz; σFR = 1.2 Hz). B: Sample raster plot of spike times for each *in vitro* MC across a series of highly correlated (C_in = 0.8) inputs added to step current injections. Color index corresponds to PRCs displayed in A. C: Component of highly correlated input injected into each MC. D, top: Distribution of spike probabilities for a single cell (cell 2; cyan) or across the entire subset of heterogeneous MCs (sum; black), condensed from raster plots using 8-ms time bins. Note the clear periodicity in spike probability for both the single cell and the heterogeneous subset. Bottom: Absolute difference in spike probabilities between the single cell and the heterogeneous subset. E: Model PRCs estimated from 27 *in vitro* MCs, with the mean parameterized PRC shown in black. F: Sample raster plot of spike times for 2 sets of 27 noisy phase oscillators.
simulated with equal firing rates (set to the mean rate recorded in vitro, 35 Hz) and driven by highly correlated input ($C_{in} = 0.8$). Each of the 27 homogeneous oscillators (black ticks) was modeled with the mean PRC (in A; black). Each of the 27 heterogeneous oscillators (colored ticks) was modeled with the PRC of corresponding color in E, G: Component of highly correlated input driving each noisy phase oscillator. H, top: Distribution of spike probabilities for homogeneous (black) and heterogeneous (red) populations of noisy phase oscillators. Bottom: Absolute difference in spike probabilities between homogeneous and heterogeneous populations.

2.4.6. Investigating the impact of single PRC component variation on correlation-induced synchronization

Intrinsic biophysical diversity within MCs in vitro, as measured by PRC heterogeneity, thus significantly affects correlation-induced synchronization of neural oscillations. This heterogeneity within MC PRCs is captured in our parameterized PRC model and arises from variance in three components: overall amplitude, sinusoidal node, and the balance between phase advance and delay regimes (Figure 2.3). In our next experiment, we thus examined how diversity within each of these PRC components independently impacts correlation-induced synchronization. Understanding which aspects of PRC shape have the greatest effect on synchronization will ultimately help identify the biophysical properties underlying the overall effect of cellular diversity on synchronization.

We simulated a homogeneous population of noisy phase oscillators with firing rates and model PRC parameters matching the mean values observed in vitro. We then varied a single PRC parameter throughout its observed in vitro range (Figure 2.10A,D,G), calculated the net difference in resulting PRCs between oscillators (Figure 2.10B,E,H), and integrated the resulting spike time coherence from 15–75 Hz between each pair of simulated oscillators for perfectly correlated input (Figure 2.10C,F,I). It is important to note that this simple approach neglects the significant interdependence observed among the distribution of PRC component parameter values for the population of recorded MCs (Figure 2.3). Given the low to moderate ($R^2 = 0.33 – 0.49$) relationships observed among these parameters, however, results of this analysis should still largely hold for MCs and generalize to any population of neurons exhibiting noisy periodic activity (CV_ISI < 0.4) and largely or wholly independent variance among the three PRC components.

In keeping with our in vitro results, nearly all of the resulting PRCs were type II, with the exception of the highest values of either the sinusoidal (Figure 2.10D) or exponential (Figure 2.10G) component parameters. In general, the greatest degree of synchrony was observed along the diagonal of each coherence plot (Figure 2.10C,F,I), which corresponds to the dark blue diagonal demarcating zero PRC difference in Figure 2.10B,E,H. This reaffirms the impact of PRC heterogeneity on correlation-induced synchronization. However, neither the degree of synchrony observed between identical oscillators nor the effect of PRC differences on synchrony was uniform across the range of parameter values considered. For variation in the amplitude component parameter, small levels of PRC heterogeneity strongly desynchronized oscillators with low-amplitude PRCs (Figure 2.10C, bottom left corner), whereas synchronization of oscillators with high-amplitude PRCs tolerated substantially greater PRC heterogeneity (Figure 2.10C, top right quadrant). For variation in the sinusoidal component parameter, high levels of synchrony were only observed between oscillators with similarly low sinusoidal component parameters (Figure 2.10F, bottom left quadrant), even among oscillators with highly similar
PRCs (Figure 2.10F, top right corner) and despite a reduced effect of parameter variation on total PRC differences at high sinusoidal component parameters (Figure 2.10E). Because high values of the sinusoidal component parameter shift the balance between phase advance and delay (Figure 2.10D), this result supports the facilitation of synchronization by type II vs. type I PRC dynamics (Abouzeid and Ermentrout, 2009; Galán et al., 2007; Marella and Ermentrout, 2008). Variation in the exponential component parameter, which also determines the balance between phase advance and delay (Figure 2.10G), had a somewhat smaller effect on stochastic synchronization (Figure 2.10I). Tolerance of synchrony to variation in the exponential component parameter likely arose due to 1) the minimal impact parameter variation had on total PRC differences (Figure 2.10H) and 2) the reduction in overall PRC amplitude and window of responsive phases (Figure 2.10G). Altogether, the impact of PRC heterogeneity on stochastic synchronization thus most strongly depends on PRC type and amplitude.

Figure 2.10. Individual PRC model components independently modulate stochastic synchrony in model neurons
Noisy phase oscillators were simulated with equal firing rates (set to the mean rate recorded in vitro, 35 Hz) and driven by perfectly correlated input (\( C_{in} = 1 \)), in addition to independent white noise, as PRC model parameters were set to their mean values or independently varied throughout their full range of values determined from in vitro recordings. A,D,G: Evolution of model PRCs with independent variation of amplitude (A), sinusoidal (D), or exponential (G) component parameters. B,E,H: The net difference in model PRC shape between oscillators with independent variation of PRC component parameters, as at left. C,F,I: Correlation-induced spike time
coherence integrated from 15–75 Hz (and averaged over 5 instantiations) between oscillators with independent variation of PRC component parameters, as at left and middle.

2.4.7. Comparison of in vitro and simulation results with theoretical predictions

In keeping with our theoretical predictions (see Experimental procedures), our in vitro and simulation results thus far demonstrate that both PRC heterogeneity and firing rate divergence significantly impact correlation-induced synchronization of neural oscillators. To provide more direct comparison between our experimental results and theoretical predictions, we also examined phase difference densities and spike time correlograms from our in vitro and simulation data (Figure 2.11). Instantaneous MC phase was estimated from in vitro voltage traces by linearly interpolating phase across each ISI (Figure 2.11A). Shown clearly for two random in vitro MCs with heterogeneous PRCs (Figure 2.11B) and similar firing rates given perfectly correlated input, the in vitro phase difference density and spike time correlogram closely agreed (Figure 2.11C), revealing a peak in the distribution with a slight phase shift from \( \phi = 0 \) indicative of considerable oscillatory synchrony similar to our theoretical predictions for two other random sample MCs (Figure 2.1B). Furthermore, the same result was derived by solving our novel analytic theory (Eq. 5) for the phase difference density (Figure 2.11C) using model PRCs parameterized from the sample MCs (Figure 2.11B). This example demonstrates that our theoretical framework can accurately capture the impact biophysical diversity among real neurons has on synchronization of periodic firing.

Given this agreement between our mathematical theory and experimental observations, we would expect that the reduction in synchrony imposed by cellular heterogeneity and firing rate differences among in vitro MCs (Figure 2.7) should also manifest in a flattening of the in vitro phase difference density, as predicted by our theory (Figure 2.1). Across the population of 27 MCs for which analyses of oscillatory synchrony were performed, we first observed that the reduction in maximum output coherence imposed by intrinsic biophysical diversity (Figure 2.7E2) indeed translated into a measurable flattening of the average phase difference density and spike time correlograms (Figure 2.11D). Moreover, we again note the close qualitative relationship observed between the phase difference density and spike time correlogram (Figure 2.11D), as established by our theory (Eq. 4). Our estimate of in vitro MC phase also recapitulated the phenomenon of correlation-induced synchrony in terms of phase difference densities, shown in Figure 2.11E for the homogeneous MC population, as well as the pronounced (although not complete) flattening of the phase difference density with increasing firing rate divergence (Figure 2.11F), as predicted by our theory (Figure 2.1C,D). Equivalent results were also found using our reduced neural simulations (Figure 2.1G–I). In total, we thus observed close agreement among our theoretical predictions, which are in terms of phase difference densities, and our electrophysiological and simulation data, lending considerable credence to our core finding that both PRC and firing rate heterogeneity place important constraints on stochastic synchronization.
Figure 2.11. PRC heterogeneity and firing rate differences modulate phase difference densities of \textit{in vitro} MCs and model neurons

A: Linear interpolation of spike phase from voltage traces of 2 sample \textit{in vitro} MCs injected with perfectly correlated input ($C_{in} = 1$) firing at similar mean rates (38.5 and 37.5 Hz). B: Estimated
PRC (solid lines) and corresponding parameterized model PRC (dashed lines) of MCs shown in A. C: Experimental (solid line) and theoretical (dashed line) phase difference density (normalized to an integral of 1; primary axis; red) and experimental spike time correlogram (secondary axis; blue) across a single period (inverse of the mean firing rate of the 2 MCs) for 2 sample MCs shown in A. D,G: Mean phase difference density and mean spike time correlogram, as plotted in C, across the populations of in vitro MCs (D) and simulated noisy phase oscillator models (G) given perfectly correlated input. Homogeneous populations (solid lines) were evaluated using within-cell comparisons (n = 27 MC comparisons and n = 27 model neuron comparisons). Heterogeneous populations (dashed lines) were evaluated using between-cell comparisons (|ΔFR| ≤ 5 Hz; n = 85 MC comparisons and n = 91 model neuron comparisons). E,H: Mean phase difference densities between homogeneous MCs (E; n = 27 within-cell comparisons) and homogeneous model neurons (H; n = 27 within-cell comparisons) given different levels of input correlation (thin traces, ± SE). F,I: Mean phase difference densities between heterogeneous MCs (|ΔFR|: (0.5) Hz, n = 85; |ΔFR|: (5,10] Hz, n = 73; |ΔFR|: (10,15] Hz, n = 59; |ΔFR|: (15,20] Hz, n = 53) and heterogeneous model neurons (|ΔFR|: (0.5) Hz, n = 91; |ΔFR|: (5,10] Hz, n = 74; |ΔFR|: (10,15] Hz, n = 59; |ΔFR|: (15,20] Hz, n = 61) firing at different rates (thin traces, ± SE) given perfectly correlated input.

2.5. Discussion

2.5.1. Overview
Analysis of the mechanisms of oscillatory synchrony has been an important contribution of mathematical theory and modeling to neuroscience. Although the impact of heterogeneity on coupling-induced oscillatory synchrony has been extensively studied, models of correlation-induced synchrony (including our own previous work) have typically not taken into account the fact that neurons, even those of a single molecular type, are not identical to each other but rather differ in their intrinsic properties (Marder and Goaillard, 2006). Here, for the first time, we examine the degree to which biophysical diversity and firing rate differences in a population of real neurons influence correlation-induced oscillatory synchrony. The neuronal heterogeneity examined, which may be due to differences in ion channel expression, membrane properties, and morphology, will also impact the neuronal response to stimuli and the coding properties of populations (Padmanabhan and Urban, 2010). Here, we first measured the degree of diversity in a specific population of neurons (main OB MCs) recorded in vitro via whole cell current-clamp electrophysiology. We then analyzed the output of these neurons to determine how the measured biophysical diversity influences oscillatory synchronization of MCs in response to correlated fluctuating inputs, such as might be received from common synaptic partners. Specifically, we used MC recordings to estimate PRCs and thus quantified the response of periodically firing neurons to transient inputs. We found that MCs exhibit a broad range of type II PRCs that could be well characterized by a relatively simple phenomenological equation with three parameters. From our in vitro data, we estimated the density of MCs in the space defined by these PRC parameters and used this estimate to predict how MC diversity impacts correlation-induced oscillatory synchrony. Collectively, our experimental findings, together with the results of simulations based closely on the recordings of in vitro MCs, provide good agreement with predictions of novel analytic theory also described here.

2.5.2. Heterogeneity in mechanisms of oscillatory synchrony
Previous work has demonstrated that heterogeneity in networks of oscillating neurons can reduce synchrony induced by synaptic or gap junction coupling (Golomb and Rinzel, 1993; Wang and
Buzsáki, 1996; for review, see Kopell and Ermentrout, 2002). Coupling in such networks has generally been modeled as “all-to-all” such that each cell is coupled to every other cell. In a majority of cases, heterogeneity reduced the ability of the neurons to synchronize, with large networks often undergoing a phase transition to a synchronized state as heterogeneity is reduced (Kuramoto, 2003). Like our present results, the way in which neurons synchronize (such as via synaptic inhibition) can make the neuronal synchrony more or less susceptible to heterogeneity (Chow, 1998; White et al., 1998). Gap junctions, through their ability to average the potentials of connected neurons, can reduce the effects of heterogeneity (Kopell and Ermentrout, 2004). Even when neurons have identical biophysical properties, heterogeneity in the number of inputs can impair oscillatory synchrony (Skinner et al., 2005). Analysis of the effects of heterogeneity on correlation-induced synchronization of oscillating neurons has previously been limited to study of the effects of firing rate and input heterogeneity on transient synchronization of neurons (Brody and Hopfield, 2003; Markowitz et al., 2008).

2.5.3. Heterogeneity of mitral cells as oscillators
Our previous work has shown that MCs are heterogeneous in their intrinsic properties and that this source of cellular diversity significantly impacts encoding of stimulus features (Padmanabhan and Urban, 2010). Physiological heterogeneity will also impact the ability of real neurons to synchronize. To analyze these effects, we have treated neurons (in this case, MCs) as noisy oscillators and considered how two different physiological components of heterogeneity impact correlation-induced neural synchrony. We examined heterogeneity in intrinsic firing rate or natural frequency and heterogeneity in the neuronal response to input, as captured by the STA and PRC. Because we specifically examined oscillatory synchrony among periodically firing neurons, the diversity in neuronal response to input was best captured by differences in PRCs. PRC shape and magnitude in homogeneous populations significantly influence correlation-induced synchronization (Abouzeid and Ermentrout, 2009; Galán et al., 2007; Marella and Ermentrout, 2008). We now demonstrate that both firing rate and PRC heterogeneity place important constraints on stochastic synchronization. Specifically, physiological diversity encompassed within PRC heterogeneity resulted in up to a 30% reduction in the maximum output synchrony in vitro, whereas moderate firing rate differences (~10 Hz) similarly imposed up to a 25% reduction. Combined, these two sources of heterogeneity yielded up to a 40% reduction in the maximum output synchrony in vitro. There are a number of ways to understand the impact of heterogeneity in this context. From the standpoint of generating synchronous spiking in a heterogeneous population, up to a ~30% larger population would be required to generate the same number of synchronized spikes as a homogeneous population, and an even larger population would be required given moderate firing rate differences among the heterogeneous neurons. Alternatively, the total impact of intrinsic biophysical diversity among this class of principal neurons is approximately equivalent to a decrease in input correlation by as much as 0.2. This is similar to the range of input correlations observed between neurons in a number of brain areas (Cohen and Kohn, 2011), indicating a large role for cell-to-cell heterogeneity in regulating oscillatory synchrony.

Given this result, we predict that the degree of PRC heterogeneity among MCs (or among any cell type engaged in periodic firing) will significantly impact the degree of stochastic synchronization possible for a given level of firing rate heterogeneity. Such changes to PRC heterogeneity could arise through activity-dependent changes in channel expression,
internalization and recycling, and posttranslational modifications or through changes in channel (in)activation kinetics via neuromodulation and could occur largely independently of (or on a slower timescale than) moment-to-moment changes in driving synaptic input and consequent periodic firing rate. Such a mechanism could thereby enhance synchrony among neurons subject to similar activity levels or neuromodulatory input and decrease synchrony among differentially activated or modulated neurons. Modifying neuronal diversity thus provides a novel mechanism of modulating output synchrony without necessarily altering the average properties of the neurons involved. For example, cholinergic input, which modulates numerous voltage-dependent channels in the hippocampus and cortex and strongly facilitates synchronous network oscillations (Cobb and Davies, 2005), can also homogenize PRCs into a single dynamical type (Stiefel et al., 2008). Conversely, prolonged febrile seizures in rodents precipitate a long-term increase in the variance (but not mean) of resting membrane potential of CA1 stratum oriens interneurons (Aradi and Soltesz, 2002), likely by altering overall input and leak conductances. Such diversification of interneuronal responsiveness should in turn limit the degree of oscillatory synchrony possible and thus may serve as a homeostatic response to epileptic network rhythms. Modulation of oscillatory synchrony due to altered average properties across a population may thus be enhanced or countered by modifications in the variance of those properties. Such changes in neuronal heterogeneity may even contribute to the pathological alterations in oscillatory synchrony observed in a variety of neurological disorders, including schizophrenia, epilepsy, and autism (Uhlhaas and Singer, 2006).

2.5.4. Biophysical components of PRC heterogeneity
In this study, we employed a three-parameter phenomenological model to classify in vitro MC PRC heterogeneity. This simple model affords multiple advantages in the study of how cellular diversity impacts correlation-induced synchrony. First, the proposed model makes no assumptions about underlying ion channel distributions and thus can be generalized to any type of neuron firing in a roughly periodic manner. Second, the proposed model can reproduce the periodicity displayed by many neuronal PRCs while minimizing the potential of over-fitting phasic perturbation data with an expanded Fourier series (Galán et al., 2005; Netoff et al., 2012; Torben-Nielsen et al., 2010). Third, the proposed model enables PRC heterogeneity to be decomposed into variation in tractable PRC features (i.e., amplitude, sinusoidal node, and phase delay/advance balance, rather than abstract spline or harmonic coefficients) and then related to other cellular properties, such as firing rate. Finally, heterogeneity in each of these tractable model features can be directly related to differences in conductance and/or time constants of ion channels to provide critical biophysical insight into cellular diversity. For example, increasing the activation rate of delayed rectifying potassium channels or increasing the inactivation rate of sodium channels selectively amplifies phase delays in a firing rate-dependent manner (Fink et al., 2011), equivalent to a decrease in the exponential component parameter of the PRC model at low sinusoidal component parameter values. Likewise, increasing the conductance of slow low-threshold potassium currents can shift the node between phase delay and advance regimes to later phases while selectively reducing the amplitude of phase advances (Ermentrout et al., 2001; Gutkin et al., 2005; Pfeuty et al., 2003; Stiefel et al., 2008; 2009), equivalent to a decrease in the sinusoidal component parameter of the PRC model coupled to an increase in the exponential component parameter. In general, enhancing depolarizing currents yields greater type I PRC dynamics (equivalent to increasing sinusoidal and exponential component parameters), whereas enhancing hyperpolarizing currents yields greater type II PRC dynamics (equivalent to
decreasing sinusoidal and exponential component parameters) (for review and further examples, see Ermentrout et al., 2012). Modulating a cell’s electrotonic compactness, such as by changing input resistance, provides the simplest (although not only) means of purely modulating PRC amplitude.

2.5.5. Conserved contributions of heterogeneity in oscillatory dynamics to olfactory encoding

Beyond providing a plastic substrate for influencing neuronal synchrony, how might heterogeneity in oscillatory dynamics contribute to olfactory processing in general? During the initial stages of olfaction, odors trigger widespread synchronized oscillatory activity in the OB of mammals (Adrian, 1942; Eeckman and Freeman, 1990; Kashiwadani et al., 1999) and lower vertebrates (Friedrich and Laurent, 2001), and the homologous AL of invertebrates (Ito et al., 2009; Laurent and Davidowitz, 1994; Stopfer et al., 1997; Tanaka et al., 2009). Within this network rhythm, odors can drive transient noisy periodic firing in MC and PN (for example, see Cang and Isaacson, 2003; Friedrich et al., 2004; Ito et al., 2009; Kashiwadani et al., 1999; Laurent et al., 1996; Margrie and Schaefer, 2003), with a fraction of MC and PN spikes phase-locked to the network local field potential (Buonviso et al., 2003; Friedrich et al., 2004; Ito et al., 2009; Kashiwadani et al., 1999; Laurent and Davidowitz, 1994; Tanaka et al., 2009). Each odor evokes a stimulus- and concentration-specific pattern of phasic MC/PN activation and oscillatory synchronization that progressively diverges across time from initial glomerular input patterns (Bathellier et al., 2008; Friedrich et al., 2004; Friedrich and Laurent, 2001; Niessing and Friedrich, 2010; Stopfer et al., 2003; Wehr and Laurent, 1996; Yaksi and Friedrich, 2006), thereby encoding stimulus-specific information while the total change in firing rate across the MC/PN population is relatively constant and odor-independent (Kay and Stopfer, 2006). Moreover, despite the odor and concentration independence of synchronized MC/PN spike phase within the network local field potential (Friedrich et al., 2004; Laurent and Davidowitz, 1994; Stopfer et al., 2003; Wehr and Laurent, 1996), the proportion of synchronized spikes each MC fires can be odor dependent and uncorrelated with the total odor-evoked MC firing rate (Friedrich et al., 2004), thereby providing a second channel of stimulus-specific information. Heterogeneity in the propensity of oscillating MCs to synchronize (i.e., PRC diversity) thus may directly contribute to at least two facets of olfactory encoding: first, by diversifying the order with which MCs synchronize their activity (and thereby propagate activity to higher brain regions) to construct odor-specific activity patterns; and second, by modulating the fraction of synchronized spikes each MC fires, as the PRC strictly determines the timing (and not number) of spikes fired. Firing rate-dependent modulation of MC PRC dynamics could also provide an additional variable by which stimulus-specific information may be conveyed to downstream targets via synchronized oscillatory activity. Given that MCs are subject to both correlated input from common feedforward and lateral interactions, as well as inhibitory coupling via granule cells, how coupling- and correlation-induced mechanisms of oscillatory synchrony interact in olfactory encoding will ultimately prove a key area of future investigation. As a caveat, it is important to note that odors can also evoke aperiodic ($CV_{ISI} > 0.4$) firing in MCs (for example, see Friedrich and Laurent, 2004; Kay and Laurent, 1999; Rinberg et al., 2006) and that the full role of oscillatory synchrony in olfaction remains uncertain.
2.6. Appendix

2.6.1. Relating the phase difference density to spike time correlation

Let \( Y \) be a pulse-like function that converts the phase of a neural oscillator to a spike [e.g., \( Y = \delta \), where \( \delta \) is the Dirac delta function]. We implicitly make the function periodic. Thus we create “spike trains”, \( y_j t = Y \theta_j t \), from the phases \( \theta_j \) of the oscillators. The cross-correlation (with mean firing rate subtracted) is then

\[
C_{ij} \tau = 1 \ 2T \int_{-\tau}^{\tau} y_i t - y_j t + \tau - y_j \ dt.
\]

As \( T \to \infty \) this is an average, and thus

\[
C_{ij} \tau = \frac{\pi}{\pi} \Pr \{ t = \xi, \theta_j t + \tau = \eta \} \ Y \xi Y \eta - Y d\xi d\eta.
\]

By definition, \( \theta_j - \theta_i = \phi \) so that to lowest order, \( \theta_j t + \tau \approx \theta_j t + \tau + \theta_i t + \tau + \phi \) (where we assume that the frequency of the oscillator is 1). The probability \( \Pr \{ \theta_i, \theta_j \} \) can be expressed in terms of \( \theta_i \) and \( \phi = \theta_j - \theta_i \). For small noise, the density is uniform in \( \theta_i \) so that

\[
\Pr \{ \theta_i, \phi \} \approx P_s \phi 2\pi.
\]

Thus we can now write

\[
C_{ij} \tau \approx 1 \ 2\pi \int_{-\pi}^{\pi} P_s \phi Y \xi - Y Y \xi + \tau + \phi - Y d\xi d\phi
\]

\[
= 1 \ 2\pi \int_{-\pi}^{\pi} P_s \phi Y \xi Y \xi + \tau + \phi d\xi d\phi - Y^2.
\]

For \( Y \), a Dirac delta function, we can explicitly obtain

\[
C_{ij} \tau = 1 \ 2\pi \int_{-\pi}^{\pi} P_s \phi \delta \xi \delta \xi + \tau + \phi d\xi d\phi - 2\pi -^2
\]

\[
= P_s - \tau - 1 \ 2\pi 2\pi.
\]

Notice that since we have assumed weak noise and only looked at the lowest order effects, the cross-correlation is periodic and does not decay over time. Pfeuty et al. (2005) have performed a similar calculation for coupled oscillators.

2.6.2. Deriving the phase difference density for two heterogeneous uncoupled oscillators driven by correlated noise

To formally explore the impact of oscillator heterogeneity on correlation-induced synchronization of uncoupled oscillators, we start with two white noise-driven uncoupled oscillators under the assumptions that the noise and heterogeneities are small. After reduction to a pair of phase equations (Teramae et al., 2009), we obtain the Ito differential equations:
where \( A_j \) is heterogeneity in, say, currents, \( \Delta_j \) are the PRCs, \( W \) is the common (correlated) part of the white noise stimulus and \( W_j \) is the independent part of the noise, and \( \sigma \) is the magnitude of the partially correlated noise. What is the stationary density of the phase or time difference, \( \phi = \theta_2 - \theta_1 \)? To find this, we make a simple change of coordinates. Let \( \eta = \theta_1 \) and let \( \phi = \theta_2 - \theta_1 \). We can then rewrite the stochastic equations as

\[
\begin{align*}
    d\eta &= 1 + a_1 \eta + \sigma^2 2 \Delta_1' \eta \Delta_1 \eta dt + \sigma \Delta_1 \eta dW + \frac{1}{1 - \sigma} dW_1, \\
    d\phi &= a_2 \phi + \eta - a_1 \eta + \sigma^2 2 \Delta_2' \phi + \eta \Delta_2 \phi + \eta + \Delta_1 \eta \eta dt \\
        &\quad + \sigma \frac{1}{1 - \sigma} \Delta_2 \phi + \eta + \Delta_1 \phi dW + \frac{1}{1 - \sigma} \Delta_2 \phi + \eta dW_2 - \Delta_1 \eta dW_1.
\end{align*}
\]

Following Gardiner (1985), we can write the associated Fokker-Planck equation for the probability density, \( P \eta, \phi, t \). Because we are only interested in the steady state, \( \partial P / \partial t = 0 \), we obtain the following equation:

\[
0 = -\partial f_1 \eta P \eta \partial \eta - \partial f_2 \eta, \phi P \partial \phi + \sigma^2 2 \Delta_1 \eta \Delta_1 \eta P + 2 \partial^2 \partial \eta \partial \phi + m_{11} \eta \eta P + m_{12} \eta, \phi P + m_{22} \eta, \phi P,
\]

where

\[
\begin{align*}
    f_1 \eta &= 1 + a_1 \eta + \sigma^2 2 \Delta_1 \eta \Delta_1 \eta, \\
    f_2 \eta, \phi &= a_2 \phi + \eta - a_1 \eta + \sigma^2 2 \Delta_2 \phi + \eta \Delta_2 \phi + \eta - \Delta_1 \eta \Delta_1 \eta, \\
    m_{11} \eta &= \Delta_1^2 \eta, \\
    m_{12} \eta, \phi &= c \Delta_1 \eta \Delta_2 \eta + \phi - \Delta_1^2 \eta, \\
    m_{22} \eta, \phi &= \Delta_1^2 \eta + \Delta_2^2 \eta + \phi - 2c \Delta_1 \eta \Delta_2 \eta + \phi.
\end{align*}
\]

We want to obtain a simplified expression for the marginal density, \( P_s \phi := \int_0^{2\pi} P \eta, \phi \ d\eta \). For weak noise (\( \sigma \) small) and small heterogeneities (\( a_j \) small), the density of \( \eta \) is nearly uniform, so we can approximate the full density as \( P \eta, \phi = P_s \phi \ 2\pi \). With this approximation, we can integrate the Fokker-Planck equation with respect to \( \eta \) and use the fact that the boundary conditions are periodic to obtain a simple differential equation for the marginal density:

\[
0 = -d \beta P_s \phi + \sigma^2 2 d^2 d\phi^2 + \alpha_1 + \alpha_2 - 2c \Phi P_s \phi,
\]

where

\[
\begin{align*}
    \alpha_1 &= \alpha_2 = \frac{1}{2} (D_1 + D_2), \\
    \beta &= \frac{1}{2} \left( \frac{D_1 + D_2}{2} + \Sigma_1 \right), \\
    \sigma^2 &= \frac{1}{2} (D_1 + D_2), \\
    \phi &= \frac{1}{2} \left( \frac{D_1 + D_2}{2} + \Sigma_1 \right), \\
    D_1 &= \Delta_1^2, \\
    D_2 &= \Delta_2^2, \\
    \Sigma_1 &= \Delta_1 \Delta_2.
\end{align*}
\]
\[
\beta = 1 \int_{0}^{2\pi} a_2 \eta - a_1 \eta \, d\eta \\
\alpha_1 = 1 \int_{0}^{2\pi} \Delta_1^2 \eta \, d\eta \\
\alpha_2 = 1 \int_{0}^{2\pi} \Delta_2^2 \eta \, d\eta \\
h \phi = 1 \int_{0}^{2\pi} \Delta_1 \eta \Delta_2 \eta + \phi \, d\eta.
\]

In previous studies (e.g., Goldobin et al., 2010), the quantity \( \alpha \) is proportional to the effective diffusion constant for the ISI of the noisy oscillator. That is, the variance of the ISI is proportional to \( \sigma^2 \alpha \), where \( \sigma \) is the magnitude of the noise. We note that \( \alpha_1 + \alpha_2 \geq 2h \phi \) with equality only if \( \Delta_1 \theta = \Delta_2 \theta \) and \( \phi = 0 \). This is explained by the following: \( f x - g x^2 dx \geq 0 \) with equality only if \( f x = g(x) \). Expanding this yields \( f x^2 dx + g x^2 dx \geq 2 f x g x dx \). Substituting \( f = \Delta_1 x \) and \( g = \Delta_2 x + \phi \) gives \( \Delta_1 x^2 dx + \Delta_2 x + \phi^2 dx \geq 2 \Delta_1 x \Delta_2 x + \phi dx \).

2.7. Author contributions

Shawn D. Burton (S.D.B.), G. Bard Ermentrout (G.B.E.), and Nathaniel N. Urban (N.N.U.) designed research; S.D.B. performed electrophysiological experiments and computer simulations; G.B.E. developed novel analytical theory; S.D.B., G.B.E., and N.N.U. analyzed data; S.D.B., G.B.E., and N.N.U. wrote the manuscript.

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3. Greater excitability and firing irregularity of tufted cells underlies distinct afferent-evoked activity of olfactory bulb mitral and tufted cells

3.1. Abstract

Mitral and tufted cells, the two classes of principal neurons in the mammalian main olfactory bulb, exhibit morphological differences but remain widely viewed as functionally equivalent. Results from several recent studies, however, suggest that these two cell classes may encode complementary olfactory information in their distinct patterns of afferent-evoked activity. To understand how these differences in activity arise, we have performed the first systematic comparison of synaptic and intrinsic properties between mitral and tufted cells. Consistent with previous studies, we found that tufted cells fire with higher probability and rates and shorter latencies than mitral cells in response to physiological afferent stimulation. This stronger response of tufted cells could be partially attributed to synaptic differences, as tufted cells received stronger afferent-evoked excitation than mitral cells. However, differences in intrinsic excitability also contributed to the differences between mitral and tufted cell activity. Compared to mitral cells, tufted cells exhibited twofold greater excitability and peak instantaneous firing rates. These differences in excitability probably arise from differential expression of voltage-gated potassium currents, as tufted cells exhibited faster action potential repolarization and afterhyperpolarizations than mitral cells. Surprisingly, mitral and tufted cells also showed firing mode differences. While both cell classes exhibited regular firing and irregular stuttering of action potential clusters, tufted cells demonstrated a greater propensity to stutter than mitral cells. Collectively, stronger afferent-evoked excitation, greater intrinsic excitability and more irregular firing in tufted cells can combine to drive distinct responses of mitral and tufted cells to afferent-evoked input.

3.2. Introduction

Mitral cells (MCs) and tufted cells (TCs), the two classes of principal neurons in the mammalian main olfactory bulb (MOB), are distinguished by their distinct morphology and axonal projections, but whether they are also functionally different remains controversial (for review, see Macrides et al., 1985; Mori and Sakano, 2011). Results from several recent studies, however, suggest that these two cell classes may encode complementary olfactory information in distinct patterns of odor-evoked activity. Compared to MCs, TCs respond to lower odor concentrations (Igarashi et al., 2012; Kikuta et al., 2013), exhibit greater odor concentration invariance in their activity (Fukunaga et al., 2012; Igarashi et al., 2012), fire earlier in the sniff cycle (Fukunaga et al., 2012; Igarashi et al., 2012) and exhibit higher odor-evoked firing rates (Nagayama et al., 2004; Griff et al., 2008). Differentiating what olfactory information is encoded by MCs vs. TCs will first require understanding the origin of their distinct odor-evoked activity.

The differences in odor-evoked activity between MCs and TCs may result from differences in synaptic properties, intrinsic biophysical properties or both. Synaptic differences are likely to be a critical factor, as TCs receive more effective monosynaptic input from olfactory sensory
neurons (OSNs) (Gire et al., 2012) and weaker lateral inhibition (Ezeh et al., 1993; Christie et al., 2001; Phillips et al., 2012) than MCs. Whether these synaptic differences are sufficient to explain the differences in odor-evoked activity between MCs and TCs has not been examined, however. Furthermore, no study to date has investigated whether the two classes of MOB principal neurons differ in their intrinsic biophysical properties. Indeed, most studies of “tufted cell” physiology have focused exclusively on external tufted cells (ETCs), a distinct population of rhythmically bursting glutamatergic interneurons (e.g., see Hayar et al., 2004b; Liu and Shipley, 2008b; De Saint Jan et al., 2009) responsible for driving the indirect, long-lasting depolarization (LLD) of MCs and TCs following direct OSN input (De Saint Jan et al., 2009; Gire and Schoppe, 2009; Najac et al., 2011; Gire et al., 2012).

Equally important to the strength and timing of odor-evoked activity is the temporal pattern of action potentials evoked by sensory input (Schaefer and Margrie, 2007). Multicellular in vivo recordings have established that olfactory experience evokes precise, odor-specific spatiotemporal patterns of firing in principal neurons across the MOB (for review, see Friedrich, 2006; Bathellier et al., 2010). Diversity in principal neuron firing modes is a critical factor contributing to the encoding of stimulus-specific information (Padmanabhan and Urban, 2010; Angelo and Margrie, 2011; Tripathy et al., 2013) and regulation of neuronal synchrony (Burton et al., 2012) in these population activity patterns. In vivo recordings have shown that a subset of MOB principal neurons exhibit regular, tonic firing characterized by low interspike interval (ISI) coefficients of variation (CV$_{ISI}$), while other principal neurons exhibit irregular firing of action potential clusters (i.e., “stuttering”) with high CV$_{ISI}$ (Buonviso et al., 2003; Margrie and Schaefer, 2003; Schaefer et al., 2006; Bathellier et al., 2008; Cury and Uchida, 2010; Carey and Wachowiak, 2011; Shusterman et al., 2011). Systematic investigation in vitro has confirmed that MC populations exhibit both tonic and stuttering firing modes and has provided some detail about the mechanisms by which these firing modes are generated (Chen and Shepherd, 1997; Desmaisons et al., 1999; Friedman and Strowbridge, 2000; Balu et al., 2004; Schaefer et al., 2006; Padmanabhan and Urban, 2010; Angelo and Margrie, 2011; Fadool et al., 2011; Tucker et al., 2013). Equivalent investigation of TC firing modes is currently lacking and will be critical in understanding how TC activity contributes to MOB population activity patterns.

Here, we describe mechanisms by which MCs and TCs may encode distinct olfactory information. We first demonstrate that the differential firing responses of MCs and TCs to afferent-evoked input observed in vivo are maintained in vitro, facilitating more detailed exploration of their origin. Voltage-clamp recordings confirm that TCs receive stronger afferent-evoked excitation than MCs. Surprisingly, however, this synaptic difference is poorly correlated with the differences between MC and TC afferent-evoked firing rates. To identify other factors contributing to the different afferent-evoked firing rates of MCs and TCs, we thus perform the first systematic comparison of MC and TC biophysical properties. Critically, TCs are intrinsically twice as excitable as MCs, and this difference in excitability provides a better prediction of the distinct afferent-evoked firing rates of MCs and TCs than the strength of their afferent-evoked excitation. Our results thus suggest that both synaptic and, in particular, intrinsic cellular properties underlie the greater odor-evoked TC firing rates observed in vivo. We additionally find that TCs exhibit both tonic and stuttering firing modes, but fire significantly more irregularly than MCs, emphasizing the important contribution of high-frequency action potential clusters to TC coding. The amplitude of membrane potential sag mediated by
hyperpolarization-activated currents directly correlates with firing regularity in TCs, similar to MCs (Angelo and Margrie, 2011), but does not directly predict the greater firing irregularity of TCs vs. MCs. Collectively, our results thus identify several key functional differences through which the two classes of MOB principal neurons may transform convergent sensory input into complementary olfactory information.

3.3. Experimental procedures

3.3.1. Ethical approval
All experiments were completed in compliance with the guidelines established by the Institutional Animal Care and Use Committee of Carnegie Mellon University.

3.3.2. Animals
Multiple strains of mice with the C57BL/6 background were used in this study with no difference in results between strains (data not shown). Strains included wild type C57BL/6 (n = 8), Thy1-YFP-G (n = 14) (Feng et al., 2000), V2R-GFP (n = 1) (Del Punta et al., 2002) and M72-GFP (n = 15) (Potter et al., 2001).

3.3.3. Slice preparation
Postnatal day 13–20 mice of both sexes were anaesthetized with isoflurane and decapitated into ice-cold oxygenated dissection solution containing (in mM): 125 NaCl, 25 glucose, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 3 MgCl₂ and 1 CaCl₂. Brains were rapidly isolated and acute horizontal, sagittal and oblique slices (310 μm thick) of the MOB were prepared using a vibratome (VT1200S; Leica, Nussloch, Germany, or 5000 mz-2; Campden, Lafayette, IN, USA). Slices recovered for 15–30 min in ~37°C oxygenated Ringer solution that was identical to the dissection solution except for lower Mg²⁺ concentrations (1 mM MgCl₂) and higher Ca²⁺ concentrations (2 mM CaCl₂). Slices were then stored in room temperature oxygenated Ringer solution until recording.

3.3.4. Cell classification
MOB principal neurons were identified by: (1) large cell body size, (2) cell body position within the MC layer (MCL) or external plexiform layer (EPL), (3) the presence of an apical dendrite projecting toward the glomerular layer (GL) and (4) the presence of at least one lateral dendrite, consistent with classical classification schema (e.g., see Macrides and Schneider, 1982; Mori et al., 1983; Kishi et al., 1984). Principal neurons were classified as MCs if >50% of their cell body resided within the MCL. Principal neurons with cell bodies located only partially within the MCL (i.e., <50% of the cell body) represent “displaced MCs” (Mori et al., 1983; Kishi et al., 1984) and “internal TCs” (Ghosh et al., 2011; Igarashi et al., 2012), and were not targeted for recording due to their ambiguous identity as MCs or TCs without full axonal tracing. Principal neurons with cell bodies residing completely within the EPL (i.e., 0% within the MCL) were classified as TCs. Under this classification scheme, our MC dataset included clear examples of both type I (e.g., see Figure 3.4, mitral cells 02, 04, 06, 07, 18) and type II (e.g., see Figure 3.4, mitral cells 01, 11, 13, 32, 35) MCs that extend their lateral dendrites into the deep and superficial EPL, respectively (Orona et al., 1984), as well MCs with more ambiguous lateral dendrite depths (e.g., see Figure 3.4, mitral cells 08, 19, 20, 21, 31). Furthermore, our resulting TC dataset included cells ranging from deep (e.g., see Figure 3.5, tufted cells 06, 07, 09) to

50
superficial (e.g., see Figure 3.5, tufted cells 12, 26, 27) TCs. Importantly, none of the cells included in the TC dataset exhibited the rhythmic bursting characteristic of ETCs (e.g., see Hayar et al., 2004b; Liu and Shipley, 2008b; De Saint Jan et al., 2009).

3.3.5. Electrophysiology
Slices were continuously superfused with 37°C oxygenated Ringer solution. Cells were visualized using infrared differential interference contrast video microscopy. Whole-cell recordings were made from individual cells using electrodes filled with (in mM) 120 potassium gluconate, 2 KCl, 10 HEPES, 10 sodium phosphocreatine, 4 Mg-ATP, 0.3 Na3GTP, 0–0.2 EGTA, 0–0.025 Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA) and 0.2% Neurobiotin (Vector Labs, Burlingame, CA, USA). The liquid junction potential was 12–14 mV and was not corrected for. Cell morphology was reconstructed under a 100× oil-immersion objective and analyzed with Neurulucida (MicroBrightField, Inc., Williston, VT, USA). In all reconstructed cells shown, the MCL is bracketed by light grey contours and the division between the GL and EPL is shown by a dark grey contour. All cells included in this dataset exhibited spontaneous LLDs (Carlson et al., 2000) and/or intact apical tufts upon reconstruction. A minority of MCs sent a second, thin dendrite to co-terminate with their main apical dendritic tuft in the glomerular layer. These secondary glomerular projections were not included in our quantification of cell morphologies. Data were low-pass filtered at 4 kHz and digitized at 10 kHz using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA) and an ITC-18 acquisition board (Instrutech, Mineola, NY, USA) controlled by custom software written in Igor Pro (WaveMetrics, Lake Oswego, OR, USA).

For extracellular stimulation, a monopolar glass electrode was filled with Ringer solution and connected to a stimulus isolation unit (World Precision Instruments, Sarasota, FL, USA) controlled by transistor–transistor logic pulses from the ITC-18 acquisition board. Stimulus intensity was adjusted until all-or-nothing LLDs (either following or occluding direct monosynaptic OSN input) were reliably (~95% success rate) evoked on each trial. These stimulus intensities and the position of the stimulation electrode (olfactory nerve layer (ONL) vs. GL) differs from our previous study examining MC vs. TC response latencies to minimal glomerulus stimulation (Giridhar and Urban, 2012). For conciseness, we use the phrase “afferent-evoked” to refer to the activity and synaptic input of MCs and TCs arising from the combined direct monosynaptic OSN input and indirect polysynaptic ETC input (i.e., the glomerular LLD) evoked by activating OSN afferents in the ONL.

For measurements of action potential properties (except for afterhyperpolarization (AHP) duration; see below), pipette capacitance was neutralized and series resistance ($R_s$) was stringently minimized (MC: 11.7 ± 1.5 MΩ, range: 8.4–13.0 MΩ, n = 10; TC: 13.1 ± 1.5 MΩ, range: 9.6–15.5 MΩ, n = 12) and compensated for using the MultiClamp Bridge Balance operation. For measurements of afferent-evoked activity and input, pipette capacitance was neutralized and $R_s$ (MC: 20.0 ± 3.9 MΩ, range: 16.4–26.7 MΩ, n = 6; TC: 15.8 ± 3.8 MΩ, range: 12.6–22.9 MΩ, n = 7) was compensated for using the MultiClamp Bridge Balance operation in current clamp and compensated for ≥60% in voltage clamp. $R_s$ was maintained below 40 MΩ (MC: 20.1 ± 7.4 MΩ, range: 8.4–32.5 MΩ, n = 35; TC: 22.3 ± 9.6 MΩ, range: 9.6–39.0 MΩ, n = 28) and compensated for using the MultiClamp Bridge Balance operation for all other measurements, including AHP duration ($T_{AHP} 50\%$ ), which did not significantly vary with $R_s$ in
Electrode resistance was comparable for TC and MC recordings (MC: $6.3 \pm 1.0 \, \text{M} \Omega$, range: 4.7–8.9 M$\Omega$, n = 41; TC: $6.7 \pm 1.1 \, \text{M} \Omega$, range: 4.7–8.6 M$\Omega$, n = 35). Resting membrane potential ($V_{\text{rest}}$) was determined immediately after break in. For measurements of action potential and spike train properties, current was injected to normalize $V_m$ to $-58 \, \text{mV}$ and ionotropic synaptic transmission was blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), DL-2-amino-5-phosphonopentanoic acid (DL-APV, 50 μM) and gabazine (10 μM) (with the exception of Figure 3.3 where ionotropic synaptic transmission was not blocked) after determination of $V_{\text{rest}}$ and the presence of spontaneous LLDs. For measurements of afferent-evoked spiking, current was injected to normalize $V_m$ to $-50 \, \text{mV}$. Voltage clamp recordings were performed at $-60 \, \text{mV}$. Afferent-evoked activity was recorded in response to a train of extracellular stimulation pulses delivered to the ONL and averaged over 3–10 trials per stimulation frequency, with an inter-trial interval of 20 s.

### Data analysis

Afferent-evoked firing rates were calculated by dividing the total number of action potentials evoked in each stimulation cycle by the duration of each stimulation cycle. Afferent-evoked firing latencies were calculated as the time of afferent stimulation to the time of the first action potential (on suprathreshold trials). These latencies were then normalized by the total duration of the stimulation cycle and linearly converted to phase (measured in radians). For analysis of afferent-evoked synaptic currents, the rapid and delayed current peaks and charge transfer were calculated after subtracting the baseline current (measured as the current 10 ms preceding the stimulation). Charge transfer was calculated by integrating currents across the duration of each stimulation cycle (e.g., across each 250 ms window following afferent stimulation at 4 Hz). On some stimulation cycles in voltage-clamped TCs, the short-latency afferent-evoked current was strong enough to generate an escaped action potential; these trials were not considered in our analysis.

Membrane time constant ($\tau_m$), input resistance ($R_{\text{input}}$) and capacitance ($C_m$) were calculated from hyperpolarizing step current injections as previously described (Golowasch et al., 2009). Sag amplitude was measured as in previously published methods (Angelo and Margrie, 2011). Briefly, cells were injected with a series of 2 s hyperpolarizing steps ranging from 0 to $-300 \, \text{pA}$ in steps of $-50 \, \text{pA}$. The sag amplitude of the cell was then calculated as the initial response subtracted from the steady state response for whichever current injection yielded a steady state response closest to $-90 \, \text{mV}$. For cells in which membrane potential reached a minimum and then depolarized until the end of the step current injection (e.g., see Figure 3.12B,D), the initial response was calculated as the minimum voltage reached during the step current injection. For cells in which membrane potential continued to hyperpolarize throughout the duration of the step current injection (e.g., see Figure 3.12A,C), the initial response was calculated as the voltage at 100 ms after the beginning of the hyperpolarizing current injection (the time at which cells with positive sag typically reached their minimum). Firing rate-current (FI) curves were calculated from injections of 2 s depolarizing steps ranging from 0 to 300 pA in steps of 50 pA, consistent with the physiological range of LLD amplitudes observed (Figure 3.2; Carlson et al., 2000; Gire et al., 2012). Spike times and action potential threshold ($V_{\text{threshold}}$) were measured by defining the onset of an action potential as the time at which the voltage derivative exceeded 20 mV ms$^{-1}$. For each cell, action potential properties were calculated from the first action potential evoked by the weakest suprathreshold input (i.e., “rheobase”). Action potential amplitude was measured as the
difference between the voltage at action potential onset and peak. This amplitude was then used to calculate the full action potential width at half-maximal amplitude (FWHM). Slopes of action potential rising and falling phases were calculated as the respective maximum and minimum slopes achieved during an action potential. AHP amplitude was calculated as the minimum voltage reached within 10 ms after an action potential subtracted from \( V_{\text{threshold}} \). \( T_{AHP \ 50\%} \) was calculated by measuring the time of AHP start (when the action potential falling phase reached \( V_{\text{threshold}} \)) to the time at which the AHP had decayed to 50% of its maximal amplitude. The peak instantaneous firing rate was calculated as the inverse of the minimum ISI recorded during step current injections. FI curve gain was calculated as the maximum linear slope of the FI relationship. Spike latencies were measured from the time of step current onset to the first spike time at rheobase input.

Measurements of afferent-evoked activity were compared between MCs and TCs (and across stimulation cycles) using a two-way analysis of variance (ANOVA) with post hoc Tukey’s test. Measurements of firing regularity (i.e., CV\(_{\text{ISI}}\)) were compared between MCs and TCs (and across step current amplitudes) using a two-way ANOVA with post hoc Tukey’s test. All other statistical comparisons were made using linear regression and the non-parametric Wilcoxon rank sum test. Values are reported as mean ± SD unless otherwise noted. All analyses were performed in MATLAB (MathWorks, Natick, MA, USA).

3.4. Results

3.4.1. MCs and TCs exhibit different afferent-evoked activity in vitro

Previous studies have explored the firing response of MCs to the combined monosynaptic OSN input and polysynaptic ETC input (i.e., the glomerular LLD) evoked by afferent stimulation \textit{in vitro} (e.g., see Carlson et al., 2000; Schoppa, 2006b; De Saint Jan et al., 2009; Gire and Schoppa, 2009; Najac et al., 2011; Gire et al., 2012; Shao et al., 2012; 2013) but have not examined the equivalent afferent-evoked firing response of TCs \textit{in vitro}. Thus, whether the differential afferent-evoked MC vs. TC activity observed \textit{in vivo} is maintained \textit{in vitro} is not known. Critically, the maintenance of \textit{in vivo} MOB activity patterns in acute slices is not guaranteed, given the substantial excitatory (Balu et al., 2007; Boyd et al., 2012; Markopoulos et al., 2012), inhibitory (Gracia-Llanes et al., 2010; Nunez-Parra et al., 2013) and neuromodulatory (Petzold et al., 2009; Devoe and Linster 2012) centrifugal input to the MOB that is disrupted in acute slices. Such centrifugal input can influence not only the strength of afferent input (for review, see McGann, 2013) but also may regulate levels of tonic inhibition in the MOB (e.g., see Labarrera et al., 2013), which may differentially influence MC vs. TC afferent-evoked activity \textit{in vivo}. Thus, to explore the mechanisms underlying differential afferent-evoked MC vs. TC activity observed \textit{in vivo}, we first examined whether similar afferent-evoked activity patterns are maintained \textit{in vitro}.

To simulate physiological sniff-coupled afferent-evoked input (Cang and Isaacson, 2003; Margrie and Schaeffer, 2003; Schaefer et al., 2006; Phillips et al., 2012), we stimulated the ONL with a train of five 100 μs constant current pulses (10–100 μA) at 2, 4 and 8 Hz while recording from individual MCs and TCs located slightly caudal to the extracellular stimulation electrode (Figure 3.1A–C). These stimulation frequencies were selected to explore the response of MOB principal neurons to both passive respiratory and active sniffing frequencies of rodents (for
review, see Wachowiak, 2011). For each recording, the stimulus intensity was increased until the first pulse of each train reliably (~95% success rate) evoked afferent-evoked input in the form of an all-or-nothing LLD (Carlson et al., 2000; Gire and Schoppa, 2009) and then kept constant for all trials.

Compared to MCs, TCs exhibited higher afferent-evoked firing rates on suprathreshold trials (Figure 3.1D), were more likely to fire on any given trial (Figure 3.1E) and fired earlier in the stimulation cycle (Figure 3.1F) across all stimulation frequencies examined. Indeed, across all trials and stimulation cycles (including both sub- and supra-threshold cycles), TC firing rates were on average 4.4, 3.8 and 2.9 times greater than MC firing rates across 2, 4 and 8 Hz stimulation frequencies, respectively. These results agree with classical studies examining the in vivo response of MCs and TCs to a single ONL stimulation pulse (Schneider and Scott, 1983; Wellis et al., 1989; Ezeh et al., 1993). Furthermore, these results parallel analogous studies examining the odor-evoked activity of MCs and TCs (Nagayama et al., 2004; Griff et al., 2008; Igarashi et al., 2012; Fukunaga et al., 2012; Kikuta et al., 2013). In particular, the 2- to 3-fold greater afferent-evoked firing rates observed in TCs relative to MCs on supra-threshold trials in vitro (Figure 3.1D) closely matches the 2- to 3-fold greater odor-evoked firing rates observed in TCs relative to MCs in vivo (Nagayama et al., 2004). Likewise, the shorter afferent-evoked firing latencies of TCs relative to MCs in vitro agree with the shorter odor-evoked firing latencies of TCs relative to MCs in vivo. Interestingly, however, the ~25–50 ms difference in in vitro latencies (Figure 3.1F) is consistently less than the ~150–200 ms difference in odor-evoked latencies at low odor concentrations (Fukunaga et al., 2012; Igarashi et al., 2012). This discrepancy in absolute latencies can be partially attributed to differences in stimulation methods (synchronous ONL stimulation vs. asynchronous odor-binding kinetics), and also to potential differences in the inhibitory tone of the MOB circuit under in vitro and in vivo conditions, as Fukunaga et al. (2012) have elegantly demonstrated that interruption of fast inhibition disrupts differences in odor-evoked latencies between MCs and TCs.

Collectively, our results thus confirm that the acute MOB slice provides a viable preparation for exploring mechanisms driving distinct MC vs. TC afferent-evoked activity – in particular, differences in afferent-evoked firing rates. Moreover, these results demonstrate that centrifugal input is not necessary to generate stronger afferent-evoked activity in TCs compared to MCs, suggesting that centrifugal input may regulate temporal aspects of olfactory processing predominantly downstream of M/TC transformation of afferent input (Oswald and Urban, 2012a). Intriguingly, the increasing fraction of subthreshold responses from MCs (but not TCs) across sequential stimulation cycles (Figure 3.1E) also predicts that TC firing may contribute more to total MOB activity patterns than MC firing during prolonged odor exposures. This finding also provides indirect evidence supporting the hypothesis that MCs receive greater levels of afferent-evoked (probably periglomerular cell (PGC)-mediated) inhibition than TCs (Fukunaga et al., 2012; see Discussion).
Figure 3.1. Differential responses of MCs and TCs to afferent input are maintained in vitro.

A,B: Morphology of a representative MC (A) and TC (B).

C: Example afferent-evoked firing response (top) of the representative cells shown in A,B in response to 2, 4 and 8 Hz ONL stimulation. Raster plots (middle) show the firing response across successive trials (first row for...
each cell shows the example spiking response plotted at the top). Timing of ONL stimulation is plotted at the bottom. **D**: Mean afferent-evoked firing rate on suprathreshold trials was significantly greater in TCs than in MCs (2 Hz: \( P = 6.9 \times 10^{-5} \); 4 Hz: \( P = 4.1 \times 10^{-5} \); 8 Hz: \( P = 2.0 \times 10^{-4} \)). **F**: Percentage of subthreshold trials was significantly higher in MCs than in TCs (2 Hz: \( P = 5.2 \times 10^{-10} \); 4 Hz: \( P = 6.1 \times 10^{-6} \); 8 Hz: \( P = 8.8 \times 10^{-5} \)). **G**: First-spike latency on suprathreshold trials was significantly shorter in TCs than in MCs (2 Hz: \( P = 5.5 \times 10^{-5} \); 4 Hz: \( P = 6.0 \times 10^{-10} \); 8 Hz: \( P = 2.0 \times 10^{-6} \)). Data in D–G were recorded from six MCs and seven TCs.

3.4.2. Differences in afferent-evoked excitation and excitability between MCs and TCs contribute to differences in afferent-evoked firing rates

Gire et al. (2012) have recently demonstrated that TCs receive stronger afferent-evoked excitation than MCs following brief electrical ONL or optogenetic OSN stimulation. This previous study, however, did not assess whether this differential afferent-evoked excitation is sufficient to explain the stronger afferent-evoked firing response of TCs compared to MCs. To address this question, we again stimulated the ONL but voltage clamped MCs and TCs at −60 mV (i.e., near the reversal potential for Cl\(^-\)) to record their excitatory synaptic input. In agreement with Gire et al. (2012), we observed significantly larger synaptic currents in TCs than in MCs (Figure 3.2). Specifically, ONL stimulation transferred on average 1.9, 1.8 and 2.1 times more charge to TCs than MCs across 2, 4 and 8 Hz stimulation frequencies, respectively (Figure 3.2D). The greater charge transferred to TCs compared to MCs was predominantly due to a significantly larger short-latency, rapidly decaying current in TCs than in MCs (Figure 3.2E), consistent with an effectively stronger monosynaptic connection of OSNs to TCs than to MCs (Gire et al., 2012). TCs additionally demonstrated modestly larger delayed peak currents (>30 ms after stimulation) driven by the glomerular LLD (Figure 3.2F). Critically, these synaptic differences are not due to differences in apical dendrite length and filtering, as dual somatodendritic recordings have established that LLD events are propagated to the soma of MOB principal neurons with minimal attenuation (Carlson et al., 2000; Gire et al., 2012).
Figure 3.2. TCs receive greater effective afferent input than MCs
A,B: Morphology of a representative MC (A) and TC (B). C: Afferent-evoked synaptic input of the representative cells shown in A and B in response to 2, 4 and 8 Hz ONL stimulation. Five trials of each stimulation frequency are shown. Timing of ONL stimulation is plotted at the bottom. D: Average charge transferred by afferent input during each stimulation cycle was significantly greater in TCs than in MCs (2 Hz: $P = 3.5 \times 10^{-3}$; 4 Hz: $P = 0.025$; 8 Hz: $P = 6.5 \times 10^{-3}$). E: Average peak amplitude of afferent input <4 ms after ONL stimulation was significantly greater in TCs than in MCs (2 Hz: $P = 5.8 \times 10^{-5}$; 4 Hz: $P = 9.5 \times 10^{-3}$; 8 Hz: $P = 1.2 \times 10^{-3}$). F: Average peak amplitude of afferent input >30 ms after ONL stimulation was significantly greater in TCs than in MCs (2 Hz: $P = 5.6 \times 10^{-5}$; 4 Hz: $P = 7.9 \times 10^{-3}$; 8 Hz: $P = 0.015$). Data in D–F were recorded from six MCs and seven TCs.

TCs thus receive stronger afferent-evoked excitation than MCs. However, this difference in excitation is smaller than the differences observed in afferent-evoked MC vs. TC firing rates (compare Figure 3.1 and Figure 3.2). The greater TC firing rates may arise from a non-linear relationship between synaptic input and firing rate that amplifies the difference in afferent-evoked excitation between MCs and TCs. Alternatively, some other factor, such as intrinsic biophysical differences in excitability, may contribute to the differential afferent-evoked firing rates of MCs and TCs. To distinguish between these possible mechanisms, we first looked for a direct relationship between afferent-evoked firing rate and the strength of afferent-evoked excitation across MCs and TCs (Figure 3.2). To discount any confounding effects of using multiple sequential stimulation pulses (e.g., failure of a synaptic response at the end of a train of stimulation pulses), we separately examined both the response to the first stimulation pulse in each stimulus train (Figure 3.2A,D) and the response to the full stimulus train (Figure 3.2B,E). A significant correlation existed between afferent-evoked firing rates and the rapid peak amplitude of afferent-evoked excitation (Figure 3.2B), confirming that the strength of afferent-evoked excitation (as measured by the rapid peak current amplitude) contributes to the distinct afferent-evoked firing rates of MCs and TCs. Indeed, there was no clear difference between MC and TC afferent-evoked firing rates normalized by rapid peak current amplitudes (Figure 3.2C). The relationship between firing rates and rapid peak current amplitudes was quite weak ($R^2 = 0.04$), however, and surprisingly no significant relationship was observed between another measure of afferent-evoked excitation strength (total charge transferred) and firing rate (Figure 3.2D–F). These results thus suggest that other factors in addition to the strength of afferent-evoked excitation contribute to the difference in afferent-evoked firing rates between MCs and TCs.
Figure 3.3. Differences in afferent input and excitability contribute to the higher afferent-evoked firing rates of TCs vs. MCs

A,B: Average firing rates (including subthreshold trials; e.g., see Figure 3.1) plotted against the average rapid peak current (e.g., see Figure 3.2) evoked by ONL stimulation at 2, 4 and 8 Hz for MCs (n = 6) and TCs (n = 7). The responses to the first stimulation pulse in a train of five stimulation pulses are plotted in A. The responses to all stimulation pulses are plotted in B. Rapid peak currents weakly but significantly predicted firing rates across MCs and TCs (B; black line; linear regression: $P = 3.5 \times 10^{-3}; R^2 = 0.04$). This relationship proved insignificant when only considering the response to the first stimulation pulse in each train, however (A; dashed line; linear regression: $P = 0.54$).

C: Average firing rate normalized by the rapid peak current evoked by ONL stimulation at 2, 4 and 8 Hz for MCs and TCs. Error bars denote SEM. D–F: As in A–C but examining the relationship between average afferent-evoked firing rates and the total charge transferred by ONL stimulation. No significant correlation existed between rate and charge transferred in response to the first stimulation pulse (D; dashed line; linear regression: $P = 0.96$) or in response to the full stimulus train (E; dashed line; linear regression: $P = 0.52$). TCs exhibited a strong trend toward higher afferent-evoked firing rates than MCs when normalizing by the total charge transferred by afferent stimulation (F; $P = 0.08$, two-way ANOVA).

G,H: As in A,B and D,E but examining the relationship between average afferent-evoked firing rates and the FI curve gain measured in each MC and TC. FI curve gain significantly predicted the afferent-evoked firing rate evoked by the first ONL stimulation pulse (G; black line; linear regression: $P = 0.0$
1.3 × 10^{-4}; R^2 = 0.33) and the entire ONL stimulation train (H; black line; linear regression: P = 5.7 × 10^{-18}; R^2 = 0.32). I: FI curves of MCs and TCs measured from the response to somatic step current injections.

We therefore next considered whether differences in overall excitability between MCs and TCs might provide a better prediction of afferent-evoked firing rate than the strength of afferent-evoked excitation. To measure excitability, we recorded the firing rates evoked by 2 s somatic step current injections ranging from 0 to 300 pA in amplitude and calculated the gains of the resulting FI curves (Figure 3.3J). Indeed, a significant and comparatively strong relationship existed between FI curve gains and the afferent-evoked firing rates following a single stimulation pulse (Figure 3.3G) or a train of stimuli (Figure 3.3H). This relationship could not be explained by any correlation between FI curve gain and the rapid peak current amplitude of afferent-evoked excitation (data not shown). Thus, these results demonstrate that differences in excitability contribute to the differences in afferent-evoked firing rates between MCs and TCs, and in turn suggest that TCs exhibit greater excitability than MCs. It is important to note, however, that these gain measurements were performed in the presence of intact synaptic transmission, and thus do not provide a pure measure of intrinsic excitability, as both MC and TC firing recruits recurrent inhibition (for review, see Schoppa and Urban, 2003). These results thus motivated a more controlled comparison of the intrinsic biophysical properties of MCs and TCs to better understand the factors contributing to the afferent-evoked activity of MOB principal neurons.

3.4.3. TCs are more excitable than MCs
Currently, the intrinsic biophysical properties of MCs, but not TCs, are well characterized (cf. http://neuroelectro.org/neuron/129/ and http://neuroelectro.org/neuron/131/). Thus, to determine the contribution of intrinsic biophysical differences to the distinct afferent-evoked activity of MCs and TCs, we systematically compared the passive, action potential and spike train properties of MCs and TCs under blockade of fast synaptic transmission.

TCs demonstrated significantly lower C_m and a weak trend toward higher R_{input} than MCs (Table 3.1, Figure 3.7K,M). These results are consistent with prior findings that TCs have smaller somata and fewer and shorter lateral dendrites than MCs (Macrides and Schneider, 1982; Orona et al., 1984; Igarashi et al., 2012; Kikuta et al., 2013). Reconstruction and analysis of 30 MCs and 21 TCs from our in vitro data set confirmed these morphological differences (Table 3.2; Figure 3.4 and Figure 3.5). We additionally observed no significant difference in total process length, total process volume or convex hull volume between MC and TC apical dendritic tufts (Table 3.3; Figure 3.4 and Figure 3.5), suggesting that: (1) the stronger afferent input to TCs is not due to greater overlap of TC dendrites with OSN axons within the glomerular compartment, and (2) both TC and MC apical dendritic tufts are well positioned to synaptically interact with the multitude of juxtaglomerular interneurons. Additionally, pairwise regression analysis between morphological and intrinsic biophysical properties of MCs and TCs revealed that MC R_{input} depends strongly on apical, but not lateral, dendrite volume, even though lateral dendrite volume and soma area strongly regulate MC C_m, as expected (Figure 3.6). This result complements previous reports of high ion channel densities in MC apical dendrites (for review, see Migliore and Shepherd, 2002).
Figure 3.4. MC morphology
Reconstructed morphologies of 30 MCs recorded for analysis of intrinsic MC biophysical properties. The MCL is bracketed by light grey contours and the division between the GL and EPL is shown by a dark grey contour. Scaling is equivalent for Figure 3.4 and Figure 3.5.
Figure 3.5. TC morphology
Reconstructed morphologies of 21 TCs recorded for analysis of intrinsic TC biophysical properties. The MCL is bracketed by light grey contours and the division between the GL and EPL is shown by a dark grey contour. Scaling is equivalent for Figure 3.4 and Figure 3.5.
Figure 3.6. MC and TC intrinsic biophysical properties are largely independent of morphological properties

Linear regression analysis was performed between physiological and morphological properties for MCs (n=30) and TCs (n=21). Significant relationships (with Bonferroni correction for multiple comparisons) are plotted as black (MCs) and grey (TCs) lines. MC $R_{\text{input}}$ significantly decreases with increasing apical, but not lateral, dendrite volume ($p=4.0\times10^{-4}$; $R^2=0.37$). In contrast, MC $C_m$ significantly increases with increasing soma area ($p=3.5\times10^{-5}$; $R^2=0.46$) and lateral dendrite volume ($1.2\times10^{-4}$; $R^2=0.41$). No significant relationships were observed between TC physiological and morphological properties.

Table 3.1. Passive membrane properties of MCs vs. TCs

<table>
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<th>Mitral cells</th>
<th>Tufted cells</th>
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<td>$V_{\text{rest}}$ (mV)</td>
<td>$-53.9 \pm 4.0$ (20)</td>
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<td>$R_{\text{input}}$ (MΩ)</td>
<td>94.3 ± 40.5 (35)</td>
<td>111.8 ± 51.6 (28)</td>
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<td>$\tau_m$ (ms)</td>
<td>21.3 ± 9.4 (35)</td>
<td>18.8 ± 8.6 (28)</td>
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<tr>
<td>$C_m$ (pF)</td>
<td>236.4 ± 94.6 (35)</td>
<td>188.8 ± 110.1 (28)**</td>
</tr>
<tr>
<td>Sag amplitude (mV)</td>
<td>2.0 ± 2.6 (35)</td>
<td>4.4 ± 6.1 (28)</td>
</tr>
</tbody>
</table>

**$p < 0.01$. Values reported are mean ± SD ($n$).

Table 3.2. Somatodendritic morphological properties of MCs vs. TCs

<table>
<thead>
<tr>
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<th>Mitral cells</th>
<th>Tufted cells</th>
</tr>
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<tbody>
<tr>
<td>Soma area ($\mu$m²)</td>
<td>349.0 ± 106.4 (30)</td>
<td>236.2 ± 93.1 (21)**</td>
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<td>Lateral dendrites</td>
<td></td>
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<tr>
<td>$\Sigma$ Length ($\mu$m)</td>
<td>2766.0 ± 1792.2 (30)</td>
<td>1624.8 ± 642.6 (21)**</td>
</tr>
<tr>
<td>$\Sigma$ Volume ($\mu$m³)</td>
<td>3433.8 ± 2221.7 (30)</td>
<td>1937.4 ± 838.8 (21)**</td>
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<td>Apical dendrites</td>
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<tr>
<td>$\Sigma$ Length ($\mu$m)</td>
<td>258.3 ± 60.6 (30)</td>
<td>144.4 ± 44.3 (21)**</td>
</tr>
<tr>
<td>$\Sigma$ Volume ($\mu$m³)</td>
<td>3025.0 ± 1588.3 (30)</td>
<td>1235.0 ± 627.0 (21)**</td>
</tr>
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</table>

**$p < 0.01$; ***$p < 0.001$. Values reported are mean ± SD ($n$).

Table 3.3. Tuft morphological properties of MCs vs. TCs

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<thead>
<tr>
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<th>Mitral cells</th>
<th>Tufted cells</th>
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<tbody>
<tr>
<td>$\Sigma$ Length ($\mu$m)</td>
<td>967.6 ± 464.2 (30)</td>
<td>838.7 ± 356.7 (21)</td>
</tr>
<tr>
<td>$\Sigma$ Volume ($\mu$m³)</td>
<td>816.9 ± 479.7 (30)</td>
<td>599.2 ± 411.4 (21)</td>
</tr>
<tr>
<td>Convex hull ($\mu$m³)</td>
<td>49,040.8 ± 34,375.5 (30)</td>
<td>38,601.4 ± 21,749.2 (21)</td>
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</tbody>
</table>

Values reported are mean ± SD ($n$).

TCs exhibited shorter duration action potentials than MCs (Table 3.4, Figure 3.7E.H). This difference was entirely due to a faster repolarization of TC action potentials (Table 3.4, Figure 3.7F,I,J), suggesting that MCs and TCs differ in their expression of voltage-gated potassium channels. Consistent with this hypothesis, TCs exhibited significantly faster AHP kinetics than MCs, and further tended to exhibit larger AHP amplitudes and a slower action potential rising phase than MCs (Table 3.4, Figure 3.7F,G,I,J).
Table 3.4. Action potential properties of MCs vs. TCs

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<tr>
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<th>Mitral cells</th>
<th>Tufted cells</th>
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<tbody>
<tr>
<td>V_{threshold} (mV)</td>
<td>$-42.2 \pm 3.0 \ (10)$</td>
<td>$-42.5 \pm 2.9 \ (12)$</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>$76.2 \pm 5.4 \ (10)$</td>
<td>$72.1 \pm 5.5 \ (12)$</td>
</tr>
<tr>
<td>FWHM (ms)</td>
<td>$1.06 \pm 0.20 \ (10)$</td>
<td>$0.87 \pm 0.10 \ (12)^*$</td>
</tr>
<tr>
<td>Rising slope (mV ms$^{-1}$)</td>
<td>$237.9 \pm 48.4 \ (10)$</td>
<td>$197.9 \pm 62.5 \ (12)$</td>
</tr>
<tr>
<td>Falling slope (mV ms$^{-1}$)</td>
<td>$-72.2 \pm 20.4 \ (10)$</td>
<td>$-91.4 \pm 13.0 \ (12)^*$</td>
</tr>
<tr>
<td>AHP (mV)</td>
<td>$14.8 \pm 3.2 \ (10)$</td>
<td>$16.8 \pm 3.3 \ (12)$</td>
</tr>
<tr>
<td>T_{AHP 50%} (ms)</td>
<td>$58.2 \pm 77.5 \ (35)$</td>
<td>$20.5 \pm 20.1 \ (28)^{**}$</td>
</tr>
</tbody>
</table>

$^*$p < 0.05; **p < 0.001. Values reported are mean ± SD ($n$).

Figure 3.7. Passive properties of MCs vs. TCs
A–D: Morphology (A,B) and voltage responses (C,D) of a representative MC (A,C) and TC (B,D) to step current injections. E: Waveform of the first action potentials evoked by the weakest suprathreshold input (black suprathreshold traces in C,D). F: Phase plot of membrane potentials during the weakest suprathreshold input. G: AHPs following the action potentials plotted in E. H:
Average action potential waveform. Thin lines denote mean ± SEM. I: Phase plot of average action potential derivative vs. average action potential waveform across MC and TC populations. J: Average temporal evolution of membrane potential derivatives during the first action potential evoked by the weakest suprathreshold input. Averages taken over 10 MCs and 12 TCs in H–J. K–M: Distributions of $R_{\text{input}}$ (K), $\tau_m$ (L) and $C_m$ (M) calculated from hyperpolarizing step current injections (e.g., as plotted in C,D) over 35 MCs and 28 TCs.

Both classes of MOB principal neurons exhibited considerable heterogeneity in their rheobase current (Table 3.5), consistent with previous investigation of MC rheobase values (Angelo and Margrie, 2011). The lack of significant difference between MC and TC rheobase values (Table 3.5) thus suggests that the substantially higher fraction of subthreshold responses to afferent-evoked input recorded in MCs relative to TCs (Figure 3.1) is principally due to differences in the strength of afferent-evoked excitation between MCs and TCs. We likewise found no significant difference between, and a substantial degree of heterogeneity in, MC and TC first-spike latencies at rheobase (Table 3.5). This observation is consistent with the strong dependence of odor-evoked MC and TC latency differences on extrinsic, synaptic properties (Fukunaga et al., 2012).

Table 3.5. Spike train properties of MCs vs. TCs

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<th>Mitral cells</th>
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<tr>
<td>Rheobase (pA)</td>
<td>111.4 ± 55.7 (35)</td>
<td>94.6 ± 49.7 (28)</td>
</tr>
<tr>
<td>Rheobase spike latency (ms)</td>
<td>510.0 ± 486.0 (35)</td>
<td>402.3 ± 479.5 (28)</td>
</tr>
<tr>
<td>Peak instantaneous rate (Hz)</td>
<td>62.8 ± 15.9 (35)</td>
<td>120.1 ± 28.4 (28)**</td>
</tr>
<tr>
<td>FI curve gain (Hz 50 pA$^{-1}$)</td>
<td>9.8 ± 3.8 (35)</td>
<td>20.3 ± 7.2 (28)**</td>
</tr>
<tr>
<td>CV$_{\text{ISI, ~20 Hz}}$</td>
<td>0.45 ± 0.29 (35)</td>
<td>0.80 ± 0.43 (28)**</td>
</tr>
<tr>
<td></td>
<td>***$p$ &lt; 0.001. Values reported are mean ± SD ($n$).</td>
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The two classes of MOB principal neurons exhibited markedly different levels of excitability in response to somatic step current injections (Table 3.5, Figure 3.8), confirming our earlier results (Figure 3.3). Both the average FI curve gain and the peak instantaneous firing rate of TCs doubled those of MCs (Table 3.5, Figure 3.8C–E). These differences probably arise, in part, from the narrower action potentials and the faster AHP kinetics in TCs, which enable TC membrane potentials to more quickly “reset” following an action potential than MC membrane potentials. The weak trend toward higher $R_{\text{input}}$ in TCs may also partially contribute to the greater excitability of TCs vs. MCs. We note, however, that there are numerous examples in our dataset where TCs exhibit greater excitability than MCs despite equal or lower $R_{\text{input}}$ (Figure 3.8F).

In total, we thus find that in addition to extrinsic synaptic differences, the two classes of MOB principal neurons exhibit significant differences in intrinsic biophysical properties. In particular, TCs are intrinsically twice as excitable as MCs on average, and this greater excitability contributes significantly to the higher afferent-evoked firing rates observed in TCs. Moreover, differences in action potential waveforms probably influence synaptic propagation of MC vs. TC activity to MOB interneurons and downstream cortical targets (see Discussion).
Figure 3.8. TCs are intrinsically more excitable than MCs

A,B: Morphology and firing response to various step current injections of a representative MC (A) and TC (B). C,D: FI relationships for 35 MCs (C) and 28 TCs (D). Thick lines correspond to representative cells shown in A and B. Note that several TCs go into depolarization block at high step current amplitudes. E: Average FI relationships across all MCs and TCs. Error bars denote SEM. F: FI curve gain vs. $R_{input}$ across MCs and TCs. Thick-lined symbols correspond to representative cells.

3.4.4. TCs stutter more than MCs

Our results thus far have focused on the strength of afferent-evoked activity in MOB principal neurons. However, several studies have demonstrated that the odor-specific temporal patterning of action potentials also critically contributes to the encoding of olfactory information (for review, see Friedrich, 2006; Bathellier et al., 2010). Indeed, Haddad et al. (2013) recently confirmed that olfactory cortex can decode differences in spike timing of MOB principal
neurons. We thus next examined how MCs and TCs transform input into specific temporal patterns of action potentials.

Principal neurons of the MOB exhibit diverse firing modes in vivo, transforming afferent input into both regular and irregular patterns of action potentials (Buonviso et al., 2003; Margrie and Schaefer, 2003; Schaefer et al., 2006; Bathellier et al., 2008; Cury and Uchida, 2010; Carey and Wachowiak, 2011; Shusterman et al., 2011). We and others have recently demonstrated that at least a portion of this temporal patterning of MOB activity arises from the intrinsic ability of MCs to exhibit both tonic and stuttering firing modes (Padmanabhan and Urban, 2010; Angelo and Margrie, 2011; Fadool et al., 2011; Tucker et al., 2013). To investigate whether TCs exhibit similar firing mode diversity, we compared the regularity of MC and TC spike trains evoked by somatic step current injections (Table 3.5, Figure 3.9). Similar to MCs, TCs exhibited diverse firing modes (Figure 3.9A,B), with output ranging from tonic firing with varying degrees of spike frequency adaptation and narrow ISI distributions to irregular stuttering with highly skewed ISI distributions (Figure 3.9C,D) highly reminiscent of some populations of cortical and subcortical fast-spiking interneurons (for review, see Markram et al., 2004; Tepper and Bolam, 2004). Furthermore, TCs on average fired more irregularly than MCs, as measured by the CV_{ISI} (Table 3.5, Figure 3.9E–H). This difference in regularity was not just due to differences in firing rate, as TCs likewise exhibited significantly higher CV_{ISI} than MCs when we controlled for rate (Table 3.5). Moreover, CV_{ISI} and excitability (as measured by FI curve gain) were not well correlated (Figure 3.9I). These results are also consistent with a recent characterization of the firing patterns of MCs and TCs in vitro in response to spontaneous LLDs (Ma and Lowe, 2010). Indeed, we were able to confirm that the firing mode measured by somatic step current injection closely corresponds to the firing mode evoked by afferent input (Figure 3.10). Thus, we expect that TCs exhibit diverse firing modes and fire more irregularly than MCs in vivo.
Figure 3.9. TCs exhibit diverse firing modes and more irregular firing than MCs
A,B: Spike raster plots across 35 MCs (A) and 28 TCs (B) for firing responses to 2 s-step current injections coming closest to 20 Hz. Spike trains are ordered according to CV\_ISI, with minimum and maximum CV\_ISI values shown. C,D: ISI distributions of the most regular and irregular MC (C) and TC (D) spike trains shown in A,B. E: CV\_ISI across multiple step current injection amplitudes for MCs. F: Average CV\_ISI across all MCs for multiple step current injection
amplitudes. Error bars denote SEM. **G,H:** As in **E** and **F** for TCs. Note that TCs demonstrated a significantly higher $CV_{ISI}$ than MCs (compare **F** and **H**; $P = 5.8 \times 10^{-9}$, two-way ANOVA with *post hoc* Tukey’s test). Note also the close correspondence between the average $CV_{ISI}$ and $CV_2$ for MCs (**F**) but not for TCs (**H**). **I:** FI curve gain vs. $CV_{ISI}$ across MCs and TCs.

**Figure 3.10. The diversity of afferent-evoked firing modes is predicted by somatic step current injections**

**A-C:** Representative MOB principal neuron (A) exhibiting a regular firing response to both afferent stimulation (B) and somatic step current injection (C). Raster plots in **B** show the firing response across multiple successive trials of 2 Hz afferent stimulation (first row shows the example spiking response plotted at top). **D-F:** Same as **A-C**, but for a MOB principal neuron exhibiting a stuttering firing response. **G:** $CV_{ISI}$ of the firing response to afferent stimulation vs. the $CV_{ISI}$ of the firing response to somatic step current injection (evoking a ~20 Hz firing rate) for 5 MCs (black crosses) and 6 TCs (grey diamonds). Thick-lined symbols correspond to the regular and stuttering neurons shown in **A** and **D**, respectively. Solid grey diamond plots the firing response of a stuttering TC with long action potential clusters observed during somatic step current injections. These long action potential clusters generated regular patterns of action potentials within the relatively shorter afferent stimulation cycles, thus yielding a high somatic step current-evoked $CV_{ISI}$ and a low afferent evoked $CV_{ISI}$. Including this outlier, the $CV_{ISI}$ evoked by somatic step current injection significantly predicted the $CV_{ISI}$ evoked by afferent
input (solid line; linear regression: $p=0.03; R^2=0.44$). Omission of the outlier revealed a more robust relationship (dashed line; linear regression: $p=3.5\times10^{-4}; R^2=0.81$).

Interestingly, MCs exhibited a significant age-dependent decrease in firing regularity, particularly after 18 days of age (Figure 3.11). Thus, different developmental time courses may partially explain the more irregular firing of TCs than MCs in our dataset. We note, however, that even mature MCs exhibit clear differences in their stuttering firing patterns compared to TCs (see below).

![Figure 3.11. Biophysical diversity within MCs and TCs is largely independent of age](image)

Linear regression analysis was performed between animal age and $R_{\text{input}}, C_m, \tau_m, \text{sag amplitude}, T_{\text{AHP}50\%}, \text{rheobase, first spike latency at rheobase, } CV_{50\%}, \sim 20 \text{ Hz, and FI curve gain for MCs and TCs. Significant relationships (with Bonferroni correction for multiple comparisons) are plotted as black (MCs) and grey (TCs) lines. Age values have been jittered by } \sim 0.1 \text{ d in the plot of rheobase vs. age to help visualize overlapping data points. MC firing regularity significantly decreases with animal age (p=1.2\times10^{-5}; R^2=0.44). Variance in other MC and TC biophysical properties could not be explained by age differences.}

Visual inspection of spiking patterns further suggested that the way in which irregularly firing MCs and TCs stutter is different. Specifically, TCs fired clusters of high-frequency action potentials separated by long ISIs between clusters (Figure 3.9B). In contrast, MCs exhibited comparatively similar within-cluster and between-cluster ISIs (Figure 3.9A). In other words, the instantaneous firing rate of TCs departed substantially from the mean rate for each spike train,
while the instantaneous firing rate of MCs more closely tracked the mean rate. To quantify this effect, we calculated the instantaneous ISI variability normalized to the instantaneous ISI and averaged this across the spike train to yield a single value per spike train. This metric, called CV2 (Holt et al., 1996), is equivalent to CV$_{SI}$ for a regular spike train and for a perfectly random spike train (i.e., a homogeneous Poisson process) but is lower than CV$_{SI}$ for a slowly rate-modulated spike train (e.g., a spike train with highly discrete bursts). Confirming our initial observations, MCs exhibited nearly identical CV$_{SI}$ and CV2 (Figure 3.9F). In contrast, TCs exhibited a markedly higher CV$_{SI}$ than CV2 (Figure 3.9H), especially at higher input strengths. Thus, the greater overall firing irregularity of TCs compared to MCs arises from a greater propensity of TCs to fire highly discrete clusters of action potentials.

### 3.4.5. Membrane potential sag predicts stuttering in both TCs and MCs

The differences in MC vs. TC firing regularity suggest that distinct ionic mechanisms may regulate MC vs. TC firing modes. Toward this end, Angelo and Margrie (2011) recently demonstrated that differential expression of hyperpolarization-activated cation channels across MCs is related to MC firing regularity. In their study, MCs exhibiting more sag in response to a hyperpolarizing step current (i.e., larger positive sag amplitude) tended to fire more regularly than MCs exhibiting less sag. We therefore next examined the distribution of sag responses in MCs and TCs to determine if: (1) a similar relationship exists between TC firing regularity and sag, and (2) if the greater propensity of TCs to stutter is predicted by the distribution of TC sag amplitudes.

In agreement with Angelo and Margrie (2011), we observed a broad diversity of sag responses in MCs (Figure 3.12E), including both negative (Figure 3.12A) and positive (Figure 3.12B) sag amplitudes. TCs likewise exhibited a broad distribution of sag responses (Table 3.1, Figure 3.12C–E). Interestingly, sag amplitudes strongly predicted rheobase values in both classes of MOB principal neurons (Figure 3.12G), as previously reported for MCs (Angelo and Margrie, 2011). To statistically test for a relationship between sag amplitude and firing mode, we performed linear regression between sag amplitude and the CV$_{SI}$ measured at a mean rate of ~20 Hz. MCs demonstrated a significant negative relationship between sag amplitude and CV$_{SI}$ (Figure 3.12F), strengthening the findings of Angelo and Margrie (2011). In turn, TCs demonstrated an even stronger relationship between sag amplitude and CV$_{SI}$ (Figure 3.12F). Thus, sag amplitude is broadly distributed across both classes of MOB principal neurons and is predictive of firing mode. We note, however, that the greater propensity of TCs than MCs to stutter is not predicted by sag, as MC and TC sag amplitudes did not significantly differ on average (Table 3.1, Figure 3.12E). Thus, our results support the hypothesis that distinct ionic mechanisms regulate MC vs. TC firing modes.
Figure 3.12. Sag amplitude predicts MC and TC firing mode

A: Morphology and membrane potential sag and spiking responses of a representative MC with negative sag amplitude. Spiking response shows the evoked response closest to 20 Hz, where $CV_{ISI}$ is measured. B: As in A, but for a MC with positive sag amplitude. C,D: As in A,B but for two representative TCs. Note that the slow recovery from the large sag amplitude in D is sufficient to evoke several rebound action potentials. E: Distribution of sag amplitudes for 35 MCs and 28 TCs. F: $CV_{ISI}$ vs. sag amplitude. MC sag amplitude significantly predicts $CV_{ISI}$ (black line; linear regression: $P = 9.4 \times 10^{-3}; R^2 = 0.19$). TC sag amplitude likewise predicts $CV_{ISI}$ (grey line; linear regression: $P = 7.3 \times 10^{-3}; R^2 = 0.46$). Thick-lined symbols correspond to representative cells in A–D. G: Rheobase vs. sag amplitude. Sag amplitude significantly predicts rheobase in both MCs (black line; linear regression: $P = 9.4 \times 10^{-6}; R^2 = 0.45$) and TCs (grey line; linear regression: $P = 3.2 \times 10^{-3}; R^2 = 0.29$).
Figure 3.13. Depth analysis of TCs
A: Distribution of somatic depths from the GL-EPL border of reconstructed TCs (see Figure 3.5 for full reconstructions). B-F: Linear regression of somatic depth against rheobase (B; p=0.09), first spike latency at rheobase (C; p=0.24), FI curve gain (D; p=0.84), peak instantaneous firing rate (E; p=0.56), CV\textsubscript{ISI} measured at ~20 Hz (F; p=0.12).

3.5. Discussion

3.5.1. Overview
For more than a century, principal neurons of the MOB have been divided between MCs and TCs on the basis of morphological and laminar differences (for review, see Gire et al., 2013). Here, we report the first systematic comparison of MC and TC intrinsic biophysical properties, and we identify several key functional features by which these two cell classes differ. Foremost, TCs are more excitable than MCs. This difference in excitability emerges, at least partially, from narrower action potentials and faster AHP kinetics in TCs, engendering shorter refractory periods in TCs than in MCs. The greater excitability of TCs, combined with stronger afferent-evoked excitation, helps drive the stronger afferent-evoked firing responses in TCs compared to MCs. Additionally, while both MCs and TCs exhibit diverse firing modes, TCs demonstrate a greater propensity to “stutter”, i.e., to fire discrete clusters of high frequency action potentials separated by long pauses. Hyperpolarization-activated currents predict firing regularity in both MCs and TCs, but cannot directly explain the greater firing irregularity of TCs. In total, these results reveal multiple mechanisms through which the two classes of MOB principal neurons can transform convergent sensory input into complementary olfactory information.

3.5.2. Physiological role of differences in afferent-evoked activity between MCs and TCs
TCs exhibit greater excitability and stronger afferent-evoked excitation than MCs. The combination of these properties may contribute to at least three physiological roles in olfactory processing: (1) differential encoding of complementary sensory information, (2) differential...
regulation of sensory processing by changes in network states and (3) collective encoding of sensory intensity (i.e., odor concentration).

First, the combination of greater excitability and stronger afferent-evoked excitation reliably drives short latency, high rate TC firing (Figure 3.1), yielding a high fidelity afferent-evoked signal. In contrast, the lower excitability and weaker afferent-evoked excitation of MCs, combined with more extensive lateral inhibition (Ezeh et al., 1993; Christie et al., 2001; Phillips et al., 2012) and potentially stronger afferent-evoked inhibition (see below), yields lower firing rates and more variable firing latencies (Figure 3.1), attributes which foster diverse temporal patterning of odor-evoked principal neuron firing (Bathellier et al., 2008; Cury and Uchida, 2010; Shusterman et al., 2011; Kato et al., 2012). Moreover, the weaker afferent-evoked excitation and stronger inhibition of MCs additionally supports broader inhibitory molecular receptive ranges (MRRs) in MCs than in TCs (Nagayama et al., 2004; Kikuta et al., 2013). In total, these observations broadly support an emerging view (e.g., see Nagayama et al., 2010; Schaefer and Margrie, 2012; Igarashi et al., 2012; Fukunaga et al., 2012) that TCs may employ a robust labeled-line code of first-order sensory information (e.g., odor identity) while MCs employ a more flexible population-wide temporal code of second-order sensory information (e.g., odor context and valence). Of note, this hypothesis is in direct accordance with the extensive axonal projections of MCs to piriform, entorhinal and amygdaloid regions and the limited axonal projections of TCs to anterior piriform regions (Nagayama et al., 2010; Igarashi et al., 2012).

Second, differences in intrinsic excitability and afferent-evoked excitation probably also yield distinct susceptibilities of MCs and TCs to changes in network state. In particular, recent evidence shows that enhanced cholinergic signaling in the glomerular microcircuit, such as during elevated attentiveness, sharpens the odor tuning of MCs (Ma and Luo, 2012). Mechanistically, cholinergic modulation of MC tuning depends on the ability of augmented afferent-evoked inhibition to block weak afferent-evoked activity in MCs (D’Souza and Vijayaraghavan, 2012; D’Souza et al., 2013). Our current results thus suggest that TCs, which demonstrate stronger afferent-evoked excitation and greater excitability than MCs, should be less susceptible to effects of cholinergic modulation than MCs. Of note, this hypothesis may be directly examined through re-analysis of several recent datasets of MOB principal neuron activity collected across different brain states (Kato et al., 2012; Blauvelt et al., 2013; Wachowiak et al., 2013).

Finally, the interaction of afferent-evoked inhibition and differences in intrinsic excitability between homotypic MCs and TCs (i.e., MCs and TCs with apical dendritic tufts in the same glomerulus) may be critical to the encoding of stimulus intensity during sparse glomerular activation (Smear et al., 2013). Three arguments support this possibility. (1) MC activity is more strongly influenced by afferent-evoked inhibition than TC activity. MC firing is strongly regulated by afferent-evoked inhibition (Shao et al., 2012), and this inhibition is stable over sequential afferent stimulation cycles (Shao et al., 2013), in contrast to the depressing afferent-evoked excitation that we observe (Figure 3.2D; but see Cang and Isaacson, 2003). The shifting balance of inhibition and excitation probably contributes to adaptation of MC firing rates with repeated afferent stimulation (Figure 3.1E). In contrast, the stable suprathreshold response of TCs to sequential afferent stimulation cycles (Figure 3.1E) suggests that TCs are less influenced
by afferent-evoked inhibition than MCs. Indeed, Fukunaga et al. (2012) have compellingly shown that inhibition (probably mediated by PGCs) strongly modulates the latency of odor-evoked firing in MCs but not in TCs. The greater influence of afferent-evoked inhibition on MC activity could arise from greater PGC–MC connectivity or from the greater excitability of TCs rendering such inhibition less effective. (2) The strength of afferent-evoked inhibition directly regulates the odor concentration dependence of MOB principal neuron activity. In their study, Fukunaga et al. (2012) additionally demonstrated that increasing odor concentration reduces MC, but not TC, firing latencies, paralleling the effect of blocking inhibition. Moreover, the preferential regulation of MC activity by afferent-evoked inhibition and a direct link between such inhibition and concentration dependence may explain the lower odor concentration threshold and greater odor concentration invariance of TC vs. MC activity (Fukunaga et al., 2012; Igarashi et al., 2012; Kikuta et al., 2013). (3) Short latency high rate TC firing significantly recruits inhibition onto MCs. Increasing odor concentrations reduces MC firing latencies by increasing excitatory input while afferent-evoked inhibition remains constant (Margrie et al., 2001; Cang and Isaacson, 2003). This suggests that at least two pathways mediate odor-evoked excitation and inhibition onto MCs. Both OSNs and ETCs are probably important sources of odor concentration-dependent MC excitation (De Saint Jan et al., 2009; Gire and Schoppa, 2009; Najac et al., 2011; Gire et al., 2012). In turn, TCs, which exhibit odor concentration-invariant activity, are probably the primary source driving odor concentration-independent, PGC-mediated inhibition onto MCs. Consistent with this hypothesis, Livneh et al. (2014) have shown that the phase of spontaneous and odor-evoked PGC firing in vivo closely matches the phase of spontaneous and odor-evoked TC, but not MC, firing in vivo (Fukunaga et al., 2012). While TCs can also directly excite MCs (Najac et al., 2011), such lateral excitation between homotypic MOB principal neurons is heavily counterbalanced by inhibition (Urban and Sakmann, 2002) and may serve primarily to regulate spike-time synchrony (Schoppa and Westbrook, 2002). In total, changes in odor concentration thus lead to decodable differences in firing latencies between homotypic MCs and TCs through the interaction of afferent-evoked inhibition and greater TC vs. MC excitability and afferent-evoked excitation.

While speculative, the proposed mechanism for encoding odor concentration within a single glomerulus can explain recent in vivo findings (Smear et al., 2013) and motivates multiple new experiments that will be critical to our understanding of olfactory processing. Foremost among these experiments is the careful dissection of inhibitory interactions between homotypic MCs and TCs. To our knowledge, the ability of MCs and TCs to directly inhibit each other by recruiting PGCs has been widely assumed but remains to be directly demonstrated. It is also possible that the proposed afferent-evoked TC-to-MC inhibition could involve granule cells (GCs) and/or EPL interneurons (Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013), cells classically viewed to mediate “lateral inhibition” between MCs and TCs. These alternative circuit pathways are less likely to mediate the described afferent-evoked inhibition, however, given the more variable and longer latency recruitment of GCs vs. PGCs (Shao et al., 2012) and the tremendous numerical superiority of PGCs to EPL interneurons (Parrish-Aungst et al., 2007), but nevertheless require investigation. Also important will be the direct evaluation of afferent-evoked inhibition onto TCs. Finally, a causal link between TC activity and afferent-evoked inhibition onto MCs predicts a negative correlation between excitatory and inhibitory MRRs of homotypic TC and MCs, respectively – an intriguing hypothesis testable through re-analysis of recent published data (Kikuta et al., 2013).
3.5.3. **Physiological role of differences in firing regularity between MCs and TCs**

The expression of highly regular, tonic firing vs. irregular firing of discrete action potential clusters will significantly impact at least three components of neural signaling in olfaction. First, the ability of slowly modulated inputs (e.g., sniff-coupled afferent input) to phase-lock spike timing (Schaefer et al., 2006) is greater in stuttering vs. regular firing MOB principal neurons (Balu et al., 2004). The higher prevalence of stuttering in TCs that we observed thus suggests that TCs may generate a reliable afferent-evoked signal across consecutive sniffs, while MC spike timing evolves (Patterson et al., 2013). The higher prevalence of stuttering in TCs may also help to explain the greater locking of TC vs. MC firing to respiration in vivo (Phillips et al., 2012). Second, we have recently demonstrated that the temporal patterning of MOB principal neuron input to anterior piriform cortex directly controls the propagation of activity between the MOB and cortex (Oswald and Urban, 2012b). This result, together with our current findings of differences in firing regularity between MCs and TCs, suggests that TCs propagate olfactory information to anterior piriform cortex differently than MCs do. Third, and relatedly, tonic vs. stuttering firing modes probably differentially recruit MOB interneurons, such as GCs, EPL interneurons and PGCs. The impact of firing mode on GC recruitment may further lead to differential recruitment of activity-dependent lateral inhibition (Arevian et al., 2008) between stuttering vs. regularly firing pairs of MOB principal neurons. Significant differences in action potential waveforms between MCs and TCs also may abet differences in interneuron recruitment via differential activation of voltage-gated calcium channels at release sites (e.g., see King and Meriney, 2005).

3.5.4. **Ionic and dynamic mechanisms of firing mode diversity in MOB principal neurons**

Defining the mechanisms driving stuttering will be a critical step in understanding the physiological roles of firing mode diversity in MCs and TCs. In both theoretical and experimental studies, neuronal stuttering is often attributed to the interaction of fast spiking dynamics with a slower, perithreshold-activated outward current, in which outward conductance incrementally accumulates with each action potential until firing is temporarily blocked, yielding overall firing dynamics known as elliptic bursting (e.g., see Rinzel and Ermentrout, 1989; Rush and Rinzel, 1995; Balu et al., 2004; Golomb et al., 2007). This mechanism has specifically been suggested to explain stuttering in MCs through the cumulative de-inactivation of D-type potassium channels (probably representing channels consisting of Kv1.1 subunits) across a cluster of action potentials (Balu et al., 2004). In contrast to this deterministic mechanism, stuttering can also occur stochastically (with an arbitrary number of action potentials per cluster) given subthreshold oscillations and a perithreshold-activated outward current with sufficiently fast activation kinetics (Golomb et al., 2007; Stiefel et al., 2013). While both of these mechanisms are thus consistent with the activity patterns we have recorded in MCs and TCs, their functional consequences on stuttering regularity are significantly different (S. D. Burton et al., unpublished observations), motivating further investigation. Of interest, TCs demonstrated a greater propensity to stutter and faster AHP kinetics than MCs, suggesting that TC stuttering may be more dominated by stochastic dynamics than MC stuttering.

Our results additionally corroborate the relationship between membrane potential sag amplitude and MC firing regularity first identified by Angelo and Margrie (2011), and further extend this relationship to TCs. While the correlation between sag and MOB principal neuron firing mode...
thus seems robust, it is not obvious how a hyperpolarization-activated current can modulate suprathreshold activity. Critically, both hyperpolarization-activated cation channels and perithreshold-activated Kv1.3 channels are differentially expressed across MCs in an activity- and experience-dependent manner (Fadool et al., 2000; 2011; Tucker and Fadool, 2002; Angelo et al., 2012; Tucker et al., 2013), further arguing that broad biophysical diversity across principal neurons is a programmed coding feature of the MOB (Padmanabhan and Urban, 2010; Tripathy et al., 2013). Full understanding of the mechanisms driving stuttering will ultimately require a complete developmental characterization of firing modes across neonatal to adult MCs and TCs.

3.5.5. Functional classification of TCs into a single class distinct from MCs and ETCs
Ramón y Cajal originally divided TCs into internal/deep, middle and external/superficial TCs on the basis of laminar depth (for a review, see Macrides et al., 1985). Here, we have restricted our classification of TCs into a single cell class based on four recent lines of evidence arguing that TCs positioned throughout the EPL are more similar to each other than to MCs and ETCs. (1) All TCs exhibit similar intra- and extra-bulbar axonal projections that are distinct from MC projections (Ghosh et al., 2011; Igarashi et al., 2012). (2) All TCs exhibit similar phase locking to the respiratory cycle that is distinct from the phase of the respiratory cycle that MCs lock to (Fukunaga et al., 2012). (3) All TCs exhibit a stronger monosynaptic OSN input than MCs (Figure 3.2; Gire et al., 2012). (4) Unbiased principal components analyses of morphological and functional properties segregate ETCs and TCs into distinct cell populations (Antal et al., 2006). Consistent with these lines of evidence, we observed no significant correlation between TC somatic depth and FI curve gain, peak instantaneous firing rate or firing regularity (Figure 3.13). The results reported here thus define the fundamental intrinsic biophysical differences between MOB MCs and TCs.

3.6. Author contributions
Shawn D. Burton (S.D.B.) and Nathaniel N. Urban (N.N.U.) designed the research; S.D.B. performed the research; S.D.B. analyzed the data; S.D.B. and N.N.U. wrote the manuscript.

3.7. Acknowledgements
This work was supported by an Achievement Rewards for College Scientists Foundation fellowship (S.D.B.) and National Institute on Deafness and Other Communication Disorders grants F31DC013490 (S.D.B.) and R01DC005798 (N.N.U.). We thank Greg LaRocca and Jennifer Dry-Henich for excellent technical assistance and members of the Urban, Oswald and Kuhlman laboratories for helpful discussions.
4. Rapid feedforward inhibition and asynchronous excitation regulate granule cell activity in the mammalian main olfactory bulb

4.1. Abstract

Granule cell-mediated inhibition is critical to patterning principal neuron activity in the olfactory bulb, and perturbation of synaptic input to granule cells significantly alters olfactory-guided behavior. Despite the critical role of granule cells in olfaction, little is known about how sensory input recruits granule cells. Here, we combined whole-cell patch-clamp electrophysiology in acute mouse olfactory bulb slices with biophysical multicompartmental modeling to investigate the synaptic basis of granule cell recruitment. Physiological activation of sensory afferents within single glomeruli evoked diverse modes of granule cell activity, including subthreshold depolarization, spikelets, and suprathreshold responses with widely distributed spike latencies. The generation of these diverse activity modes depended, in part, on the asynchronous time course of synaptic excitation onto granule cells, which lasted several hundred milliseconds. In addition to asynchronous excitation, each granule cell also received synchronous feedforward inhibition. This inhibition targeted both proximal somatodendritic and distal apical dendritic domains of granule cells, was reliably recruited across sniff rhythms, and scaled in strength with excitation as more glomeruli were activated. Feedforward inhibition onto granule cells originated from deep short-axon cells, which responded to glomerular activation with highly reliable, short-latency firing consistent with tufted cell-mediated excitation. Simulations showed that feedforward inhibition interacts with asynchronous excitation to broaden granule cell spike latency distributions and significantly attenuates granule cell depolarization within local subcellular compartments. Collectively, our results thus identify feedforward inhibition onto granule cells as a core feature of olfactory bulb circuitry and establish asynchronous excitation and feedforward inhibition as critical regulators of granule cell activity.

4.2. Introduction

Sensory encoding in the mammalian main olfactory bulb (MOB) depends critically on granule cell (GC)-mediated inhibition to (1) decorrelate principal neuron activity downstream of overlapping sensory input (Bathellier et al., 2008; Cury and Uchida, 2010; Kato et al., 2012) and (2) temporally pattern principal neuron activity within network rhythms (Friedman and Strowbridge, 2003; Neville and Haberly, 2003; Lagier et al., 2004; Bathellier et al., 2006; Schoppa, 2006b; Lepousez and Lledo, 2013; Fukunaga et al., 2014). Consequently, disruption of GC recruitment by selective perturbation of synaptic excitation or inhibition onto GCs significantly alters MOB network rhythms and odor discrimination (Nusser et al., 2001; Abraham et al., 2010; Nunez-Parra et al., 2013). Thus, understanding how sensory input recruits GCs will be a crucial step in understanding olfaction, yet to date, no study has examined how GCs integrate synaptic excitation and inhibition.

GCs fire at low rates and long latencies in vivo (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Labarrera et al., 2013; Cazakoff et al., 2014; Fukunaga et al., 2014). This sparse, long-latency activity at least partially arises from GC intrinsic properties (Schoppa and Westbrook, 1999; Kapoor and Urban, 2006; Schoppa, 2006a;
Pressler et al., 2007; Egger, 2008; Stroh et al., 2012). Unique features of synaptic excitation onto GCs likely also regulate GC recruitment. GCs receive excitation at reciprocal dendrodendritic synapses formed with principal mitral (MCs) and tufted (TCs) cells. Several recent studies have demonstrated that physiological activation of MOB sensory afferents evokes rapid TC firing but delayed MC firing (Fukunaga et al., 2012; Gire et al., 2012; Igarashi et al., 2012; Burton and Urban, 2014). Thus, the temporal structure of synaptic excitation may play a critical role in shaping GC activity.

Synaptic inhibition onto GCs may also contribute to the low rates and long latencies observed in GCs. Surprisingly, however, how inhibition influences GC activity has received almost no attention, despite the early emergence (Carleton et al., 2003; Panzanelli et al., 2009; Arenkiel et al., 2011; Deshpande et al., 2013) and central role of GABAergic inputs in the integration and maturation of adult-born GCs (Pallotto et al., 2012). Indeed, odors drive synaptic inhibition onto GCs in vivo (Labarrera et al., 2013), and all mature GCs receive functional inhibitory inputs throughout their somatodendritic axis (Price and Powell, 1970c; Panzanelli et al., 2009; Nunez-Parra et al., 2013). However, when and how synaptic inhibition regulates GC activity is not currently known.

Here, we investigate the synaptic basis of GC recruitment using in vitro patch-clamp electrophysiology and biophysical multicompartmental modeling. We demonstrate that the sparse, long-latency GC firing observed in vivo depends on the combination of (1) asynchronous synaptic excitation and (2) rapid, synchronous feedforward GABAergic inhibition, a previously unreported feature of the MOB circuit. Critically, feedforward inhibition not only regulates GC spiking, but dendrite-targeting inhibition can attenuate depolarization within local spines, providing a candidate mechanism for regulating subcellular calcium dynamics to control the balance between dendrodendritic recurrent and lateral inhibition of M/TCs.

4.3. Experimental procedures

4.3.1. Ethical approval
All experiments were completed in compliance with the guidelines established by the Institutional Animal Care and Use Committee of Carnegie Mellon University.

4.3.2. Animals
A combination of Thy1-YFP-G (n = 15; Feng et al., 2000), C57BL/6 (n = 16), albino C57BL/6J (n = 13), and albino C57BL/6J mice crossed to gene-targeted mice expressing the H134R variant of channelrhodopsin-2 fused to enhanced yellow fluorescent protein (ChR2:EYFP) from the olfactory marker protein (OMP) locus (OMP-ChR2:EYFP; n = 24) were used in this study. OMP-ChR2:EYFP mice selectively express ChR2:EYFP in the presynaptic terminals of mature olfactory sensory neurons (OSNs; Smear et al., 2011). Only heterozygous OMP-ChR2:EYFP animals and wild-type littermates were used, because complete loss of OMP expression leads to alterations in olfactory perception (Youngentob and Margolis, 1999; Youngentob et al., 2004; Lee et al., 2011; Kass et al., 2013a,b) and deficits in OSN axonal targeting (St John and Key, 2005) and OSN activation and signaling (Buiakova et al., 1996; Ivic et al., 2000; Youngentob et al., 2003; Reisert et al., 2007; Lee et al., 2011; Kass et al., 2013b).
4.3.3. Slice preparation
Postnatal day 18-28 mice of both sexes were anesthetized with isoflurane and decapitated into ice-cold oxygenated dissection solution containing the following (in mM): 125 NaCl, 25 glucose, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 3 MgCl2, and 1 CaCl2. Brains were isolated rapidly, and acute horizontal slices (310 μm thick) of the MOB were prepared using a vibratome (5000 mz-2; Campden Instruments). Slices recovered for 30 min in ~37°C oxygenated Ringer’s solution that was identical to the dissection solution except for lower Mg2+ concentrations (1 mM MgCl2) and higher Ca2+ concentrations (2 mM CaCl2). Slices were then stored in room temperature oxygenated Ringer’s solution until recording.

4.3.4. Electrophysiology
Slices were superfused continuously with warmed oxygenated Ringer’s solution (temperature measured in bath, 32°C). Cells were visualized using infrared differential interference contrast video microscopy. Recordings were concentrated in the medial half of each horizontal MOB slice, in which individual glomeruli and OSN bundles are frequently visible and MC and TC apical dendrites run parallel to the cutting plane and are thus predominantly intact (cf. Borisovska et al., 2011). GC and deep short-axon cell (dSAC) spiking activity was recorded in current-clamp mode from holding potentials of −61.9 ± 2.3 mV (n = 31) and −66.2 ± 4.4 mV (n = 10), respectively, using electrodes (final electrode resistance of 7.4 ± 1.1 MΩ, n = 51) filled with the following: 120 mM K-glucenate, 2 mM KCl, 10 mM HEPES, 10 mM Na-phosphocreatine, 4 mM Mg-ATP, 0.3 mM Na3GTP, 0.2 mM EGTA, 0.025 mM Alexa Fluor 594 (Life Technologies), and 0.2% Neurobiotin (Vector Laboratories). Cells exhibiting resting membrane potentials greater than −50 mV (frequently with high spontaneous firing rates) were excluded from analysis. Synaptic input to GCs was recorded in voltage-clamp mode using electrodes (6.9 ± 1.2 MΩ, n = 56) filled with the following: 140 mM Cs-gluconate, 10 mM QX-314, 2 mM KCl, 10 mM HEPES, 10 mM Na-phosphocreatine, 4 mM Mg-ATP, 0.3 mM Na3GTP, 0.25 mM Alexa Fluor 594, and 0.2% Neurobiotin. TC spiking and synaptic activity was recorded using electrodes (7.1 ± 1.7 MΩ, n = 7) filled with the K-based solution. Liquid junction potentials were 12-14 mV (K-based solution) and 11 mV (Cs-based solution) and were not corrected for. Cell morphology was reconstructed under a 100× oil-immersion objective and analyzed with Neurolucida (MBF Bioscience). GCs were classified as deep GCs (dGCs; also known as “type II granule cells”) if their apical dendrites branched within or beneath the mitral cell layer (MCL) and their apical dendritic spines were visibly concentrated in the lower half of the external plexiform layer (EPL; Figure 4.1A), as described previously (Mori et al., 1983; Orona et al., 1983). Likewise, GCs were classified as superficial GCs (sGCs; also known as “type III granule cells”) if their apical dendrites branched within the EPL and their apical dendritic spines were visibly concentrated in the upper half of the EPL (Figure 4.1A). A small subset of GCs could not be classified conclusively as sGCs or dGCs and were not included in comparisons of sGCs and dGCs. As noted previously (Mori et al., 1983; Orona et al., 1983), GC soma position partially predicted GC type, with GCs in the MCL or upper granule cell layer (GCL) often exhibiting sGC morphologies and GCs in the lower GCL often exhibiting dGC morphologies. dSACs were identified by their large soma size and inframitral soma position. In addition, GCs could be distinguished easily from dSACs by their passive membrane properties measured in response to a small hyperpolarizing step current injection (Table 4.1), as well as by their action potential and spike train properties measured in response to a series of depolarizing step current injections (Table 4.2 and Table 4.3, respectively). Electrophysiological data were low-pass filtered at 4 kHz.
and digitized at 10 kHz using a MultiClamp 700A amplifier (Molecular Devices) and an ITC-18 acquisition board (InstruTech) controlled by custom software written in IGOR Pro (WaveMetrics). In current-clamp recordings, pipette capacitance was neutralized, and series resistance (GCs, $37.1 \pm 8.7 \, \text{M}\Omega$, $n = 41$; dSACs, $30.3 \pm 6.8 \, \text{M}\Omega$, $n = 10$) was compensated using the MultiClamp Bridge Balance operation. In voltage-clamp recordings, series resistance (GCs, $31.6 \pm 8.4 \, \text{M}\Omega$, $n = 10$) was typically not compensated but was monitored continuously to ensure adequate electrode access and recording quality. Gabazine (GBZ; Toecris Bioscience) was applied transiently for 1–3 min at proximal somatodendritic or distal apical dendritic domains of GCs using a patch pipette filled with Ringer’s solution, 100 μM GBZ, and 25 μM Alexa Fluor 488 to visually monitor drug diffusion. Data collection began <10 s after the onset of GBZ application.

4.3.5. OSN stimulation

For extracellular stimulation, a monopolar glass electrode was filled with Ringer’s solution and connected to a stimulus isolation unit (World Precision Instruments) controlled by transistor-transistor logic pulses from the ITC-18 acquisition board. To selectively activate OSN axons within a single glomerular microcircuit, the stimulation electrode was positioned within the olfactory nerve layer (ONL) slightly superficial and rostral to the target glomerulus, often targeting a visible OSN fiber bundle, as described previously (Borisovska et al., 2011; Najac et al., 2011; Gire et al., 2012). Glomeruli were targeted based on their visibility and proximity to the recorded cell (typically positioned directly superior to the recorded cell). The intensity of extracellular stimulation (100-μs-long pulses; 27.0 ± 20.1 μA, $n = 78$) was increased until excitatory synaptic input was observed reliably (~95% success rate) on each trial in either GCs or dSACs. Increasing the stimulation intensity past the threshold level needed to reliably evoke synaptic excitation typically resulted in a negligible increase in synaptic excitation onto GCs (data not shown), consistent with the all-or-nothing nature of glomerular long-lasting depolarizations (LLDs; Gire and Schoppa, 2009). Of note, identical methods were used previously to evoke LLDs in M/TCs and never resulted in direct excitation of M/TC apical dendrites (Burton and Urban, 2014). For clarity, stimulus artifacts have been blanked in plots showing the response of cells to extracellular OSN stimulation, except when noted. For optogenetic stimulation, slices were illuminated (10–20 ms light pulse) by a 75W xenon arc lamp passed through a YFP filter set and 60× water-immersion objective centered on a single glomerulus with either closed or open field stop, achieving uniglomerular or multiglomerular activation in OMP–ChR2 mice, respectively. In TC recordings, “on-beam” describes photostimulation of the glomerulus to which the TC sends its apical dendrite, whereas “off-beam” describes photostimulation of the glomerulus just rostral to the on-beam glomerulus (see Figure 4.6A).

4.3.6. Data analysis

Action potentials were detected with a voltage derivative threshold of 20 mV/ms. Spikelets were detected with a voltage derivative threshold of 0.6 mV/ms, minimum amplitude of 7 mV, and temporally separated from full somatic action potentials by ≥10 ms, consistent with previous studies (Zelles et al., 2006; Labarrera et al., 2013) and providing a conservative estimate of spikelet prevalence that discounts numerous “prespikes” triggering full somatic action potentials (Zelles et al., 2006). Intrinsic biophysical properties (including passive membrane, action potential, and spike train properties) were measured as described previously (Burton and Urban, 2014). Postsynaptic events were detected using a standard template-matching function in
Axograph (Clements and Bekkers, 1997). IPSCs in GCs were detected using a 51-ms-long double-exponential template with 1 ms baseline, 2 ms rise time constant, and 10 ms decay time constant, approximately matching the kinetics of IPSCs observed previously in adult-born GCs (Carleton et al., 2003). EPSCs in GCs were detected using a 20-ms-long double-exponential template with 1 ms baseline, 0.5 ms rise time constant, and 4 ms decay time constant, approximately matching the kinetics of EPSCs observed previously (Schoppa, 2006a). All events were detected with a threshold amplitude of twice the SD of the baseline noise. Spurious event detections with rise times >5 ms, decay constants >100 ms or <2 ms, or amplitudes <6.5 pA were excluded from analysis. Peristimulus time histograms (PSTHs) of EPSCs and IPSCs were calculated with a bin width of 10 ms. Feedforward inhibition was considered statistically significant if the probability of observing an IPSC within 500 ms after OSN stimulation exceeded the 95% confidence interval of the probability of observing an IPSC within 500 ms before OSN stimulation. The latency and amplitude of feedforward inhibition in each cell was then calculated as the mean first IPSC latency and amplitude, respectively, in the first 10 ms time bin showing a significant increase in IPSC probability after OSN stimulation. Overall, this provided a conservative measure of feedforward inhibition requiring accurate matching of event waveforms to a single double-exponential IPSC template. In a small subset of GCs (n = 4), feedforward inhibition was clearly visible as a reliable upward current deflection after OSN stimulation but was not matched reliably to the IPSC template because of strong coincident excitatory input and incomplete voltage-clamp. This subset of GCs was excluded from our analyses of feedforward inhibition. The highly asynchronous nature of synaptic excitation onto GCs after OSN stimulation precluded accurate matching of all excitatory event waveforms to the single double-exponential EPSC template. Therefore, we calculated the excitation latency within each GC as the time from OSN stimulation to the first point that the mean excitation waveform deviated beyond the root-mean-squared noise level. All measurements are provided as mean ± SD, unless noted otherwise. Error bars denote SEM, unless noted otherwise.

4.3.7. Simulations
GC simulations were performed in the NEURON simulation environment (Hines and Carnevale, 1997) with a time step of 10 μs (a time step of 1 μs yielded equivalent results in a subset of simulations tested). The model GC was reproduced from previously published work (Li and Cleland, 2013) and consisted of four compartments (soma, apical dendrite, gemmule/spine neck, and gemmule/spine body) with multiple experimentally derived biophysical conductances (see Figure 4.8A; Li and Cleland, 2013). Excitatory synaptic inputs consisted of the following: (1) an AMPAR-like conductance with a reversal potential of 0 mV, rise time constant of 1.0 ms, and decay time constant of 7.1 ms, matching the average spontaneous EPSC (sEPSC) kinetics observed (Table 4.4); and (2) an NMDAR-like conductance with a reversal potential of 0 mV, rise time constant of 3.18 ms, decay time constants of 57.14 and 2,000 ms, and physiological Ca2+ permeability (reproduced from Grunditz et al., 2008) calculated using a spine head surface area of 3.71 μm² (Woolf et al., 1991a). Feedforward inhibitory synaptic inputs consisted of a GABAAR-like conductance with a reversal potential of −78.9 mV (Pressler and Strowbridge, 2006), rise time constant of 1.5 ms, and decay time constant of 19.7 ms, matching the average sIPSC kinetics observed (Table 4.4). The maximum excitatory conductance (gex max) for AMPAR- and NMDAR-like inputs was set to 2 and 1 nS, respectively, paralleling previous MOB modeling studies (Migliore et al., 2010; 2014; Li and Cleland, 2013; Yu et al., 2013; 2014). The maximum inhibitory conductance was set to 2 nS to match the largest feedforward
inhibitory events that we observed (in which our voltage clamp was likely to be the most complete). Trial-to-trial spike-time reliability for suprathreshold trials was calculated as described previously (Schreiber et al., 2003), using a Gaussian kernel with 2 ms SD.

4.4. Results

4.4.1. Physiological activation of olfactory bulb glomeruli drives asynchronous subthreshold and suprathreshold excitation of GCs in vitro

Sensory input to the MOB is organized by odorant receptors into distinct glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). Each glomerulus is a large microcircuit in which diverse excitatory and inhibitory interneurons modulate the sensory-evoked excitation of principal M/TC apical dendrites by OSN afferent terminals (for review, see Wachowiak and Shipley, 2006; Cleland, 2010; Nagayama et al., 2014). Several recent studies have demonstrated that odor presentation in vivo or transient OSN stimulation in vitro triggers complex interactions within the glomerulus that generate rapid TC firing but delayed MC firing (De Saint Jan et al., 2009; Najac et al., 2011; Fukunaga et al., 2012; 2014; Gire et al., 2012; Igarashi et al., 2012; Burton and Urban, 2014). To begin to investigate the synaptic basis of GC recruitment, we first characterized the response of GCs to transient low-level stimulation of OSNs that recapitulates the physiological temporal cascade of M/TC firing within a single glomerular microcircuit (Figure 4.1B; see Experimental procedures).

GCs responded to OSN stimulation with a broad range of activity patterns, including reliable short-latency firing (Figure 4.1C,D), unreliable long-latency firing (Figure 4.1E,F), and asynchronous subthreshold depolarizations (Figure 4.1G,H). Subthreshold responses were not attributable to an inability to fire action potentials, because all GCs exhibited normal firing in response to step current injections (Figure 4.1H; Table 4.2, Table 4.3). In addition, many GCs (at least 16 of 35, given our conservative estimates; see Experimental procedures) also exhibited evoked (Figure 4.1H) and/or spontaneous (Figure 4.1D) spikelets, although few GCs (3 of 35) fired full spontaneous action potentials. These spikelets resemble strongly the dendritic branch-specific Na\(^+\)-dependent action potentials (Pinato and Midtgaard, 2005; Zelles et al., 2006) that have been observed frequently in mammalian GCs in vivo (Mori and Takagi, 1978b; Wellis and Scott, 1990; Luo and Katz, 2001; Labarrera et al., 2013) but have thus far been unreported in mammalian GCs in vitro. Such dendritic branch-specific activity is likely to play a critical role in olfactory processing by regulating the subcellular release of GABA from GC dendrites onto select M/TC populations (Zelles et al., 2006; see Discussion). In total, the broad range of activity patterns observed (Figure 4.1I–K) is distinct from previous in vitro accounts that examined GC responses to synchronous M/TC activation (Egger et al., 2005) but is identical to the broad range of odor-evoked GC activity patterns observed in vivo (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Labarrera et al., 2013; Cazakoff et al., 2014; Fukunaga et al., 2014). Collectively, the close correspondence between our results and the odor- evoked GC activity observed previously in vivo suggests strongly that the asynchronous M/TC firing generated by glomerular microcircuit interactions critically regulates odor-evoked GC activity. Having thus established the recruitment properties of GCs in vitro, we next examined the synaptic integration underlying GC recruitment.
Figure 4.1. Glomerular activation drives asynchronous subthreshold and suprathreshold excitation of GCs

A: Circuitry of the MOB. Excitatory cell classes are shown in black. Inhibitory cell classes targeting excitatory cells are shown in red. Inhibitory cell classes targeting other inhibitory cells are shown in blue. GL, Glomerular layer; IPL, internal plexiform layer; ETC, external tufted cell; PGC, periglomerular cell; sSAC, superficial short-axon cell; EPL-IN, EPL-interneuron; GL-dSAC, GL-projecting dSAC; EPL-dSAC, EPL-projecting dSAC; GCL-dSAC, GCL-projecting dSAC; IBP, intrabulbar projection; LOT, lateral olfactory tract. B: Experimental design. GC activity was monitored using whole-cell patch-clamp recordings while single glomeruli were activated by low-level stimulation of OSN axons. C: Bright-field image (top; scale bar, 100 μm) and morphological reconstruction (bottom) of a representative sGC. Gray lines in this and all subsequent reconstructions correspond to the cell layers labeled in A. M, Medial; P, posterior. D: Representative voltage trace (top) and raster plot of spike times across multiple trials (middle) after a single OSN stimulation pulse (bottom) for the sGC shown in C. The highlighted trial in this and all subsequent raster plots corresponds to the representative trace shown. Red arrow marks a spontaneous spikelet. I, F: Same as C, D for another sGC showing a barrage of asynchronous EPSPs and unreliable, long-latency firing. G, H: Same as C, D for a representative dGC showing subthreshold excitation and spikelets (inset: suprathreshold response to step current injections; calibration: 0.5 s, 20 mV/100 pA). I: Histogram of mean spike probabilities across all trials. Note that several GCs exhibit only subthreshold responses. J, K: Histograms of mean number of spikes evoked (J) and mean first-spike latencies (K) per suprathreshold trial.

Table 4.1. Passive membrane properties of GCs vs. dSACs

<table>
<thead>
<tr>
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<th>GC</th>
<th>dSAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{input}}$ (MΩ)</td>
<td>603.2 ± 363.4 (32)</td>
<td>273.4 ± 129.5 (10)**</td>
</tr>
<tr>
<td>$\tau_m$ (ms)</td>
<td>27.3 ± 13.2 (28)</td>
<td>13.0 ± 5.6 (9)**</td>
</tr>
<tr>
<td>$C_m$ (pF)</td>
<td>46.1 ± 11.7 (28)</td>
<td>49.5 ± 17.1 (9)</td>
</tr>
<tr>
<td>$V_{\text{rest}}$ (mV)</td>
<td>−71.2 ± 8.5 (40)</td>
<td>−65.9 ± 7.5 (10)</td>
</tr>
</tbody>
</table>

***p < 0.01 (two-tailed unpaired t test). Values reported are mean ± SD (n).

Table 4.2. Action potential properties of GCs vs. dSACs

<table>
<thead>
<tr>
<th></th>
<th>GC</th>
<th>dSAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{threshold}}$ (mV)</td>
<td>−28.9 ± 7.8 (31)</td>
<td>−46.0 ± 7.5 (9)***</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>55.2 ± 12.0 (31)</td>
<td>72.7 ± 9.9 (9)***</td>
</tr>
<tr>
<td>FWHM (ms)</td>
<td>1.00 ± 0.19 (31)</td>
<td>0.73 ± 0.17 (9)***</td>
</tr>
<tr>
<td>Rising slope (mV/ms)</td>
<td>167.8 ± 64.3 (31)</td>
<td>272.3 ± 82.3 (9)***</td>
</tr>
<tr>
<td>Falling slope (mV/ms)</td>
<td>−60.3 ± 13.9 (31)</td>
<td>−102.5 ± 29.5 (9)***</td>
</tr>
</tbody>
</table>

***p < 0.001 (two-tailed unpaired t test). Values reported are mean ± SD (n).

Table 4.3. Spike train properties of GCs vs. dSACs

<table>
<thead>
<tr>
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<th>GC</th>
<th>dSAC</th>
</tr>
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<tbody>
<tr>
<td>Rheobase (pA)</td>
<td>37.1 ± 21.2 (31)</td>
<td>42.8 ± 24.6 (9)</td>
</tr>
<tr>
<td>Rheobase first-spike latency (ms)</td>
<td>511.6 ± 529.6 (31)</td>
<td>182.5 ± 184.3 (9)</td>
</tr>
<tr>
<td>Gain (Hz/pA)</td>
<td>0.86 ± 0.33 (30)</td>
<td>0.41 ± 0.18 (9)***</td>
</tr>
<tr>
<td>Peak instantaneous rate (Hz)</td>
<td>53.2 ± 21.7 (31)</td>
<td>112.3 ± 64.8 (9)***</td>
</tr>
</tbody>
</table>

***p < 0.001 (two-tailed unpaired t test). Values reported are mean ± SD (n).
Table 4.4. Spontaneous synaptic event properties of GCs vs. dSACs

<table>
<thead>
<tr>
<th></th>
<th>sEPSC</th>
<th>sIPSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (Hz)</td>
<td>4.1 ± 4.3 (37)</td>
<td>1.5 ± 1.3 (37)</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>−17.0 ± 7.1 (37)</td>
<td>24.9 ± 10.5 (37)</td>
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<tr>
<td>Rise_{10-90%} (ms)</td>
<td>1.0 ± 0.3 (37)</td>
<td>1.5 ± 0.7 (37)</td>
</tr>
<tr>
<td>τ_{decay} (ms)</td>
<td>7.1 ± 3.7 (37)</td>
<td>19.7 ± 8.1 (37)</td>
</tr>
</tbody>
</table>

Values reported are mean ± SD (n).

4.4.2. GCs receive asynchronous synaptic excitation and synchronous synaptic inhibition
Odors can trigger both synaptic excitation (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Fukunaga et al., 2014) and inhibition (Labarrera et al., 2013) onto GCs in vivo. GC recruitment by sensory input to the MOB should thus reflect the integration of both excitatory and inhibitory input. Although considerable research has focused on dendrodendritic excitation of GCs (for review, see Schoppa and Urban, 2003; Egger and Urban, 2006), relatively little is known about inhibition of GCs. Synaptic inhibition onto GCs can arise from both dSACs intrinsic to the MOB (Pressler and Strowbridge, 2006; Eyre et al., 2008; Boyd et al., 2012) and extrinsic centrifugal innervation (Nunez-Parra et al., 2013), and artificial activation of either of these pathways (by tetanic GCL stimulation or optogenetic photostimulation, respectively) can dramatically reduce the firing of GCs in response to somatic step current injections. Whether odors trigger synaptic inhibition onto GCs through a top-down centrifugal mechanism or through a feedforward mechanism intrinsic to the MOB is not known. Furthermore, the temporal structure of sensory-evoked inhibition onto GCs and the influence of inhibition on GC spike timing have not been characterized. Finally, it is unclear how robust synaptic inhibition onto GCs is, given the following: (1) inhibition onto GCs in vitro has only been observed previously with tetanic GCL stimulation (Pressler and Strowbridge, 2006) or in a small fraction of GCs using repetitive ONL stimulation (Schoppa, 2006a; see Discussion); and (2) inhibition onto GCs in vivo was detected reliably in only a handful of GC-odor pairs (Labarrera et al., 2013; see Discussion).

Thus, to examine the synaptic basis of GC recruitment after physiological activation of a single glomerulus, we repeated the previous experiment (Figure 4.1B) while sequentially clamping GCs at the reversal potentials of synaptic inhibition and excitation, respectively (Figure 4.2). Low-level stimulation of OSNs triggered highly asynchronous barrages of EPSCs in both sGCs (Figure 4.2B,C) and dGCs (Figure 4.2LJ), beginning 6.3 ± 7.5 ms (n = 37) after OSN stimulation and lasting several hundred milliseconds on average (Figure 4.2D,K), consistent with the repetitive firing and distributed spike latencies observed in M/TCs (Burton and Urban, 2014). Of note, this asynchronous excitatory drive can combine with long-lasting currents mediated by metabotropic glutamate receptors (Heinbockel et al., 2007a,b), canonical transient receptor potential channels (Hall and Delaney, 2002; Egger, 2008; Stroh et al., 2012), and NMDARs (Schoppa et al., 1998) in unclamped GCs to support prolonged depolarizations and long-latency firing, consistent with our above results (Figure 4.1).
Figure 4.2. GCs integrate asynchronous synaptic excitation and synchronous synaptic inhibition

A: Bright-field image (left; scale bar, 100 μm) and morphological reconstruction (right) of a representative sGC. B: Representative excitatory current trace (top) and raster plot of EPSC times across multiple trials (middle) after a single OSN stimulation pulse (bottom) for the GC shown in A. C: PSTH of EPSC times plotted in B. Solid and dashed red lines and coloring show the mean and 95% confidence interval, respectively, of the EPSC probability 500 ms before OSN stimulation. D: Mean PSTH of EPSC times across sGCs. Shading denotes mean ± SEM. Red line
shows a single-exponential fit. E–G: Same as B–D for synaptic inhibition onto sGCs. H–N: Same as A–G for dGCs.

Strikingly, clamping GCs at the reversal potential of synaptic excitation revealed robust and reliable synaptic inhibition onto both sGCs (Figure 4.2E,F) and dGCs (Figure 4.2L,M), with a mean first-IPSC amplitude after OSN stimulation of 56.4 ± 56.4 pA (n = 32). Inhibition onto GCs was highly prevalent, with a significant increase in the probability of observing an IPSC after OSN stimulation observed in 32 of 33 total GCs recorded. In strong contrast to the asynchronous excitatory input to GCs, inhibitory input to GCs was highly synchronous (Figure 4.2G,N), typically consisting of one to two IPSCs. Furthermore, inhibition onto GCs was rapid, with a mean onset of 13.3 ± 9.7 ms, suggesting strongly that a synaptic pathway intrinsic to the MOB mediates inhibition onto GCs. Indeed, inhibition followed the onset of excitation with a mean lag of 6.9 ± 7.0 ms (n = 32), consistent with a canonical monosynaptic excitation and disynaptic feedforward inhibition circuit. Given the high prevalence and polysynaptic nature of this inhibition and the inevitable disruption of some synaptic connections during the slicing procedure, our results thus collectively demonstrate for the first time that essentially all GCs receive reliable, short-latency, and synchronous synaptic inhibition after glomerular activation.

Repetitive sniffing dynamically modulates the strength of sensory input to the MOB in the behaving animal (for review, see Wachowiak, 2011). Thus, to evaluate the relevance of synaptic inhibition onto GCs in a more physiological context, we additionally examined whether the observed synaptic inhibition onto GCs is reliably evoked by patterned OSN stimulation mimicking theta frequency sniffing. Indeed, short-latency inhibition was reliably and synchronously evoked onto GCs by each of five 4 Hz OSN stimulation pulses (Figure 4.3E), yielding robust and highly phasic increases in IPSC probabilities (Figure 4.3F,G). In contrast, 4 Hz OSN stimulation evoked highly asynchronous synaptic excitation onto GCs (Figure 4.3B), with uniformly elevated EPSC probabilities throughout the entire stimulation train (Figure 4.3C,D). Synchronous synaptic inhibition onto GCs is thus not only robust and reliable in the acute slice preparation but also likely plays a critical role in regulating GC recruitment during olfactory-guided behavior.
Figure 4.3. Synaptic inhibition onto GCs is reliable across physiological theta frequency sniff rhythms
A: Bright-field image (left; scale bar, 100 μm) and morphological reconstruction (right) of a representative sGC. B: Representative excitatory current trace (top) and raster plot of EPSC times across multiple trials (middle) after five OSN stimulation pulses at 4 Hz (bottom) for the GC shown in A. C: PSTH of EPSC times plotted in B. Solid and dashed red lines and coloring show the mean and 95% confidence interval, respectively, of the EPSC probability 500 ms before the
first OSN stimulation pulse. D: Mean PSTH of EPSC times across GCs. Shading denotes mean ± SEM. E–G: Same as B–D for synaptic inhibition onto GCs.

4.4.3. dSACs mediate feedforward inhibition onto proximal somatodendritic and distal apical dendritic domains of GCs

The rapid timescale and robust nature of the inhibition evoked onto GCs by OSN stimulation suggests strongly that interneurons intrinsic to the MOB mediate feedforward synaptic inhibition onto GCs. dSACs are the only known class of MOB interneurons that monosynaptically inhibit GCs (Pressler and Strowbridge, 2006; Eyre et al., 2008; see Discussion). Thus, we hypothesized that glomerular activation recruits dSACs to mediate the observed synaptic inhibition onto GCs. Of note, this model requires that physiological activation of a single glomerulus recruits dSAC firing that is consistent with the rapid and reliable nature of the observed inhibition onto GCs (Figure 4.2). However, at present, dSAC recruitment has only been examined using direct glomerular stimulation in a subset of dSACs termed “Blanes cells” held at depolarized potentials perithreshold for firing (Pressler and Strowbridge, 2006). Therefore, to test our hypothesis, we next characterized the response of dSACs at resting membrane potentials (Table 4.1) to transient low-level stimulation of OSNs (Figure 4.4A).

Consistent with our hypothesis, dSACs with axons arborizing across the EPL and bearing clear bouton-like structures (Figure 4.4B,D) exhibited rapid and highly reliable first-spike latencies after transient OSN stimulation (Figure 4.4C,E). Similar activity was likewise observed in a subset of glomerular layer (GL)-projecting dSACs (Figure 4.4F,G) known to form en passant synapses onto GCs in the EPL (Eyre et al., 2008). Critically, the first spike latency across all dSACs (16.4 ± 11.2 ms, n = 10) – including spiny Blanes cells (Figure 4.4D) as well as other dSAC types (Price and Powell, 1970b; Pressler and Strowbridge, 2006; Eyre et al., 2008; Pressler et al., 2013; Figure 4.4B,F) – coincided with the onset of the observed synaptic inhibition onto GCs (Figure 4.4H), supporting strongly the hypothesis that dSACs mediate rapid feedforward inhibition onto GCs. Interestingly, dSAC first-spike latencies consistently followed TC first-spike latencies but preceded MC first-spike latencies recorded under equivalent conditions (Figure 4.4H; Burton and Urban, 2014). This temporal structure suggests that TCs (and/or external TCs, which likewise exhibit rapid recruitment; Gire et al., 2012) provide a strong source of synaptic excitation to EPL- and GL-projecting dSACs, consistent with the extensive overlap of EPL- and GL-projecting dSACs and TC axon collaterals in the internal plexiform layer and superficial GCL (Kishi et al., 1984; Orona et al., 1984; Liu and Shipley, 1994; Eyre et al., 2008).
**Figure 4.4. Glomerular activation drives reliable short-latency dSAC firing**

**A:** Experimental design. dSAC activity was monitored using whole-cell patch-clamp recordings while single glomeruli were activated by low-level stimulation of OSN axons. **B:** Bright-field image (top; scale bar, 100 μm; inset: axonal boutons in the EPL) and morphological reconstruction (bottom; axon colored red) of a representative EPL-dSAC. **C:** Representative voltage trace (top) and raster plot of spike times across multiple trials (middle) after a single OSN stimulation pulse (bottom) for the EPL-dSAC shown in **B**. Note the highly reliable short-latency firing after OSN stimulation. **D–G:** Same as **B,C** for a second EPL-dSAC (**D,E**) and a representative GL-dSAC (**F,G**). Inset: GL-dSAC firing (or depolarization on a subthreshold trial) followed OSN stimulation (note the stimulus artifact) with a nonzero latency, consistent with
synaptic excitation and non-antidromic stimulation by the extracellular stimulation electrode positioned within the ONL. **H**: Cumulative distributions of the mean first-spike latencies of M/TCs (from Burton and Urban, 2014) and dSACs and the onset of inhibition onto GCs after OSN stimulation. IPL: Internal plexiform layer; EPL-dSAC: EPL-projecting dSAC; GCL-dSAC: GCL-projecting dSAC.

Ultrastructural analyses have demonstrated that dSACs form GABAergic synapses onto both distal apical dendritic and proximal somatodendritic domains of GCs (Eyre et al., 2008). This suggests that the inhibitory synaptic currents recorded in GCs (Figure 4.2 and Figure 4.3) reflect GABAergic inputs to either distal apical dendritic or proximal somatodendritic domains or both. Determining the subcellular localization of synaptic inhibition onto GCs is of critical importance, given that dendritic inhibition could significantly attenuate dendritic Ca\(^{2+}\) influx (for review, see Higley, 2014) and GABA release onto M/TCs (for review, see Schoppa and Urban, 2003; Egger and Urban, 2006) and inhibit dendritic spike generation (Mori and Takagi, 1978b; Wells and Scott, 1990; Luo and Katz, 2001; Pinato and Midtgaard, 2005; Zelles et al., 2006; Labarrera et al., 2013; Figure 4.1), whereas somatic inhibition could inhibit the generation of full action potentials by dendritic spikes (Luo and Katz, 2001; Pinato and Midtgaard, 2005; Zelles et al., 2006; Simões-de-Souza et al., 2014) and attenuate GC excitation by centrifugal fibers (Balu et al., 2007) and M/TC axon collaterals (Schoppa, 2006a).

Therefore, we next characterized the subcellular localization of feedforward synaptic inhibition onto GCs using a pharmacological approach equivalent to several previous studies examining the subcellular features of synaptic excitation onto GCs (Halabisky and Strowbridge, 2003; Schoppa, 2006a). Specifically, we stimulated OSNs as above while focally puffing the GABA\(_A\)R antagonist GBZ near distal apical dendritic or proximal somatodendritic domains of GCs (Figure 4.5A; see Experimental procedures). Focal application of GBZ near proximal somatodendritic domains robustly, reversibly, and rapidly decreased feedforward inhibition onto GCs (Figure 4.5B–E), with five of five tested GCs exhibiting >30% reduction in the peak inhibitory current within the first trial after the onset of GBZ application (Figure 4.5G) and >40% on average across multiple trials (Figure 4.5F). In turn, focal application of GBZ near distal apical dendritic domains exhibited more heterogeneous effects, with some GCs exhibiting a clear and immediate decrease in feedforward inhibition (Figure 4.5B,C) and other GCs exhibiting little to no decrease (Figure 4.5D,E). Across seven tested GCs, three exhibited >30% reduction in the peak inhibitory current on average (with two GCs exhibiting >20% reduction in the first trial after the onset of GBZ application), whereas four exhibited minimal reductions (Figure 4.5F,G). Given the limitations of our experimental design, in which GBZ was applied focally near a single dendritic branch, and that each GC extends numerous secondary apical dendritic branches that can be separated by >100 \(\mu \text{m}\) (Figure 4.1, Figure 4.2, Figure 4.3), our results suggest that GCs receive feedforward inhibition on both proximal somatodendritic domains and throughout some or all of their distal apical dendritic branches, consistent with the ultrastructural localization of dSAC synapses onto GCs (Eyre et al., 2008). In total, our analyses of dSAC recruitment (Figure 4.4) and the subcellular localization of inhibition onto GCs (Figure 4.5) demonstrate collectively that dSAC-mediated feedforward inhibition onto GCs is well positioned to influence both GC firing and dendritic integration and GABA release (see below).
Figure 4.5. Feedforward inhibition onto GCs is localized to proximal somatodendritic and distal apical dendritic domains

A: Experimental design. Feedforward inhibitory currents were recorded in GCs (clamped at the reversal potential of synaptic excitation) while focally puffing the GABA<sub>A</sub>R antagonist GBZ (with Alexa Fluor 488) near distal apical dendritic or proximal somatodendritic domains. B:
Inhibitory current traces across multiple trials (top) after a single OSN stimulation pulse (bottom) in a representative GC, with transient application of GBZ near the soma (Bi; scale bar, 20 μm) or an apical dendritic branch (Bii). Note that GBZ (imaged just before puff termination) remained strongly localized throughout the duration of the puff application. C: Time course of peak inhibitory currents before, during (shaded regions), and after GBZ application. Gaps in data correspond to periods during which the puffing pipette was positioned and data were not collected. Note that both proximal ("prox") somatodendritic and distal ("dist") apical dendritic GBZ application reduced strongly the peak inhibitory currents recorded. D,E: Same as B,C for a second representative GC showing a strong reduction in peak inhibitory currents during proximal somatodendritic but not distal apical dendritic GBZ application. F: The decrease in mean peak inhibitory current during proximal or distal GBZ application relative to the mean peak current preceding GBZ application. Red lines and points correspond to the representative cells shown in B–E. Both proximal (p = 1.1 × 10⁻³, one-tailed t test; n = 5) and distal (p = 0.027, one-tailed t test; n = 7) GBZ application yielded average reductions in peak inhibitory currents that were significantly greater than zero. G: Same as F but plotting the decrease in peak inhibitory current for the trial immediately following the onset of GBZ application, at which point GBZ diffusion away from the site of application was at a minimum. Proximal GBZ application led to an immediate reduction in the peak inhibitory current (p = 4.5 × 10⁻³, one-tailed t test; n = 5), whereas distal GBZ application did not lead consistently to an immediate reduction in the peak inhibitory current (p = 0.11, one-tailed t test, n = 7). IPL: Internal plexiform layer. *p < 0.05; **p < 0.01.

4.4.4. Excitation and feedforward inhibition onto GCs scale in strength across different levels of sensory input

Thus far, we have examined the synaptic input onto GCs after physiological activation of a single glomerulus. However, we have shown previously that multiple neighboring glomerular microcircuits can activate individual GCs (Kapoor and Urban, 2006; Arevian et al., 2008). This result and the relatively broad odor tuning of GCs (Tan et al., 2010) suggest collectively that GCs receive excitatory synaptic input from multiple glomerular microcircuits. Consequently, the strength of synaptic excitation onto GCs should scale with the strength of sensory input to the MOB, with stronger sensory inputs (e.g., higher odor concentrations) activating more glomeruli than weaker sensory inputs (e.g., lower odor concentrations; Rubin and Katz, 1999; Meister and Bonhoeffer, 2001; Wachowiak and Cohen, 2001; Spors and Grinvald, 2002; Bozza et al., 2004; Spors et al., 2006; Fletcher et al., 2009). Whether feedforward synaptic inhibition onto GCs can likewise scale with sensory input strength will have critical consequences on GC recruitment by weak vs. strong sensory inputs. Thus, we next sought to determine the relative scaling of excitation vs. inhibition onto GCs downstream of weak (i.e., uniglomerular) vs. strong (i.e., multiglomerular) sensory input to the MOB.

To achieve controllable activation of one or many glomeruli, we turned to an optogenetic strategy for stimulating OSNs. Acute slices were prepared from gene-targeted OMP-ChR2:EYFP mice that selectively express ChR2:EYFP in mature OSNs (Smear et al., 2011). To confirm that a single glomerulus could be optogenetically activated comparable with our previous experiments using extracellular OSN stimulation, we performed control recordings from TCs as we photostimulated on- or off-beam glomeruli (Figure 4.6A; see Experimental procedures). Indeed, photostimulation of on-beam glomeruli evoked highly reproducible LLD events (Figure 4.6C,E,F) and firing (Figure 4.6D,G) that was directly comparable with results obtained using extracellular OSN stimulation within individual glomeruli (Burton and Urban, 2014). In contrast,
photostimulation of neighboring off-beam glomeruli evoked no excitatory currents (Figure 4.6C,E,F) or firing (Figure 4.6D,G) in TCs.

Figure 4.6. Optical activation of single glomeruli in gene-targeted OMP-ChR2:ELYFP mouse brain slices
A: Experimental design. B: Bright-field (top left; scale bar, 100 μm) and epifluorescent (top right) images and morphological reconstruction (bottom) of a representative TC. C: Example current traces after brief targeted photostimulation (blue line) of the on-beam glomerulus (left) or rostrally neighboring off-beam glomerulus (right) for the TC shown in B. D: Representative voltage traces (top) and raster plot of spike times across multiple trials (bottom) after brief targeted photostimulation (blue line) of the on-beam (left) or rostrally neighboring off-beam (right) glomerulus for the TC shown in B. E–G: On-beam glomerular photostimulation evoked significantly greater peak excitatory currents (E; p = 0.020, one-tailed paired t test; n = 7), excitatory charge transfer (F; p = 0.025, one-tailed paired t test; n = 7), and spike rates (G; p = 0.012, one-tailed paired t test; n = 6) in TCs than off-beam glomerular photostimulation. Red lines and points correspond to the representative cell shown in B–D. H: Cumulative distributions of TC first-spike latencies after optical or extracellular stimulation of OSN axons within single glomerular microcircuits. Extracellular stimulation data from Burton and Urban (2014). IPL: internal plexiform layer. *p < 0.05.

Having thus confirmed that we can optogenetically activate individual glomeruli, we next examined the synaptic input to GCs after uniglomerular vs. multiglomerular photostimulation (Figure 4.7A; see Experimental procedures). As above (Figure 4.2), activation of a single glomerulus evoked an asynchronous barrage of excitatory events, as well as rapid and reliable synaptic inhibition (Figure 4.7Ci). Strikingly, expanding our photostimulation to multiple glomeruli increased dramatically the strength of synaptic excitation onto GCs (Figure 4.7Ci). Across all GCs, the peak excitatory current after multiglomerular vs. uniglomerular photostimulation increased 1.8 ± 0.9-fold, from −87.8 ± 45.2 to −144.5 ± 88.3 pA (Figure 4.7D). As a caveat, this increase in excitation strength onto GCs may arise partially from stronger illumination and activation of a single glomerulus at the center of our photostimulation. In particular, we suspect that TCs, which are more strongly driven by direct OSN input than MCs (Gire et al., 2012; Burton and Urban, 2014), may fire more in response to multiglomerular vs. uniglomerular photostimulation. However, we note that displacing the multiglomerular photostimulation from centering on the originally photostimulated glomerulus to a neighboring glomerulus did not alter the excitation evoked onto GCs (data not shown). Moreover, in a subset of recordings (n = 4), we additionally confirmed that GCs received excitation from multiple neighboring glomerular microcircuits by sequentially photostimulating individual glomeruli (Figure 4.7C). In total, our results (1) verify and extend our previous findings (Kapoor and Urban, 2006; Arevian et al., 2008) by directly showing that GCs receive convergent synaptic excitation from multiple glomerular microcircuits and (2) confirm that the strength of synaptic excitation onto GCs scales with the strength of sensory input to the MOB.

Of great interest, GCs also received feedforward inhibition from multiple neighboring glomerular microcircuits (Figure 4.7C), and multiglomerular photostimulation dramatically increased the peak inhibitory current onto GCs by 2.6 ± 1.8-fold, from 78.5 ± 72.8 to 156.2 ± 96.7 pA (Figure 4.7D). Moreover, on a cell-by-cell basis, feedforward inhibition scaled in strength with excitation (Figure 4.7E). Strikingly, this increase in the strength of synaptic input to GCs occurred with no apparent change in kinetics, with both uniglomerular and multiglomerular photostimulation evoking highly asynchronous excitation and synchronous inhibition (Figure 4.7F,G). Collectively, our results strongly suggest that asynchronous synaptic excitation and synchronous feedforward inhibition are critical regulators of GC recruitment by both weak and strong sensory input to the MOB.
A  uni-gglomerular stimulation

B  multi-gglomerular stimulation

D  inhibition,

E  excitation

C1  uni

CII  uni

CIII  uni

CIV  uni

F  uni-gglomerular stimulation

G  multi-gglomerular stimulation

H  voltage (mV)

I  spikes

J  latency (ms)
Interestingly, the scaling of feedforward inhibition with excitation also suggests that GCs may exhibit primarily odor concentration-invariant firing in vivo, consistent with multiple previous examples (Wellis and Scott, 1990, their Fig. 5; Luo and Katz, 2001, their Fig. 8; Cang and Isaacson, 2003, their Fig. 10). Therefore, to begin to investigate the dependence of GC recruitment on the strength of sensory input to the MOB, we additionally examined the spiking response of GCs to uniglomerular vs. multiglomerular photostimulation. Across six GCs exhibiting suprathreshold responses to glomerular photostimulation, uniglomerular photostimulation evoked 0.8 ± 0.7 spikes per trial (Figure 4.7H,1), with varying first-spike latencies (Figure 4.7J) and spike probabilities (data not shown), consistent with the response of GCs to extracellular OSN stimulation within a single glomerulus (Figure 4.1). In the same GCs, expanding our photostimulation to multiple glomeruli evoked 2.4 ± 2.1 spikes per trial (Figure 4.7H,1). Although significant, this increase in firing is quite modest given the ~55 pA average increase in peak excitation (Figure 4.7D), which alone would generate up to an ~47 Hz increase in firing rate if injected at the soma (Table 4.3). Our results thus provide initial support for the hypothesis that feedforward inhibition scales with excitation to normalize GC firing across different levels of sensory input. Critically, such normalization will act to maintain GC activity in a range that is sensitive to the cooperative activation of M/TCs (Arevian et al., 2008; Urban and Arevian, 2009), supporting decorrelation of M/TCs via activity-dependent lateral inhibition over a large odor concentration range. Moreover, our results importantly motivate a more systematic investigation of the odor concentration dependence of GC activity in vivo.
4.4.5. *Feedforward inhibition regulates GC spike timing*

To further understand how asynchronous excitation and synchronous feedforward inhibition regulate GC activity, we performed a series of simulations using a biophysical multicompartmental GC model (Figure 4.8A) that recapitulates a wide array of physiological GC activity patterns (Li and Cleland, 2013). To simulate synaptic excitation onto the model GC after activation of a single glomerulus, we delivered 10-30 excitatory events per trial, based on previous estimates that each GC dendritic tree is decorated with 100-300 large spines or “gemmules” (Woolf et al., 1991b) and that ~10% of gemmules receive excitatory input from a single glomerular microcircuit (Egger et al., 2005). Each excitatory event (mediated by both AMPAR and NMDAR conductances; see Experimental procedures) was delivered to the gemmule body compartment (Figure 4.8A), corresponding to the site of reciprocal dendrodendritic synapses (Rall et al., 1966). To examine the influence of the temporal structure of excitation on GC firing, excitatory event times were either drawn randomly from a distribution matching the mean PSTH of EPSC times (Figure 4.8B) or were made artificially synchronous. To examine the influence of feedforward inhibition on GC firing, single inhibitory synaptic events were delivered to the soma and distal apical dendritic compartments (Figure 4.8A), matching previous ultrastructural localization of dSAC-GC synapses (Eyre et al., 2008) and our functional localization of feedforward inhibition onto GCs (Figure 4.5). Inhibition followed the onset of the mean experimental excitation time course (or the onset of the artificially synchronous excitation) by 6.9 ms (Figure 4.8B), matching the mean delay observed between excitation and inhibition (see above). Finally, to ensure the robustness of our simulation results, excitatory event strength (and therefore the excitation/inhibition ratio) was also systematically varied by ±20% across simulations.

Artificially synchronous excitation reliably evoked 2.0 ± 0.3 spikes per trial (range, 1–3) over a narrow distribution of short first-spike latencies (7.83 ± 0.08 ms; Figure 4.8C; data not shown). This activity pattern deviates substantially from the broader distributions of spike numbers and latencies observed *in vivo* in response to odors (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Labarrera et al., 2013; Cazakoff et al., 2014; Fukunaga et al., 2014) and *in vitro* in response to physiological glomerular activation (Figure 4.1) but matches previous accounts of GC activity *in vitro* in response to synchronous M/TC activation (Egger et al., 2005). Inclusion of feedforward inhibition reduced the number of spikes per trial to 1.0 ± 0.2 (range, 1–2) but had no effect on the first-spike latency (Figure 4.8C), as expected from the delayed onset of inhibition relative to excitation. In strong contrast to the GC activity patterns evoked by artificially synchronous excitation, physiologically asynchronous excitation evoked a much broader distribution of 1.3 ± 0.8 spikes per trial (range, 0–6) over a wide distribution of mean first-spike latencies (125.9 ± 43.9 ms; Figure 4.8D–F), paralleling our experimental observations (Figure 4.1) and the odor-evoked GC activity observed *in vivo* (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Labarrera et al., 2013; Cazakoff et al., 2014; Fukunaga et al., 2014).
Figure 4.8. Feedforward inhibition and asynchronous excitation interact to reduce the temporal fidelity of GC firing

A: Biophysical multicompartmental GC model properties (see Experimental procedures; Li and Cleland, 2013). B: Ten to 30 excitatory events were delivered either synchronously or asynchronously with probabilistic latency drawn from the average PSTH of EPSC times after
extracellular OSN stimulation. Inset: Zero to one inhibitory events were delivered to the distal apical dendritic and proximal somatodendritic domains with latency 6.9 ms after the onset of probabilistic (or artificially synchronous) excitation. C: Representative simulation with 20 artificially synchronous excitatory events ($g_{ex}$ max = 110%) and feedforward inhibition (blue trace) or no inhibition (black trace). D: Representative simulation (top) and raster plot of spike times across multiple simulations (middle) with 20 physiologically asynchronous excitatory events ($g_{ex}$ max = 110%) and feedforward inhibition (blue trace) or no inhibition (black trace) after simulated glomerular activation (bottom). E: Mean first-spike latency (averaged across 50 trials) for varying numbers and strengths of asynchronous excitatory inputs with feedforward inhibition (right) or without (left). F: Distribution of mean first-spike latencies for all permutations shown in E, G, H: Same as E,F for suprathreshold trial-to-trial spike-time reliability. I: Bath application of GBZ significantly reduced GC first-spike latencies evoked by extracellular OSN stimulation (p = 0.039, one-tailed paired t test; n = 5), matching simulation predictions (E,F). J: Change in mean suprathreshold trial-to-trial spike-time reliability vs. change in mean first-spike latencies after bath application of GBZ. GCs exhibiting strong reductions in first-spike latencies likewise exhibited increased spike-time reliabilities, approximately matching simulation predictions (G,H). *p < 0.05.

Strikingly, inclusion of single inhibitory somatic and distal apical dendritic events substantially increased and broadened the distribution of first-spike latencies (194.5 ± 61.4 ms; Figure 4.8D–F). Moreover, this change in spike timing substantially reduced the trial-to-trial spike-time reliability (Figure 4.8G,H). In agreement with our simulation results, we found that blocking feedforward inhibition onto GCs with bath application of GBZ (10 μM) consistently reduced GC first-spike latencies from 145.4 ± 95.8 to 78.1 ± 106.4 ms in response to extracellular OSN stimulation (Figure 4.8I). Although bath application of GBZ did not consistently increase spike-time reliability as predicted (0.23 ± 0.34 vs. 0.23 ± 0.20, before vs. after GBZ application), the GCs most strongly affected by GBZ (exhibiting reductions in first-spike latency >80 ms) exhibited a consistent increase in spike-time reliability (Figure 4.8J). Thus, even with network-wide changes in activity triggered by bath application of GBZ, at least a subset of the experimentally recorded GCs directly matched our simulation results. In total, the results of our simulations, which are broadly consistent with our experimental recordings, reveal that feedforward inhibition strongly delays GC firing. Importantly, feedforward inhibition can thereby complement other mechanisms regulating spike timing in GCs. In particular, GCs express A-type potassium channels, which blunt rapid excitation to delay firing (Schoppa and Westbrook, 1999; Kapoor and Urban, 2006; Schoppa, 2006a) but inactivate during sustained excitatory input, such as from theta frequency glomerular activation (Schoppa, 2006a). Thus, feedforward inhibition, which is recruited reliably across theta frequency glomerular activation (Figure 4.3), likely plays a dominant role in regulating the respiratory coupling of GC spike timing (Cazakoff et al., 2014) and subthreshold depolarization (Youngstrom and Strowbridge, 2015) during bouts of sustained odor sampling. Of great interest, our results further demonstrate that feedforward inhibition can reduce the overall temporal fidelity of firing when excitatory input is strongly asynchronous. Strikingly, this desynchronizing role directly contrasts with the ability of feedforward inhibition to enhance spiking precision when excitatory input is only weakly asynchronous (see Discussion). Furthermore, by broadening GC spike latencies, feedforward inhibition will enhance the decorrelation of principal neuron activity (Giridhar and Urban, 2012) and thus likely plays a critical role in complex olfactory-guided behavior (e.g., difficult odor discriminations).
4.4.6. Feedforward inhibition regulates subcellular GC activity and Ca\(^{2+}\) dynamics

Although our simulations have thus far demonstrated that feedforward inhibition influences GC spike timing (Figure 4.8), previous data suggest that GC dendrites are also capable of releasing GABA independent of somatic action potentials (Isaacson and Strowbridge, 1998). Indeed, the low odor-evoked firing rates of GCs in vivo (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Labarrera et al., 2013; Cazakoff et al., 2014; Fukunaga et al., 2014) together with the substantial evidence of isolated dendritic spikes in GCs in vivo (Mori and Takagi, 1978b; Wellis and Scott, 1990; Luo and Katz, 2001; Labarrera et al., 2013) and in vitro (Pinato and Midtgaard, 2005; Zelles et al., 2006; Figure 4.1) and the recent demonstration of gemmule-localized voltage-gated sodium currents (Bywalez et al., 2015) suggest that a large proportion of GC-mediated inhibition of M/TCs arises from highly localized dendritic branch- (or gemmule-) specific GABA release independent of somatic action potentials, comparable with the “parallel processing” performed by amacrine cells in the retina (Grimes et al., 2010). Thus, feedforward inhibition targeting distal apical dendritic domains of GCs is likely of greatest importance in regulating GC activity at the subcellular level. Therefore, we next examined what influence feedforward inhibition onto apical dendrites has on local gemmule activity evoked by individual excitatory inputs.

Specifically, we monitored the membrane potential and total intracellular Ca\(^{2+}\) concentration within the gemmule body compartment of our model GC while delivering single excitatory and inhibitory events with varying temporal lags (Figure 4.9B), consistent with our observation that individual excitatory events both preceded and followed feedforward inhibition (Figure 4.2, Figure 4.3, Figure 4.7). Critically, we observed that a single inhibitory event targeting the distal apical dendrite at the base of the gemmule (Figure 4.9A) triggered a substantial reduction (up to ~30%) in the peak gemmule depolarization (Figure 4.9C) and a more moderate reduction (up to ~15%) in the peak Ca\(^{2+}\) concentration change (Figure 4.9D) evoked by a coincident excitatory event at the gemmule body (Figure 4.9A). Of note, the drop in local Ca\(^{2+}\)-dependent GC GABA release caused by coincident feedforward inhibition is expected to be even greater than the observed decrease in total Ca\(^{2+}\) concentration (Figure 4.9D) given the strong coupling of GABA release to Ca\(^{2+}\) influx specifically through local NMDARs (Chen et al., 2000; Halabisky et al., 2000) and the localization of metabotropic GABA\(_B\)Rs to presynaptic GC compartments (Isaacson and Viten, 2003). These results thus confirm that even a single inhibitory event can markedly attenuate local dendritic depolarization, Ca\(^{2+}\) influx, and consequent GABA release to disinhibit select principal neurons (see Discussion).
4.5. Discussion

4.5.1. Overview
GC-mediated inhibition is critical to patterning sensory-evoked M/TC activity, yet how sensory input recruits GC activity remains incompletely understood. Therefore, we have examined systematically how GCs integrate synaptic excitation and inhibition downstream of sensory input to the MOB. We show for the first time that OSN activation not only triggers highly asynchronous excitation onto GCs but also short-latency, synchronous GABAergic inhibition via a canonical disynaptic M/TC–dSAC feedforward circuit (Figure 4.10A). Moreover, each GC receives both excitation and feedforward inhibition from multiple glomerular microcircuits. Thus, as the strength of sensory input to the MOB increases and more glomeruli are activated, both excitation and feedforward inhibition onto GCs increase, maintaining a balanced synaptic drive and normalizing GC firing. We further reveal that feedforward inhibition interacts with highly asynchronous excitation to desynchronize GC firing, in marked contrast to the role of feedforward inhibition in synchronizing firing in other brain regions. Additionally, feedforward inhibition attenuates depolarization in local dendritic compartments to promote independent processing between dendritic compartments. Collectively, our results identify feedforward inhibition onto GCs as a core feature of MOB circuitry and establish asynchronous excitation and feedforward inhibition as critical regulators of GC activity.

Indirect evidence of feedforward inhibition onto GCs has been observed previously in the salamander olfactory bulb (Wellis and Kauer, 1994), which, together with our current findings, suggests strongly that feedforward inhibition onto GCs is a robust and conserved feature of olfactory circuits. However, of note, two previous studies failed to observe robust feedforward inhibition onto GCs. First, Schoppa (2006a) observed bicuculline-sensitive events in only 2 of 12 GCs tested. These recordings were obtained under high intracellular Cl− concentrations, however, making it likely that the coincident EPSCs evoked by glomerular activation occluded any IPSCs, which would also be downward-going events under these conditions. We further note that this previous study evoked synaptic input onto GCs using trains of ONL stimulation pulses collectively lasting ~30 ms. Thus, the short-latency feedforward inhibition that we observed, which predominantly occurred within 30 ms of OSN stimulation, was also likely occluded by the patterned ONL stimulation. Second, Labarrera et al. (2013) observed odor-evoked IPSCs in only ~20% of GC-odor pairs in the anesthetized mouse (compared with the ~70% of pairs exhibiting odor-evoked excitation). However, it is likely that some odor-evoked IPSCs were missed as a result of coincident excitation and incomplete voltage clamp of distal dendritic branches, similar to our own observations in the acute slice preparation (see Experimental procedures). Furthermore, a large fraction of the odor-evoked excitation observed in their study likely originated from centrifugal inputs to GCs (Balu et al., 2007; Boyd et al., 2012; Markopoulos et al., 2012), which may not be directly coupled to the feedforward inhibitory circuits characterized in our study. Thus, we expect that, although GCs typically do not exhibit net inhibitory responses
to odors in vivo, feedforward inhibition nevertheless plays a widespread and critical role in regulating GC spike timing and subcellular activity in vivo.

Figure 4.10. Feedforward inhibition onto GCs is a core feature of MOB circuitry that regulates GC-mediated inhibition of principal neurons

A: Canonical circuit diagram of GC synaptic inputs. Sensory input to the MOB activates M/TCs, which monosynaptically and asynchronously excite GCs and disynaptically and synchronously inhibit GCs via dSAC-mediated feedforward inhibition. B: The subcellular organization of excitation and feedforward inhibition can regulate local GC depolarization and consequent GABA release to control the balance between dendrodendritic recurrent and lateral inhibition of M/TCs. Activation of “glomerulus A” may drive excitation (MCₐ) and feedforward inhibition (dSACₐ) onto different GC dendritic branches (Bi), activating local GABA release while inactivating remote GABA release, yielding stronger recurrent inhibition of MCₐ than lateral inhibition of MCᵦ and potentially disinhibiting MCᵦ. Conversely, activation of glomerulus A may drive excitation and feedforward inhibition onto the same GC dendritic branch (Bii), inactivating local GABA release while activating remote GABA release to preferentially drive lateral inhibition. Feedforward inhibition onto any GC dendritic branch (Bi,Bii), and particularly onto proximal somatodendritic domains (Bi), can attenuate propagation of dendritic depolarization to the soma, supporting local dendritic spikes and independent, parallel processing between different GC dendritic branches.

4.5.2. Feedforward inhibition regulates the balance between recurrent and lateral inhibition of principal neurons

Understanding how feedforward inhibition onto GCs influences sensory processing in the MOB will ultimately require precise knowledge of the subcellular organization of excitation and feedforward inhibition onto each GC. For example, activity of one M/TC may evoke excitation and feedforward inhibition onto distinct GC dendritic branches, resulting in strong recurrent inhibition but attenuated lateral inhibition (i.e., net “homotypic inhibition”; Figure 4.10Bi). Of note, such organization likewise provides a potential mechanism for lateral disinhibition between M/TCs. Alternatively, activity of one M/TC may evoke excitation and feedforward inhibition onto the same dendritic branch, leading to attenuated recurrent inhibition but strong lateral inhibition (i.e., net “heterotypic inhibition”; Figure 4.10Bii). In addition to shifting the balance between recurrent and lateral inhibition, feedforward inhibition onto GC dendrites and proximal somatodendritic domains (Figure 4.10Bii) will limit the propagation of dendritic depolarization to the soma, fostering dendritic branch-specific spiking and compounding the parallel processing capacity of each GC. Moreover, the termination of ~10% of dSAC synapses in the EPL directly onto GC gemmules (Eyre et al., 2008) may further enhance the compartmentalization of
feedforward inhibition (Chiu et al., 2013; Higley, 2014) to promote subcellular modulation of GABA release from individual GC gemmules.

4.5.3. Feedforward inhibition enables dynamic modulation of olfactory bulb network rhythms

GC-mediated inhibition of principal neurons strongly drives gamma frequency network rhythms in the MOB (Friedman and Strowbridge, 2003; Neville and Haberly, 2003; Lagier et al., 2004; Bathellier et al., 2006; Schoppa, 2006b; Lepousez and Lledo, 2013; Fukunaga et al., 2014). We hypothesize that feedforward inhibition onto GCs in turn suppresses MOB network rhythms in at least two ways. First, feedforward inhibition reduces GABA release from GCs and will thereby decouple M/TC and GC activity. Second, feedforward inhibition interacts with asynchronous synaptic excitation to reduce the temporal fidelity of GC firing. This finding is in marked contrast to canonical models in which feedforward inhibition enhances spike-time precision (Pinto et al., 1996; Pouille and Scanziani, 2001) and depends on the strongly asynchronous time course of synaptic excitation onto GCs. Specifically, rapid feedforward inhibition suppresses GC firing during the initial, relatively synchronous phase of excitation, allowing the later, more asynchronous phase of excitation to instead drive firing, yielding lower spike-time precision. By thus reducing the temporal fidelity of GC firing, feedforward inhibition will desynchronize odor-evoked firing across GCs and thereby disrupt the synchronization of M/TCs by coincident inhibition (Galán et al., 2006; Schoppa, 2006b). Blocking feedforward inhibition onto GCs (e.g., through dSAC-specific neuromodulation) should thus strengthen M/TC–GC interactions and enhance GC synchronization, promoting the synergistic synchronization of M/TCs linked by lateral inhibition (Marella and Ermentrout, 2010). Indeed, consistent with our hypothesis, deletion of the main GABA_A R β subunit expressed in GCs significantly enhances MOB oscillatory power during olfactory-guided behavior (Nusser et al., 2001). Thus, regulating dSAC activity represents a novel mechanism for dynamically modulating MOB network rhythms.

4.5.4. Inclusion of dSACs in future models of olfaction

Here, we show that dSACs respond to OSN stimulation with rapid and reliable firing that is coincident with feedforward inhibition onto GCs. Although our results thus argue strongly that dSACs mediate feedforward inhibition onto GCs, it remains possible that other neuron types also contribute to this inhibition. In particular, transsynaptic viral tracing studies have identified both dSACs and EPL interneurons (EPL-INs) as presynaptic to GCs (Arenkiel et al., 2011; Deshpande et al., 2013; Miyamichi et al., 2013; Garcia et al., 2014). Note, however, that ultrastructural (Toida et al., 1996; Lepousez et al., 2010) and functional (Huang et al., 2013; Kato et al., 2013) analyses have observed no direct synapses between EPL-INs and GCs, suggesting that GCs infect EPL-INs via tripartite-like connections with M/TCs (Garcia et al., 2014). Other EPL-INs that are somatostatin-, parvalbumin-, vasointestinal peptide-, and corticotropin-releasing hormone-negative may mediate feedforward inhibition onto GCs, but these will represent a small fraction of MOB neurons compared with dSACs (Eyre et al., 2009).

Current models of olfaction generally do not include dSACs. However, the prevalence, connectivity patterns, and functional roles of dSACs argue compellingly for their integration into future models. Specifically, dSACs comprise a large population of MOB interneurons (Price and Powell, 1970b), numbering ~13,500 per rodent MOB or three to four principal neurons per dSAC (Eyre et al., 2009), comparable with the principal neuron/interneuron ratio in the neocortex (Markram et al., 2004). Additionally, dSACs form extensive axonal arbors, conferring
a high degree of connectivity onto other MOB interneurons (Eyre et al., 2009; Miyamichi et al., 2013), including adult-born neurons (Arenkiel et al., 2011; Deshpande et al., 2013). The unique properties of dSAC inputs are also likely of critical importance. Notably, dSACs receive the strongest centrifugal excitation from the piriform cortex of all MOB cell types (Boyd et al., 2012). Moreover, our current results suggest that dSACs may be recruited preferentially by TCs, thus identifying an additional circuit feature by which MCs and TCs mediate parallel processing of olfactory information (Fukunaga et al., 2012; Igarashi et al., 2012; Adam et al., 2014; Burton and Urban, 2014; Otazu et al., 2015).

Of utmost importance, several findings have now demonstrated that dSACs govern multiple aspects of MOB activity. Foremost, we have shown that dSAC-mediated feedforward inhibition normalizes GC spike numbers and regulates GC spike timing and subcellular activity and can thereby control the decorrelation and temporal patterning of M/TC activity across different levels of sensory input. Through centrifugal excitation, dSACs can likewise regulate GC activity via feedback inhibition (Boyd et al., 2012; Oswald and Urban, 2012a), providing a substrate for top-down modulation of olfactory processing (Restrepo et al., 2009). Additionally, GABAergic input from dSACs is essential for the integration and maturation of adult-born GCs (Pallotto et al., 2012), which can significantly influence olfaction (for review, see Lepousez et al., 2013). Subsets of dSACs likely regulate olfaction in numerous other ways as well, including via state-dependent and persistent firing (Pressler and Strowbridge, 2006, Pressler et al., 2013) and through unique axonal projections, including GL-projecting dSACs (Eyre et al., 2008; 2009) and cortical-projecting dSACs (Kosaka and Kosaka, 2007b; 2010; Eyre et al., 2008).

4.6. Appendix

4.6.1. Functional differences between morphological subtypes of GCs regulate sensory-evoked GC activity

Classical morphological studies suggest that GCs can be subdivided into superficial GCs (sGCs), which innervate the superficial EPL, and deep GCs (dGCs), which innervate the deep EPL (Orona et al., 1983; Mori et al., 1983). This putative morphological subdivision of GCs suggests that preferential connectivity may exist between sGC apical dendrites and TC lateral dendrites in the superficial EPL and dGC apical dendrites and MC lateral dendrites in the deep EPL (Figure 4.1A; Mori et al., 1983; Orona et al., 1983; 1984). Of great interest, several recent studies have demonstrated that TCs fire at shorter latencies and higher rates than MCs following glomerular activation (Nagayama et al., 2004; Griff et al., 2008; Fukunaga et al., 2012; Igarashi et al., 2012; Burton and Urban, 2014). We therefore hypothesized that sGCs are more strongly recruited than dGCs following glomerular activation, reflecting preferential sGC-TC and dGC-MC connectivity and stronger TC vs. MC sensory-evoked activity.

Supporting our hypothesis, GC soma position correlates with GC subtype (Orona et al., 1983; Mori et al., 1983) and odor-evoked activity in vivo (Wellis and Scott, 1990). Specifically, GCs located in the MCL and upper GCL tend to exhibit sGC morphologies and suprathreshold odor responses while GCs located in the lower GCL tend to exhibit dGC morphologies and subthreshold odor responses (Orona et al., 1983; Mori et al., 1983; Wellis and Scott, 1990). Whether these differences in odor-evoked activity in vivo reflect functional differences between GC subtypes and M/TC connectivity remains unclear, however, as: 1) different odors activate
multiple glomeruli in distinct spatiotemporal patterns, which can evoke a complex array of convergent excitation and inhibition onto individual GCs (Burton and Urban, 2015), and 2) GC activity in vivo is strongly influenced by centrifugal input (Balu et al., 2007; Markopoulos et al., 2012; Boyd et al., 2012) and anesthesia (Kato et al., 2012; Cazakoff et al., 2014).

Therefore, to more directly test our above hypothesis, we recorded the excitatory synaptic input and spiking responses of GCs to activation of single nearby glomeruli in acute MOB slices, with post-hoc recovery of Neurobiotin-filled cell morphologies. To enable across-cell comparisons of synaptic input and spiking responses, we used low stimulation intensities to capitalize on the well-established all-or-none nature of glomerular activation (Carlson et al., 2000; Gire and Schoppa, 2009).

Consistent with previous morphological accounts (Orona et al., 1983; Mori et al., 1983), GCs exhibited distinct sGC or dGC morphologies upon visual inspection (Figure 4.11A,D; Figure 4.11A,B). Indeed, reconstruction and visual classification of a large subset of recorded GCs and analysis of the spatial distribution of gemmules – the site of reciprocal dendrodendritic synapse formation (Rall et al., 1966) – confirmed that sGCs preferentially innervate the superficial EPL while dGCs preferentially innervate the deep EPL (Figure 4.11G). Moreover, somatic depth significantly – but incompletely – predicted GC subtype (Figure 4.11A,D; Figure 4.11A,B; Table 4.5), as previously observed (Orona et al., 1983; Mori et al., 1983). To determine whether the observed morphological differences reflect subtypes of GCs rather than a continuum, we additionally performed unbiased clustering of GCs. Specifically, clustering of GCs by the Euclidean distances among their normalized gemmule distributions (using Ward’s method) and application of the gap statistic method yielded 3 distinct clusters: dGCs, sGCs, and a small group of sGCs with prominent innervation of the deep glomerular layer (Figure 4.12C). Moreover, these clusters closely aligned with our original classification by visual inspection, with 19 of 19 sGCs and 9 of 11 dGCs correctly assigned (Figure 4.12C). Our results therefore quantitatively confirm the morphological subdivision of GCs into sGCs and dGCs.

<table>
<thead>
<tr>
<th><strong>Table 4.5. Morphological properties of sGCs vs. dGCs</strong></th>
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<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Soma area (μm²)</td>
</tr>
<tr>
<td>Soma depth from MCL (μm)</td>
</tr>
<tr>
<td>Basal dendrites, Σ length (μm)</td>
</tr>
<tr>
<td>Basal dendrites, Σ volume (μm³)</td>
</tr>
<tr>
<td>Apical dendrites, Σ length (μm)</td>
</tr>
<tr>
<td>Apical dendrites, Σ volume (μm³)</td>
</tr>
<tr>
<td>Apical dendrites, gemmules</td>
</tr>
</tbody>
</table>

Values reported are mean ± standard deviation (*n*). ***p<0.001; n.s., not significant (two-tailed unpaired *t* test).
Figure 4.11. Synaptic and intrinsic differences regulate sGC vs. dGC recruitment following glomerular activation
A: Reconstructed morphologies and distribution of apical dendritic gemmules across the MCL (black bars), EPL (grey bars), and GL (red bars) of 3 representative sGCs. Grey and red ticks represent the EPL midpoint and the gemmule distribution mean, respectively. B,C: Spiking response (B) and excitatory synaptic input (C) of the 3 sGCs shown in A following activation of a single glomerulus superficial to the targeted GC. D-F: Same as A-C for 3 representative dGCs.

G: Distribution of apical dendritic gemmules across reconstructed sGCs and dGCs. H: A greater proportion of sGCs than dGCs fired in response to glomerular activation (Chi-square test, \(p=4.2\times10^{-3}\)). I: Excitatory input to sGCs exhibited larger peak currents (rank-sum test, \(p=8.5\times10^{-3}\)) and charge transferred (rank-sum test, \(p=0.046\)) than excitatory input to dGCs. No difference in excitation latency was observed (6.6 ± 11.6 vs. 9.7 ± 11.4 ms; rank-sum test, \(p=0.12\)). Scalebar: 0.2 s/10 pA (inset: 40 ms/20 pA). J,K: sGCs and dGCs showed significantly different firing rate-current (FI) curves in response to somatic step current injection (2-way ANOVA, \(p=4.1\times10^{-3}\)). Individual (J) and mean (K) FI curves shown. Dashed lines show diminished firing due to depolarization block. L: sGC action potentials exhibited more hyperpolarized thresholds (unpaired \(t\) test, \(p=4.8\times10^{-3}\)), larger amplitudes (unpaired \(t\) test, \(p=1.1\times10^{-4}\)), and faster rising slopes (unpaired \(t\) test, \(p=2.9\times10^{-3}\)) than dGC action potentials. Inset: action potential phase plot. Scalebar: 30mV/100 mVms\(^{-1}\); dashed lines show origin. Shaded regions show mean ± SEM.

In agreement with our hypothesis, a strikingly higher percentage of sGCs than dGCs fired in response to activation of a single glomerulus (Figure 4.11B,E,H) due, at least partially, to stronger excitatory synaptic input to sGCs than dGCs (Figure 4.11I). As a caveat, we note that the greater recruitment of sGCs following glomerular activation may arise as an artifact of our acute slice preparation. Specifically, as TC circuitry is closer to any given glomerulus than MC circuitry, TC-mediated input to GCs (likely sGCs) may be better preserved than MC-mediated input to GCs (likely dGCs) in the acute slice, leading to stronger sGC excitation and recruitment following glomerular activation. Three lines of evidence argue against this possibility, however, and instead support greater feedforward recruitment of sGCs as a physiological feature of the MOB circuit. First, our in vitro observation of greater sGC firing following glomerular activation (Figure 4.11B,E,H) corresponds well with the previous in vivo observation of stronger odor-evoked activity in putative sGCs (Wellis and Scott, 1990). Second, examination of GC biophysical properties revealed several intrinsic differences supporting greater recruitment of sGCs than dGCs, including a more hyperpolarized action potential threshold in sGCs (Table 4.6) and greater intrinsic excitability in sGCs in response to somatic step current injections (Figure 4.11J,K; Table 4.7), despite equivalent somatodendritic sizes (Figure 4.12D; Table 4.5) and passive membrane properties (Table 4.8) between sGCs and dGCs. Third, analysis of spontaneous synaptic activity revealed no difference in event frequency or amplitude between sGCs and dGCs (Table 4.9). Critically, recordings of spontaneous synaptic activity were performed in the absence of TTX and thus contain some degree of action potential-dependent input, which likely originates from intact presynaptic cells. Therefore, equal spontaneous event frequencies between sGCs and dGCs suggests that their respective presynaptic circuits are comparably intact. Moreover, equal spontaneous event amplitudes suggests a comparable contribution of larger action potential-dependent and smaller action potential-independent events between sGCs and dGCs, again consistent with comparably intact presynaptic circuits.

In total, our results thus suggest that sGCs are more strongly recruited than dGCs following activation of a single glomerulus due to stronger excitatory input and greater intrinsic excitability. Critically, this finding also provides the first functional evidence (albeit indirect) that sGCs and dGCs exhibit preferential connectivity with TCs and MCs, respectively. Our
results thus further identify a novel synaptic mechanism supporting parallel processing of sensory information by MCs and TCs.

Figure 4.12. Morphological analysis of sGCs and dGCs
A: Reconstructed morphologies and distribution of apical dendritic gemmules across the MCL (black bars), EPL (grey bars), and GL (red bars) for 19 sGCs whose response to single glomerular activation was examined (see Figure 4.11). Grey and red ticks represent the EPL midpoint and the
gemmule distribution mean, respectively. **B**: Same as A for 11 dGCs. **C**: Dendrogram of clustered GC morphologies. Line colors correspond to the 3 significant clusters. Numbers correspond to the reconstructed morphologies shown in **A, B**, with number colors reflecting GC classification by visual inspection (sGC: red; dGC: black). Inset: projection of GCs across the first 2 principal components (74.0% of total variance) calculated from the normalized gemmule distributions. **D**: Sholl analysis of GC dendritic morphologies. sGCs and dGCs exhibited no significant difference in apical (p=0.21) or basal (p=0.56) dendritic complexity (unpaired t-tests of area under Sholl curves). Shaded regions show mean ± SEM.

**Table 4.6. Action potential properties of sGCs vs. dGCs**

<table>
<thead>
<tr>
<th></th>
<th>sGC</th>
<th>dGC</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>V_{threshold} (mV)</td>
<td>–31.9 ± 6.3 (19)</td>
<td>–23.8 ± 5.7 (8)</td>
<td>4.8×10^{-3} **</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>60.6 ± 8.8 (19)</td>
<td>44.3 ± 7.6 (8)</td>
<td>1.1×10^{-4} ***</td>
</tr>
<tr>
<td>FWHM (ms)</td>
<td>0.97 ± 0.14 (19)</td>
<td>1.09 ± 0.29 (8)</td>
<td>0.16 (n.s.)</td>
</tr>
<tr>
<td>Rising slope (mV ms^{-1})</td>
<td>195.9 ± 53.5 (19)</td>
<td>109.4 ± 33.9 (8)</td>
<td>2.9×10^{-4} ***</td>
</tr>
<tr>
<td>Falling slope (mV ms^{-1})</td>
<td>–63.2 ± 12.8 (19)</td>
<td>–53.8 ± 17.2 (8)</td>
<td>0.13 (n.s.)</td>
</tr>
</tbody>
</table>

Values reported are mean ± standard deviation (n). **p<0.01; ***p<0.001; n.s., not significant (two-tailed unpaired t test).

**Table 4.7. Spike train properties of sGCs vs. dGCs**

<table>
<thead>
<tr>
<th></th>
<th>sGC</th>
<th>dGC</th>
<th>P value</th>
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<tbody>
<tr>
<td>Rheobase (pA)</td>
<td>36.3 ± 20.9 (19)</td>
<td>46.2 ± 22.6 (8)</td>
<td>0.28 (n.s.)</td>
</tr>
<tr>
<td>Rheobase first-spike latency (ms)</td>
<td>543.6 ± 542.9 (19)</td>
<td>209.0 ± 164.9 (8)</td>
<td>0.10 (n.s.)</td>
</tr>
<tr>
<td>Gain (Hz pA^{-1})</td>
<td>0.86 ± 0.33 (19)</td>
<td>0.91 ± 0.34 (7)</td>
<td>0.74 (n.s.)</td>
</tr>
<tr>
<td>Peak instantaneous rate (Hz)</td>
<td>59.3 ± 23.5 (19)</td>
<td>42.4 ± 15.2 (8)</td>
<td>0.074 (n.s.)</td>
</tr>
</tbody>
</table>

Values reported are mean ± standard deviation (n). n.s., not significant (two-tailed unpaired t test).

**Table 4.8. Passive membrane properties of sGCs vs. dGCs**

<table>
<thead>
<tr>
<th></th>
<th>sGC</th>
<th>dGC</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>R_{input} (MΩ)</td>
<td>599.8 ± 397.6 (20)</td>
<td>499.7 ± 248.9 (8)</td>
<td>0.52 (n.s.)</td>
</tr>
<tr>
<td>r_m (ms)</td>
<td>26.6 ± 14.6 (17)</td>
<td>26.1 ± 11.5 (7)</td>
<td>0.94 (n.s.)</td>
</tr>
<tr>
<td>C_m (pF)</td>
<td>46.0 ± 12.2 (17)</td>
<td>48.9 ± 11.5 (7)</td>
<td>0.60 (n.s.)</td>
</tr>
<tr>
<td>V_{rest} (mV)</td>
<td>–71.3 ± 6.8 (22)</td>
<td>–65.6 ± 10.0 (8)</td>
<td>0.085 (n.s.)</td>
</tr>
</tbody>
</table>

Values reported are mean ± standard deviation (n). n.s., not significant (two-tailed unpaired t test).
### Table 4.9. Spontaneous synaptic event properties of sGCs vs. dGCs

<table>
<thead>
<tr>
<th></th>
<th>sGC</th>
<th>dGC</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>(sEPSPs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>7.4 ± 3.0 (21)</td>
<td>6.6 ± 2.0 (8)</td>
<td>0.45 (n.s.)</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>1.2 ± 0.5 (21)</td>
<td>0.9 ± 0.3 (8)</td>
<td>0.09 (n.s.)</td>
</tr>
<tr>
<td>Rise&lt;sub&gt;20-80%&lt;/sub&gt; (ms)</td>
<td>2.7 ± 0.6 (21)</td>
<td>2.6 ± 0.3 (8)</td>
<td>0.86 (n.s.)</td>
</tr>
<tr>
<td>( \tau_{\text{decay}} ) (ms)</td>
<td>19.4 ± 3.9 (21)</td>
<td>26.4 ± 15.8 (8)</td>
<td>0.066 (n.s.)</td>
</tr>
<tr>
<td><strong>(sEPSCs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>4.2 ± 4.2 (26)</td>
<td>4.0 ± 4.8 (10)</td>
<td>0.87 (n.s.)</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>-18.0 ± 7.1 (26)</td>
<td>-14.8 ± 7.1 (10)</td>
<td>0.24 (n.s.)</td>
</tr>
<tr>
<td>Rise&lt;sub&gt;10-90%&lt;/sub&gt; (ms)</td>
<td>1.0 ± 0.2 (26)</td>
<td>1.2 ± 0.4 (10)</td>
<td>0.035 *</td>
</tr>
<tr>
<td>( \tau_{\text{decay}} ) (ms)</td>
<td>7.1 ± 4.0 (26)</td>
<td>6.9 ± 3.3 (10)</td>
<td>0.97 (n.s.)</td>
</tr>
<tr>
<td><strong>(sIPSCs)</strong></td>
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<td>Frequency (Hz)</td>
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<td>Amplitude (pA)</td>
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<td>Rise&lt;sub&gt;10-90%&lt;/sub&gt; (ms)</td>
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<td>( \tau_{\text{decay}} ) (ms)</td>
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<td>20.9 ± 7.3 (10)</td>
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Values reported are mean ± standard deviation (n). *p<0.05; n.s., not significant (two-tailed unpaired t test).

### 4.7. Author contributions

Shawn D. Burton (S.D.B.) and Nathaniel N. Urban (N.N.U.) designed research; S.D.B. performed research; S.D.B. analyzed data; S.D.B. and N.N.U. wrote the manuscript.

### 4.8. Acknowledgements

This work was supported by National Institute on Deafness and Other Communication Disorders Grants F31DC013490 (S.D.B.), R01DC005798 (N.N.U.), and R01DC011184 (N.N.U.) and the Pennsylvania Department of Health Commonwealth Universal Research Enhancement Program (N.N.U.). We thank Greg LaRocca and the Mellon Institute Centralized Vivarium staff for excellent technical assistance and members of the Urban, Barth, Gittis, Kuhlman, Ermentrout, and Oswald laboratories for helpful discussions.
5. Olfactory bulb deep short-axon cells mediate widespread inhibition of tufted cell apical dendrites

5.1. Abstract

In the main olfactory bulb (MOB), the first station of sensory processing in the olfactory system, GABAergic interneuron signaling shapes principal neuron activity to regulate olfaction. Lack of known selective markers for MOB interneurons has strongly impeded cell type-selective investigation of interneuron function, however. Here, we identify the first selective marker of glomerular layer-projecting deep short-axon cells (GL-dSACs) and systematically investigate the structure, intrinsic physiology, sensory recruitment, neuromodulation, synaptic output, and functional role of GL-dSACs in the MOB circuit. GL-dSACs are located in the internal plexiform layer where they integrate centrifugal cholinergic input with highly convergent feedforward sensory input. GL-dSAC axons arborize extensively across the glomerular layer to provide highly divergent yet selective output onto interneurons and principal tufted cells. GL-dSACs are thus capable of shifting the balance of principal tufted vs. mitral cell activity across large expanses of the MOB in response to diverse sensory and top-down neuromodulatory input.

5.2. Introduction

The mammalian brain contains a vast diversity of GABAergic inhibitory interneurons. The identification of cell type-selective molecular markers has fostered tremendous insight into how distinct interneurons shape sensory processing and behavior (Kubota, 2014; Wester and McBain, 2014; Letzkus et al., 2015). In the main olfactory bulb (MOB), inhibitory circuits precisely regulate the activity of principal mitral and tufted cells (M/TCs) to drive olfactory-guided behavior (Schoppa and Urban, 2003; Arevian et al., 2008; Fukunaga et al., 2012; 2014; Gschwend et al., 2015). Selective markers for many MOB interneurons have not been identified (Eyre et al., 2009), however, impeding investigation of cell type-selective interneuron function.

Sensory input to the MOB is organized into odorant receptor-specific glomeruli, where olfactory sensory neuron (OSN) axons and external tufted cells (ETCs) drive sensory activation of M/TC apical dendritic tufts in the superficial layers of the MOB (Nagayama et al., 2014). Deeper in the MOB, the inframitral layers contain a large class of GABAergic interneurons classically known as deep short-axon cells (dSACs) whose functional roles remain almost entirely unknown. dSACs can be subdivided into granule cell layer (GCL)-, external plexiform layer (EPL)-, and glomerular layer (GL)-projecting dSACs (GCL/EPL/GL-dSACs) (Eyre et al., 2008). Of great interest, GL-dSAC axons arborize across multiple glomeruli, and thus may significantly regulate sensory-evoked MOB activity (Eyre et al., 2008). Moreover, GL-dSACs are concentrated in the internal plexiform layer (IPL), a region of dense innervation by external tufted cell (ETC) and TC axon collaterals (Macrides et al., 1985; Liu and Shipley, 1994), glutamatergic and GABAergic centrifugal feedback (Boyd et al., 2012; Markopoulos et al., 2012; Nunez-Parra et al., 2013), and neuromodulatory input (Linster and Fontanini, 2014), and are thus poised to coordinate widespread MOB activity patterns across distinct brain states.
Here, we identify nicotinic acetylcholine receptor (nAChR) subunit α2 (chrna2) as the first selective marker of GL-dSACs. Using Chrna2-Cre mice, we demonstrate that GL-dSACs integrate highly convergent sensory input from ETCs of several glomeruli and selectively inhibit the apical dendritic tufts of ETCs and TCs across multiple glomeruli. Strikingly, GL-dSACs neither receive MC input nor provide output onto MCs. GL-dSAC activity is also regulated by direct centrifugal cholinergic input. In total, the uniquely divergent yet selective connectivity of GL-dSACs supports a novel lateral inhibitory circuit between ETCs and TCs that is modulated by top-down cholinergic input.

5.3. Experimental procedures

5.3.1. Animals and virus injections

All experiments were completed in compliance with the guidelines established by the Institutional Animal Care and Use Committee of Carnegie Mellon University and the University of Pittsburgh. Chrna2-Cre mice (line OE29) were obtained from GENSAT (Gong et al., 2007) and used as heterozygous F1-2 hybrid crosses (on a mixed FVB/N, Crl:CD1[ICR], and C57BL/6 background) with C57BL/6 and Ai3 mice (Madisen et al., 2010). Approximately one-third of Chrna2-Cre mice exhibited markedly denser Cre-dependent labeling and were not used in this study. For Cre-dependent Chr2:mCherry expression, ~1 μL of AAV1-EF1a-DIO-hChR2(H134R)-mCherry (University of Pennsylvania Vector Core) was unilaterally injected into the MOB or forebrain at P2-3 (Cheetham et al., 2015). For Cre-dependent EGFP expression, ~1 μL of AAV1-CAG-flex-GFP (University of North Carolina Vector Core) was unilaterally injected into the adult MOB.

5.3.2. Immunohistochemistry

Mice were anesthetized with intraperitoneal injection of 0.4 mL ketamine (20 mg/mL)/xylazine (3.3 mg/mL), then transcardially perfused with 1% NaCl in 0.1 M phosphate buffer (PB) followed by paraformaldehyde (4% in PB). Brains were extracted, postfixed in paraformaldehyde overnight, sunk in sucrose (30% w/v in PB), then cryogenically sliced into 25 μm-thick sagittal sections with a sliding microtome (Leica, SM2000R). Sections were incubated with Triton X-100 (0.1% in PB) and normal donkey serum (NDS; 2% in PB) for 1 hr at room temperature, washed with PB, and then incubated for 1 hr at room temperature (unless otherwise noted) with combinations of the following primary antibodies (in PB with 2% NDS and 0.05% Tween 20): goat anti-GFP (Abcam, ab6673; 1:10000), rabbit anti-GFP (Molecular Probes, A-11122; 1:1000), rabbit anti-GABA_A_Rα1 (Alomone Labs, AGA-001; 1:1000), goat anti-ChAT (EMD Millipore, AB144P; 1:500; overnight incubation at 4C), rabbit anti-NaV1.6 (Abcam, ab83764; 1:2000; overnight incubation at 4C), sheep anti-TH (EMD Millipore, AB1542; 1:1000), mouse anti-PV (clone PARV-19; Sigma, P3088; 1:1000; overnight incubation at 4C), rabbit anti-CB (EMD Millipore, AB1778; 1:1000), rat anti-m2AChR (clone M2-2-B3; EMD Millipore, MAB367; 1:500), goat anti-SST (Santa Cruz Biotechnology, sc-7819; 1:500; overnight incubation at 4C), goat anti-OMP (Wako, 544-10001; 1:5000; overnight incubation at 4C), mouse anti-MAP2 (clone HM-2; Abcam, ab11267; 1:4000), goat anti-CR (Santa Cruz Biotechnology, sc-11644; 1:1500). Sections were then washed in PB, and incubated for 1 hr at room temperature with combinations of the following secondary antibodies raised in donkey (1:600 in PB with 2% NDS and 0.05% Tween 20): anti-goat AF488, anti-rabbit AF488, anti-rabbit AF594, anti-sheep AF594, anti-mouse AF594, anti-goat AF594, anti-rat AF594.

For chromogenic EYFP immunohistochemistry, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, BA-1000; 1:200) and then processed similarly to acute slices containing Neurobiotin-filled cells. Briefly, slices were fixed in paraformaldehyde (4% in PB) for >24 hr at 4C, quenched with H$_2$O$_2$/methanol (1%/10% in PB) for 1 hr at room temperature, washed with PB, permeabilized with Triton X-100 (2% in PB) for 1 hr at room temperature, and then incubated overnight at 4C in Vectastain ABC complex solution (Vector Laboratories; 1% Reagent A, 1% Reagent B in PB with 1% Triton X-100). Slices were then extensively washed with PB before incubation with DAB (Sigma; 0.5 mg/mL with 0.01% H$_2$O$_2$ in PB) for 10 min at room temperature.

Immunofluorescent images were collected on inverted Zeiss LSM 510 META Duoscan and 880 confocal microscopes using 40-100× oil-immersion objectives in maximum intensity projections of 0.5-1.0 μm-step z-stacks at 1024x1024 or 2048x2048 resolution. For DAB-stained tissue, images were collected on an Olympus BX51WI microscope using 10-20× air objectives and a 100× oil-immersion objective. Cell morphologies were reconstructed under a 100× oil-immersion objective and analyzed with Neurolucida (MBF Bioscience).

5.3.3. Slice preparation
Postnatal day 18-28 mice of both sexes were anesthetized with isoflurane and decapitated into ice-cold oxygenated dissection solution containing (in mM): 125 NaCl, 25 glucose, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 3 MgCl$_2$, 1 CaCl$_2$. Brains were isolated and acute horizontal or sagittal slices (310 μm thick) were prepared using a vibratome (5000mz-2; Campden or VT1200S; Leica). Slices recovered for 30 min in ~37ºC oxygenated Ringer’s solution that was identical to the dissection solution except for lower Mg$^{2+}$ concentrations (1 mM MgCl$_2$) and higher Ca$^{2+}$ concentrations (2 mM CaCl$_2$). Slices were then stored at room temperature until recording.

5.3.4. Electrophysiology
Slices were continuously superfused with 34ºC oxygenated Ringer’s solution. Cells were visualized using infrared differential interference contrast video microscopy. Current-clamp recordings were made using electrodes (4-9 MΩ) filled with (in mM): 120 K-glucuronate, 2 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 Mg-ATP, 0.3 Na$_3$GTP, 0.2 EGTA, 0.025 AF594, and 0.2% Neurobiotin (Vector Labs). Voltage-clamp recordings were made using electrodes filled with (in mM): 140 Cs-gluconate, 10 QX-314, 2 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 Mg-ATP, 0.3 Na$_3$GTP, 0.025 AF594, and 0.2% Neurobiotin. Liquid junction potentials were 12-14 mV (K$^+$-based solution) and 11 mV (Cs$^+$-based solution) and were not corrected for, except where noted. Data were low-pass filtered at 4 kHz and digitized at 10 kHz using a MultiClamp 700A amplifier (Molecular Devices) and an ITC-18 acquisition board (Instrutech) controlled by custom software written in IGOR Pro (WaveMetrics). OSN fiber bundles projecting into single glomeruli were stimulated (100 μs-long constant current pulses) using an extracellular monopolar glass electrode. For optogenetic stimulation, slices were illuminated by a 75W xenon arc lamp passed through a YFP filter set and 60× water-immersion objective centered on the recorded cell or the GL. NBQX (Tocris and Sigma), AP5 (Tocris), GBZ (Tocris), DMPP (Alomone Labs), and Mec (Tocris and Alomone Labs) were added to the bath or focially applied,
as described. Reconstructed GL-dSAC dendrites and axons are drawn in black and red, respectively.

5.3.5. Data analysis
Synaptic events were detected in Axograph, with a 1 ms-minimum event separation time. sEPSCs were detected using a 4.5 ms-long double exponential template with 0.5 ms baseline, 0.4 ms rise time constant, and 2 ms decay time constant. sIPSCs were detected using a 21 ms-long double exponential template with 1 ms baseline, 0.6 ms rise time constant, and 8 ms decay time constant. All events were detected with a threshold amplitude of 3 × SD of the baseline noise. Event amplitudes, 20-80% rise times, and decay constants were calculated using Axograph. Synaptic input latencies were defined as the interval of time from extracellular stimulation to the onset of the synaptic response, which was defined as the time at which the synaptic response reached 5% of its peak amplitude. Synaptic input durations were defined as the interval of time from the onset of the synaptic response to the time at which the synaptic response decayed to 5% of its peak amplitude. For paired GL-dSAC/MC recordings, charge transferred was calculated by integrating current traces from the end of the stimulus artifact to 1 s or 50 ms after OSN stimulation for MCs and GL-dSACs, respectively. For all other recordings, charge transferred was calculated by integrating current traces from the onset of the synaptic response to the time at which the synaptic response decayed to 5% of its peak amplitude. Stimulus artifacts are blanked for visual clarity of synaptic responses. Intrinsic biophysical properties were calculated as previously described (Burton and Urban, 2014; 2015), using a 20 mV/ms threshold for detecting action potentials. To calculate power spectral densities from spontaneous firing epochs, binary spike trains were converted to sinusoidal spike phases and analyzed using Welch’s method (500 ms windows, 50% window overlap). Values are reported as mean ± SD. Errorbars and shading denote mean ± SEM.

5.4. Results
5.4.1. Genetically targeting GL-dSACs
GL-dSACs are concentrated in the MOB IPL (Figure 5.1A), and are composed of horizontal cells and a subset of stellate Golgi cells (Eyre et al., 2008). To systematically study GL-dSACs, we therefore searched for candidate markers that are selectively expressed in IPL cells. In situ hybridization of chrna2 sparsely labels IPL cells within the MOB (Ishii et al., 2005). Consistent with this previous report and the prior morphological characterization of GL-dSACs, reporter-mediated Cre-dependent EYFP expression in Chrna2-Cre/Ai3 mice (Figure 5.1B) selectively labeled horizontal and Golgi cells within the IPL (Figure 5.1C). Putative axons with bouton-like structures could also be seen coursing profusely throughout the GL but were absent from other cell layers (Figure 5.1C). Chrna2-Cre/Ai3 mice additionally labeled a minority of TCs (Figure 5.1C) and a small number of neurons throughout other brain regions (Figure 5.2; see Experimental procedures). Localized injection of AAV expressing Cre-dependent EGFP into the MOB of adult Chrna2-Cre mice (Figure 5.1B) likewise selectively labeled horizontal and Golgi cells, with putative axons in the GL extending up to ~1 mm away from infected somata (Figure 5.1D).
Figure 5.1. Chrna2-Cre selectively labels dSACs in the IPL

A: MOB circuitry. Glutamatergic cells are shown in black. GABAergic cells targeting glutamatergic cells are shown in red. GABAergic cells putatively targeting other GABAergic cells are shown in blue. B: Experimental strategy to drive Cre-dependent expression of EYFP, EGFP, or ChR2:mCherry under the control of the chrna2 promoter. C: Cre-dependent expression of EYFP following Ai3 reporter cross. Scalebar: 100 μm. D: Cre-dependent expression of EGFP 2 weeks-post adult AAV injection into the dorsal MOB. Scalebar: 500 μm (inset: 50 μm). E: Colocalization of EYFP with dSAC marker GABA,A,Rα1. Scalebar: 50 μm.
Figure 5.2. Whole brain Chrna2-Cre expression pattern
A-D: Parasagittal section from a representative Chrna2-Cre/Ai3 mouse. Cre-dependent EYFP expression is localized to neurons in the MOB IPL, as well as to sparse neurons in the accessory olfactory bulb (A), neocortex (B), septal nucleus (C), and substantia innominata and bed nucleus stria terminalis (D), consistent with *in situ* hybridization results (Ishii et al., 2005). Scalebar: 500 μm (insets: 100 μm).
The α1 subunit of the GABA_A R is strongly expressed in GCL-located dSACs and ~50% of IPL-located dSACs, in addition to M/TCs (Eyre et al., 2008; 2009). Consistent with this finding, a large fraction (45.2%) of Chrna2-Cre-labeled neurons exhibited strong GABA_A Rα1 expression (Figure 5.1E). Moreover, of the dSACs strongly expressing GABA_A Rα1, 14.6% exhibited Chrna2-Cre-dependent labeling. Using previous cell counts (Eyre et al., 2009), we therefore estimate that Chrna2-Cre mice label 1-2,000 dSACs per MOB. While thus a small population, Chrna2-Cre-labeled dSACs represent a substantial fraction of the total dSACs within the IPL (Eyre et al., 2009).

The extensive arborization of putative axons throughout the GL precluded tracing individual Chrna2-Cre-labeled dSACs. Therefore, to better resolve cellular morphologies, we filled individual EYFP-expressing dSACs with Neurobiotin via targeted whole-cell recordings in acute slices from Chrna2-Cre/Ai3 mice (Figure 5.3A,B). Post-hoc reconstructions (Figure 5.3C,D) revealed that Chrna2-Cre-labeled dSACs extend 4.8 ± 1.5 (n=18) sparsely spiny and beaded dendrites up to ~200 μm through the IPL parallel to the MCL (Figure 5.3E) and project a single thin process superficially to arborize across the GL. Of note, processes entering the GL could only be traced a short distance due to their thin, tortuous profile and the high cell density of the GL. Chrna2-Cre-labeled dSAC morphologies nevertheless closely match the GL-dSAC morphologies previously recovered from serial thin section reconstruction (Eyre et al., 2008). Moreover, high densities of axon initial segment channel Na_v1.6 (Lorincz and Nusser, 2008) within the proximal regions of the superficial processes of Chrna2-Cre-labeled dSACs (Figure 5.3F) confirmed that Chrna2-Cre-labeled dSACs project their axons superficially into the GL. Further, Chrna2-Cre-labeled somata poorly colocalized with nonselective markers of EPL- and GCL-dSACs (Figure 5.4) and Chrna2-Cre-labeled axonal boutons in the GL poorly colocalized with superficial short-axon cell (sSAC) marker tyrosine hydroxylase (TH) (Figure 5.3G). Collectively, our results thus show that Chrna2-Cre mice selectively label GL-dSACs in the MOB, establishing the first selective marker of any dSAC subclass.
**Figure 5.3. Chrna2-Cre-labeled dSACs project axons superficially to the GL**

A,B: EYFP-expressing dSACs in Chrna2-Cre/Ai3 mice were filled with AF594 and Neurobiotin via whole-cell recordings. Insets show axon projecting through the EPL and into the GL. Scalebar: 20 μm (A); 100 μm (B). C: Reconstructed morphology of the cell shown in A,B. Scale is the same as in B. D: Additional reconstructions. Asterisk marks a Golgi cell exhibiting state-dependent burst firing. E: Dendritic Sholl analysis. Inset: mean angular distribution of reconstructed dendritic and axonal process lengths, showing horizontal dendritic extensions (black) and superficial axonal projections (red) (n=18). F: Na\textsubscript{v}1.6 localizes to proximal regions of superficially projecting processes of Chrna2-Cre-labeled dSACs (arrowhead). Scalebar: 20 μm. G: Chrna2-Cre-labeled axonal boutons poorly colocalize with TH. Scale is the same as in F.
Figure 5.4. Chrna2-Cre-labeled dSACs poorly colocalize with EPL- and GCL-dSAC markers
A-D: 0-3% of Chrna2-Cre-labeled dSACs are immunopositive for PV (A), CB (B), m2AChR (C), or SST (D). A single Chrna2-Cre-labeled dSAC coexpressing CB is shown in B. Scalebars: 50 μm.

5.4.2. GL-dSACs fire intrinsically at theta frequencies
Cell-attached recordings from EYFP-expressing GL-dSACs in acute slices (Figure 5.5A) revealed spontaneous firing at ~6 Hz that was highly regular and stable, yielding robust spectral peaks at 5-20 Hz (Figure 5.5B,C) and low interspike interval (ISI) coefficient of variation (CV_{ISI}) values (Table 5.1). Application of NBQX (10 μM), AP5 (50 μM), and Gabazine (GBZ; 10 μM) had no effect on spontaneous firing rates (Figure 5.5B,C) or regularity (Table 5.1), demonstrating that highly regular firing is an intrinsic property of GL-dSACs. Consistent with this finding, whole-cell recordings from a large population of GL-dSACs (n=33) likewise revealed highly regular and stable firing at ~8 Hz (Figure 5.5D-F,H,I; Table 5.1).

Spontaneous firing was abolished by moderate hyperpolarization from a mean membrane potential (V_m) measured during ISIs (V_{m,ISI}) of −55.0 ± 3.0 mV to −61.6 ± 2.7 mV (n=20), enabling examination of GL-dSAC excitability through somatic step current injections. The majority of GL-dSACs responded to depolarizing current with highly regular firing (Figure 5.5G,K) and relatively uniform excitability (Figure 5.5L; Table 5.1). Strikingly, while all GL-dSACs exhibited regular firing at rest (Figure 5.5F,I), a subset of GL-dSACs (23.1%) responded to weak depolarizing current (<50 pA) from hyperpolarized V_m with prominent 3-4 Hz burst
firing (Figure 5.5J), while stronger currents (>50 pA) evoked highly regular firing (Figure 5.5K). Activity in this subset of GL-dSACs thus closely matches recent characterization of state-dependent burst firing in rat Golgi cells (Pressler et al., 2013). In total, our results thus suggest that GL-dSACs likely contribute, through both regular firing and state-dependent burst firing, to the generation of theta frequency MOB rhythms.

**Figure 5.5. Intrinsic biophysical properties of GL-dSACs**

A-C: EYFP-expressing GL-dSACs fire tonically in cell-attached recordings independent of synaptic activity (5.7 ± 5.2 vs. 6.5 ± 6.3 Hz, before vs. after NBQX/AP5/GBZ application; n=10; p=0.10, paired t test). Red points denote cell shown in A,B. Inset: mean power of cell-attached spike times (n=10). D: Distribution of GL-dSAC spontaneous firing rates recorded in whole-cell mode (8.3 ± 5.6 Hz; n=33). Inset: mean power of spike times (n=33). E-G: Whole-cell recordings from a representative GL-dSAC showing regular firing at rest (F) and at a hyperpolarized V_m in response to a depolarizing step current (G). Single action potential expanded at right. H-J: An example GL-dSAC exhibiting burst firing when depolarized from a hyperpolarized V_m. Single burst expanded at right. K: GL-dSACs exhibit highly regular firing in response to strong (>50 pA) depolarizing step currents (without NBQX/AP5/GBZ, n=20; with NBQX/AP5/GBZ, n=6). L: GL-dSACs exhibit relatively uniform excitability in response to step current injections (without NBQX/AP5/GBZ, n=20; with NBQX/AP5/GBZ, n=6).
Table 5.1. Functional properties of GL-dSACs

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<td><strong>p &lt; 0.01 (two-tailed unpaired t test). Values reported are mean ± SD (n).</strong></td>
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5.4.3. GL-dSACs receive highly convergent ETC-mediated excitation

Under baseline conditions, GL-dSACs are continuously bombarded with spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs, respectively) (Figure 5.6B-D, Figure 5.7A-D; Table 5.1), identifying the IPL as a region of high synaptic activity. Physiological activation of a single nearby glomerulus via extracellular stimulation of OSN fiber bundles (Figure 5.6A) – analogous to the presentation of a low concentration odor in vivo (Mainland et al., 2014) – evoked robust, short-latency excitatory input to GL-dSACs that was graded across stimulation intensities (Figure 5.6E-H). Importantly, this robust excitatory input was observed regardless of whether the GL-dSAC axon entered the GL or was truncated in the EPL during slice preparation (Figure 5.6Q-T), excluding the possibility of direct sensory input to GL-dSAC axons within the GL. This result was further supported by the lack of colocalization between GL-dSAC axonal boutons and OMP-positive glomerular compartments (Figure 5.12A). In addition, sensory-evoked excitatory input to GL-dSACs was reliably followed by feedforward inhibition (Figure 5.7E-H).

The excitatory input evoked by glomerular activation reliably triggered 1-2 short-latency action potentials in GL-dSACs followed by a consistent pause in firing (Figure 5.6J-P). Glomerular activation thus acts to reset the phase of spontaneous firing in GL-dSACs. The high precision of this sensory-evoked response provides additional evidence that GL-dSACs may be involved in theta frequency rhythmogenesis in the MOB (see Discussion). Consistent with this model, GL-dSAC firing reliably tracked trains of OSN stimulation mimicking physiological bouts of theta frequency sniffing (Figure 5.8).
**Figure 5.6. OSN stimulation resets the spontaneous firing phase of GL-dSACs**

A: Excitatory input to GL-dSACs was recorded following activation of a single glomerulus by OSN stimulation. B: Whole-cell recording of a representative EYFP-expressing GL-dSAC. Scalebar: 20 μm. C: Example recording of sEPSCs from the cell shown in B. Inset: mean sEPSC (scalebar: 4 ms/10 pA). D: Distribution of GL-dSAC sEPSC rates (10.4 ± 8.2 Hz, n=33). Inset: distribution of sEPSC amplitudes. E: Low intensity OSN stimulation (arrowhead) evoked a short burst of EPSCs in the cell shown in B. Mean response shown. Inset: magnification of synaptic
response (scalebar: 10 ms/200 pA). Individual trials shown in lighter color. F,G: Same as E for medium (F) and high (G) intensity OSN stimulation. H,I: Mean amplitude (H), latency (H), and duration (I) of sensory-evoked excitatory input to GL-dSACs following low (2.5 ± 1.4 μA; n=12), medium (4.3 ± 2.3 μA; n=6), and high (5.3 ± 1.2 μA; n=3) intensity OSN stimulation. J: Low intensity OSN stimulation (arrowhead) evoked a single action potential and partially reset the spontaneous firing phase of the cell shown in B. Multiple trials are shown (upper), with a single trial plotted in black for clarity and a raster plot of spike times across trials (lower; highlighted trial corresponds to trial plotted in black). Inset: magnification of spiking response (scalebar: 10 ms/20 mV). K: PSTH (10 ms bins) shows a rapid peak in action potential probability following glomerular stimulation at 0 s (dashed line: 95% confidence interval of basal action potential probability). L-O: Same as J,K for medium (L,M) and high (N,O) intensity OSN stimulation. P: Mean number of action potentials evoked within 20 ms following low, medium, and high intensity OSN stimulation. Q-T: Two representative GL-dSACs with truncated axons (arrowheads) exhibiting sensory-evoked excitatory input following OSN stimulation. Morphology scalebars: 50 μm. Inset scalebar: 10 ms/50 pA.

Figure 5.7. Sensory-evoked GL-dSAC firing is regulated by feedforward inhibition
A: Whole-cell recording of a representative EYFP-expressing GL-dSAC. Scalebar: 20 μm. B: Example recording of sEPSCs and sIPSCs from the cell shown in A. Inset: magnification of mean sIPSC and sEPSC waveforms (scalebar: 5 ms/10 pA). C: GL-dSACs exhibited comparable sIPSC and sEPSC rates (10.0 ± 5.4 vs. 12.8 ± 5.2 Hz, sEPSC vs. sIPSC rate; n=16; p=0.09, paired t test).
Red points denote cell shown in **A,B. D:** GL-dSAC exhibited larger sIPSC than sEPSC amplitudes (32.2 ± 8.2 vs. 38.6 ± 8.0 pA, sEPSC vs. sIPSC amplitude; n=16; p=7.9×10^-7, paired t test). Red points denote cell shown in **A,B. E:** OSN stimulation (black triangle) evokes short-latency excitatory input followed by a barrage of IPSCs in the cell shown in **A.** Results plotted as in Figure 5.6. Inset scalebar: 10 ms/100 pA. **F-H:** Sensory-evoked inhibitory input to GL-dSACs (n=9) exhibited longer latencies (**F;** 3.8 ± 0.6 vs. 7.9 ± 3.6 ms, excitatory vs. inhibitory input; p=5.2×10^-3, paired t test) but comparable amplitude (**G;** 191.6 ± 131.3 vs. 117.1 ± 100.5 pA, excitatory vs. inhibitory input; p=0.21, paired t test) and charge transferred (**H;** 2.1 ± 1.8 vs. 2.0 ± 2.0 pC, excitatory vs. inhibitory input; p=0.96, paired t test) as sensory-evoked excitatory input. Red points denote cell shown in **A,B.**

**Figure 5.8.** **GL-dSAC firing reliably tracks theta frequency OSN stimulation**

**A:** Whole-cell recording of a representative EYFP-expressing GL-dSAC. Scalebar: 20 μm.

**B:** A train of OSN stimuli (black triangles) at 5 or 10 Hz evoked a time-locked train of depressing excitatory input to the cell shown in **A.**

**C,D:** A train of OSN stimuli (black triangles) at 5 or 10 Hz evoked single time-locked action potentials in the cell shown in **A.** Results plotted as in Figure 5.6. **E:** Mean amplitude of sensory-evoked excitatory input to GL-dSACs across 10 stimulation pulses at 5 (n=10) or 10 Hz (n=7), normalized to the first stimulation pulse response. Excitatory input to GL-dSACs exhibits substantial initial frequency-dependent depression, followed by stable responses. **F:** Mean number of action potentials evoked within 20 ms following each of 10 stimulation pulses at 5 (n=11) or 10 Hz (n=11). GL-dSACs track 5 and 10 Hz OSN stimulation with high precision, frequently emitting an action potential within 20 ms of OSN stimulation.
Sensory-evoked excitatory input to GL-dSACs decayed rapidly (Figure 5.6I). This short duration contrasts with the long-lasting M/TC-mediated excitation observed in granule cells (GCs) (Burton and Urban, 2015), and suggests that GL-dSACs and GCs are postsynaptic to distinct sources of excitation. We therefore hypothesized that ETCs mediate sensory-evoked excitatory input to GL-dSACs, consistent with the extensive overlap of ETC axons and GL-dSACs within the IPL (Figure 5.1, Figure 5.3) (Macrides et al., 1985; Liu and Shipley, 1994; Eyre et al., 2008). To test our hypothesis, we performed paired recordings of GL-dSACs and nearby MCs (Figure 5.9A-D) while stimulating OSNs at intensities perithreshold for generating an all-or-none long-lasting depolarization (LLD) in the MC (Carlson et al., 2000; Gire and Schoppa, 2009). The MC LLD is mediated by the regenerative activation of all recurrently coupled ETCs within a glomerulus (De Saint Jan et al., 2009; Gire and Schoppa, 2009). If GL-dSACs receive excitatory input from MCs, then LLD generation should be an important predictor of excitatory input to GL-dSACs. If GL-dSACs instead receive direct input from ETCs, then OSN stimulation should drive excitatory input to GL-dSACs even when LLD generation fails, reflecting the activation of some (but not all) of the ETCs within the glomerulus. Strikingly, GL-dSACs received robust excitatory input on both LLD success and LLD failure trials (Figure 5.9E,F), consistent with direct input from ETCs. Moreover, excitatory input to GL-dSACs was modestly but significantly weaker on LLD failure trials than on LLD success trials (Figure 5.9E,F), consistent with the activation of fewer ETCs. In total, our results thus demonstrate that: 1) GL-dSACs receive sensory-evoked excitatory input from ETC axons in the IPL, and 2) GL-dSACs are excited even when sensory input to the MOB fails to activate principal MCs.

ETC axons can extend long distances within the IPL (see Discussion) (Macrides et al., 1985; Liu and Shipley 1994; Belluscio et al., 2002; Lodovichi et al., 2003). It is thus possible that GL-dSACs integrate ETC-mediated excitatory input from both nearby and distant glomeruli. To examine the convergence of sensory-evoked input to GL-dSACs, we activated single glomeruli hundreds of micrometers away while recording excitatory input to GL-dSACs (Figure 5.9A). In paired recordings from GL-dSACs and nearby MCs, activation of distant glomeruli (Figure 5.9B) evoked no input to MCs (Figure 5.9E), confirming that extracellular stimulation does not activate OSN axons in passage. In contrast to MCs, GL-dSACs received robust excitatory input (Figure 5.9E), even when activating individual glomeruli >700 μm anterior to the recorded cell (Figure 5.9H-J). Equivalent results were likewise observed when activating glomeruli posterior to the recorded cell (Figure 5.9K-M). Additionally, direct stimulation of either the nearby or distant IPL (Figure 5.9G,H) evoked robust, short-latency GL-dSAC excitation (Figure 5.9J), again consistent with ETC axon-mediated input to GL-dSACs in the IPL. Collectively, our results thus show that GL-dSACs integrate highly convergent sensory-evoked excitatory input from several glomeruli, suggesting broad odor tuning in vivo.
Figure 5.9. GL-dSACs integrate ETC-mediated excitation from several glomeruli
A: Excitatory input to pairs of GL-dSACs and nearby MCs was recorded following activation of single nearby or distant glomeruli by OSN stimulation. B-D: Representative paired recording of a
MC and EYFP-expressing GL-dSAC, with activation of a nearby glomerulus (Bii) that contains the MC apical dendritic tuft (C), or a glomerulus ~520 µm anterior (Bi). Scalebar: 50 µm. E: Excitatory input to the GL-dSAC/MC pair shown in B-D following OSN stimulation (arrowhead). For nearby glomerulus activation, OSNs were stimulated at intensities perithreshold for generating a MC LLD. Results plotted as in Figure 5.6. Inset scalebar: 10 ms/50 pA. F: Mean amplitude and charge of sensory-evoked excitatory input to GL-dSAC/MC pairs (n=3) on LLD success and LLD failure trials. Excitatory input to GL-dSACs was moderately weaker on LLD failure trials (amplitude: 150.2 ± 90.8 vs. 115.5 ± 95.5 pA, LLD success vs. LLD failure; p=8.0×10^{-3}, paired t test; charge: 18.2 ± 1.9 vs. 0.9 ± 0.4 pC, LLD success vs. LLD failure; p=0.09, paired t test), while excitatory input to MCs was absent on LLD failure trials (amplitude: 80.5 ± 16.6 vs. 14.5 ± 8.7 pA, LLD success vs. LLD failure; p=0.035, paired t test; charge: 18.2 ± 3.7 pC, LLD success vs. LLD failure; p=0.030, paired t test). Red points denote cell shown in B-E. G: Excitatory input to GL-dSACs was recorded following activation of single nearby or distant glomeruli by OSN stimulation, or following stimulation of the nearby or distant IPL. H,I: Representative GL-dSAC recording, with activation of a nearby glomerulus (Hiv) or a glomerulus ~760 µm anterior (Hii), or stimulation of the nearby IPL (Hiii) or distant IPL (Hi). Scalebar: 50 µm. J: Excitatory input to the cell shown in H,I following OSN or IPL stimulation (arrowhead). Inset scalebar: 10 ms/100 pA. Mean response to nearby glomerulus activation reproduced in each inset for visual comparison. K-L: Representative GL-dSAC recording, with activation of a nearby glomerulus (Ki) or a glomerulus ~470 µm posterior (Kii). Scalebar: 50 µm. M: Excitatory input to the cell shown in K,L following OSN stimulation (arrowhead). Results plotted as in Figure 5.6. Inset scalebar: 10 ms/50 pA. Mean response to nearby glomerulus activation reproduced in each inset for visual comparison.

5.4.4. Centrifugal cholinergic fibers innervate GL-dSACs
Cholinergic signaling critically regulates olfaction (Linster and Fontanini, 2014). Understanding how cholinergic signaling influences sensory processing in the MOB has proven difficult, however, as multiple neuron types express cholinergic receptors but exhibit no or weak response to endogenous ACh release (D’Souza and Vijayaraghavan, 2014; Liu et al., 2015; Smith et al., 2015). Expression of chrna2 suggests that GL-dSACs may express nAChRs and respond to endogenous ACh release. Immunostaining for choline acetyltransferase (ChAT) revealed dense cholinergic innervation of the MOB, including pronounced innervation of the IPL (Figure 5.10A), as previously observed (Macrides et al., 1981). Within the IPL of Chrna2-Cre/Ai3 mice, EYFP-expressing GL-dSACs frequently colocalized with ChAT (Figure 5.10B), suggesting that centrifugal cholinergic fibers may target GL-dSACs.

Focal application of nicotinic agonist dimethylphenylpiperazinium (DMPP; 100 µM) (Figure 5.10C) dramatically increased the spontaneous firing rate of EYFP-expressing GL-dSACs in Chrna2-Cre/Ai3 mice (Figure 5.10D-F,H). This increase in rate was driven by an inward current, which was abolished by nicotinic antagonist mecamylamine (Mec; 20 µM) (Figure 5.10G,I). Importantly, blockade of glutamatergic and GABAAergic transmission had no effect on the response to DMPP (Figure 5.10H,I), confirming that DMPP acts directly on functional nAChRs expressed in GL-dSACs.

To test whether GL-dSACs respond to endogenous ACh release, we recorded postsynaptic currents in EYFP-expressing GL-dSACs in Chrna2-Cre/Ai3 mice while stimulating the IPL (Figure 5.10J-L). At low stimulation intensities, IPL stimulation evoked rapid, short-duration EPSCs that were largely blocked by glutamatergic antagonists (Figure 5.9G-J; data not shown).
likely reflecting ETC axon-mediated input. In the continued presence of glutamatergic and GABAergic antagonists, however, slightly increasing stimulation intensities evoked robust EPSCs in all GL-dSACs tested (Figure 5.10M,N). These currents exhibited slower kinetics than glutamatergic ETC input (compare Figure 5.9J and Figure 5.10N) and were abolished by brief bath application of Mec (Figure 5.10M). Our results thus demonstrate that GL-dSACs respond to endogenous ACh release from centrifugal cholinergic fibers in the IPL, implicating GL-dSACs as a central component in the neuromodulatory control of MOB activity.

Figure 5.10. GL-dSACs exhibit nicotinic responses to endogenous ACh release
A,B: Colocalization of ChAT-positive fibers with EYFP-expressing GL-dSACs in Chrna2-Cre/Ai3 mice. Scalebar: 50 μm. C: GL-dSAC activity was recorded during focal application of broad-spectrum nicotinic agonist DMPP. D: Whole-cell recording of a representative EYFP-
expressing GL-dSAC. Scalebar: 20 μm. E,F: Focal application of DMPP consistently increased the spontaneous firing rate of the cell shown in D. Results plotted as in Figure 5.6. Insets: magnification of 1 s-long periods of firing before and during DMPP application. G: Voltage-clamp recording of the mean inward current evoked by DMPP in the cell shown in D. Bath application of Mec abolished the inward current. H: DMPP application significantly increased GL-dSAC spontaneous firing rates (9.2 ± 3.9 vs. 22.0 ± 7.5 Hz, before vs. during DMPP application; n=5; p=0.013, paired t test). Red points denote cell shown in D-G. I: Mecamylamine blocked the DMPP-evoked inward current in GL-dSACs, leading to a significant reduction in current amplitude (325.5 ± 75.6 vs. 12.0 ± 6.1 pA, before vs. after Mec application; n=5; p=7.3x10^-4, paired t test). Red points denote cell shown in D-G. J: Synaptic input to GL-dSACs was recorded while stimulating the IPL and blocking glutamatergic and GABAergic receptors. K,L: Whole-cell recording of a representative EYFP-expressing GL-dSAC. Scalebar: 20 μm. M: IPL stimulation (10 μA) in the presence of NBQX/AP5/GBZ evoked a robust EPSC in the cell shown in K,L that was rapidly abolished by Mec application. Inset: mean synaptic response evoked by IPL stimulation (arrowhead) before and after Mec application (scalebar: 100 ms/50 pA). Individual trials shown in lighter colors. N: Mean postsynaptic nicotinic current recorded in GL-dSACs (peak: 57.7 ± 38.8 pA; decay constant: 29.2 ± 7.2 ms; n=6) following IPL stimulation (arrowhead; 10-15 μA), calculated by subtracting the mean current after Mec application from the mean current before Mec application (in the presence of NBQX/AP5/GBZ).

5.4.5. GL-dSACs innervate PGCs
To examine the synaptic output of GL-dSACs, we injected AAV into the MOB to drive Cre-dependent channelrhodopsin (ChR2):mCherry expression in GL-dSACs (Figure 5.11A, Figure 5.14; see Experimental procedures). Photostimulation with brief (10 ms) blue light pulses reliably evoked 1-4 action potentials in GL-dSACs in the presence of glutamatergic antagonists (Figure 5.11B-H), confirming the direct influence of ChR2 on GL-dSACs.

Previous ultrastructural examination of biocytin-filled GL-dSAC axon terminals identified synaptic contacts onto putative PGCs (Eyre et al., 2008). Consistent with this result, a large subset of PGCs – which were identified by their small somata and dendritic tuft (Figure 5.11LJ) – exhibited robust, short-latency (~1 ms; Figure 5.11H,M) postsynaptic currents following brief (10 ms) light pulses (Figure 5.11K). These postsynaptic currents were unaffected by glutamatergic antagonists but were abolished by subsequent application of GBZ (Figure 5.11K-N), consistent with monosynaptic release of GABA from GL-dSACs onto PGCs. Interestingly, the postsynaptic currents in PGCs reversed at a relatively depolarized potential (~44 mV; Figure 5.11N) despite the recording electrode containing a low-Cl⁻ internal solution (see Experimental procedures). This depolarized reversal potential is consistent with previous reports (Smith and Jahr, 2002; Parsa et al., 2015), however, and suggests that GABA release from GL-dSACs may act to drive PGC firing (see Discussion).
Figure 5.11. GL-dSACs innervate PGCs

A: Cre-dependent viral expression of ChR2:mCherry. Scalebar: 100 μm. B: Whole-cell fill following cell-attached recording of a representative GL-dSAC expressing ChR2:mCherry. Scalebar: 20 μm. C,D: Brief photostimulation (10 ms; blue line) evoked multiple action potentials in cell-attached recordings from the cell shown in B. Results plotted as in Figure 5.6. Inset scalebar: 10 ms/50 pA. E,F: Same as C,D for whole-cell recordings from a different GL-dSAC before and after application of NBQX and AP5. Inset scalebar: 10 ms/20 mV. G,H: Photostimulation rapidly evoked 1-4 action potentials in GL-dSACs independent of excitatory synaptic transmission (spikes: 2.8 ± 1.3 vs. 2.6 ± 1.1, before vs. after NBQX/AP5 application; n=5; p=0.48, paired t test; latency: 8.0 ± 3.4 vs. 7.9 ± 2.2 ms, before vs. after NBQX/AP5.
Approximately one-third of PGCs (i.e., type 1 PGCs) receive direct sensory-evoked input from OSNs and colocalize with OMP-positive, MAP2-negative glomerular compartments, while the remaining PGCs (i.e., type 2 PGCs) colocalize with OMP-negative, MAP2-positive glomerular compartments and receive indirect sensory-evoked input from ETCs and M/TCs (Nagayama et al., 2014; Najac et al., 2015). To investigate whether GL-dSACs target type 1 and/or 2 PGCs, we first examined the overlap between GL-dSAC axonal boutons and OMP-positive glomerular compartments. Strikingly, EYFP-expressing GL-dSAC boutons exhibited no colocalization with OMP-positive compartments (Figure 5.12A) but strong colocalization with MAP2-positive dendritic processes in the GL (Figure 5.12B), suggesting that GL-dSACs innervate type 2 PGCs.

Type 2 PGCs can be further subdivided into non-overlapping populations of calbindin- and calretinin-positive PGCs (CB-PGCs and CR-PGCs, respectively) (Nagayama et al., 2014; Najac et al., 2015). GL-dSAC boutons colocalized with both CB- and CR-PGCs (Figure 5.12C,D), suggesting that GL-dSACs innervate both CB- and CR-PGCs. To functionally investigate GL-dSAC innervation of distinct PGC subclasses, we putatively classified PGCs according to known physiological differences (Figure 5.12E-J). Specifically, CB-PGCs exhibit much greater sEPSC amplitudes and frequencies than CR-PGCs, with ~80% of CB-PGCs exhibiting an sEPSC frequency >8 Hz and 100% of CR-PGCs exhibiting an sEPSC frequency <8 Hz (Najac et al., 2015). Both putative PGC subclasses exhibited postsynaptic currents upon GL-dSAC photostimulation (Figure 5.12K,L). Interestingly, however, all putative CB-PGCs (5 of 5 cells) exhibited large (100-400 pA) postsynaptic currents, while most putative CR-PGCs (7 of 12 cells) exhibited small (10-60 pA) postsynaptic currents. Our results thus demonstrate that GL-dSACs exhibit a consistently strong influence on putative CB-PGCs, while innervation of putative CR-PGCs is weak and more variable.
Figure 5.12. GL-dSACs innervate multiple PGC subclasses
A,B: EYFP-expressing GL-dSAC axonal boutons in Chrna2-Cre/Ai3 mice innervate OMP-negative (A), MAP2-positive (B) glomerular compartments. Scalebar: 20 μm. C,D: EYFP-expressing GL-dSAC axonal boutons are closely juxtaposed with both CB-PGCs (C) and CR-PGCs (D). Scale same as in A. E: Whole-cell recording of a representative putative CB-PGC. Scalebar: 20 μm. F: Spontaneous excitatory synaptic input to the cell shown in E, exhibiting high...
sEPSC frequencies and amplitudes. **G:** Magnification of highlighted region in **F.** **H-J:** Same as **E-G** for a representative putative CR-PGC, exhibiting low sEPSC frequencies and amplitudes. **K:** Distribution of PGC sEPSC amplitudes and frequencies, color-coded by the amplitude of postsynaptic input evoked by GL-dSAC photostimulation. Dashed line at 8 Hz separates putative CR-PGCs (<8 Hz) from putative CB-PGCs (>8 Hz). Crosses correspond to the putative CB-PGC and CR-PGC shown in **E-G** and **H-J,** respectively. **L:** Mean postsynaptic input evoked by GL-dSAC photostimulation (blue square) in putative CR-PGCs and CB-PGCs. Inset: magnification of synaptic response in putative CR-PGCs (scalebar: 50 ms/10 pA). Red trace corresponds to cell shown in **H-J.**

**5.4.6. GL-dSACs innervate ETCs and TCs and can drive selective long-lasting inhibition of TCs**

We next investigated whether GL-dSAC photostimulation evoked postsynaptic currents in other MOB cell types within or connected to the GL, including ETCs, TCs, and MCs. Given previous ultrastructural data suggesting that GL-dSACs exclusively target GABAergic neurons (Eyre et al., 2008), we expected that GL-dSAC photostimulation might indirectly influence glutamatergic MOB neurons via polysynaptic pathways. Surprisingly, however, ETCs – which were identified by their large apical dendritic tuft and lack of lateral dendrites (Figure 5.13Ai,Bi) – exhibited reliable, short-latency inhibitory currents following GL-dSAC photostimulation (Figure 5.13Ci), suggesting instead that ETCs receive direct GL-dSAC input. Indeed, the latency of inhibitory input to ETCs was not significantly different than the latency of input to PGCs (Figure 5.13G). Our results thus suggest that both PGCs and ETCs receive direct, monosynaptic input from GL-dSACs.

In striking contrast to the postsynaptic currents observed in PGCs and ETCs, brief GL-dSAC photostimulation evoked robust, short-latency, long-lasting inhibitory currents in 14 of 18 TCs, including 4 of 8 superficial TCs (sTCs) located near the GL border (Figure 5.13Aii-Cii) and 10 of 10 middle TCs (mTCs) (Figure 5.13Aiii-Ciii). Of note, the 4 sTCs that failed to exhibit long-lasting inhibitory currents instead displayed synaptic responses similar to ETCs, and thus likely correspond to the subset of ETCs that bear lateral dendrites (Nagayama et al., 2014). To confirm that these distinct synaptic responses were not artifacts arising from slice-to-slice or glomerulus-to-gemolurus differences, we additionally performed paired recordings of ETCs and TCs connected to the same glomerulus (Figure 5.13Aiv,Biv) and observed identical results (Figure 5.13Civ). GL-dSAC photostimulation thus evokes highly distinct modes of inhibitory input to ETCs and TCs – the first such circuit that has been observed.

Surprisingly, MCs exhibited either no postsynaptic response to GL-dSAC photostimulation (Figure 5.13Av-Cv) or a very weak long-lasting inhibitory current (Figure 5.13D). Moreover, synaptic latencies were equivalent among PGCs, ETCs, and TCs but significantly longer in MCs (Figure 5.13G). Our results therefore show that GL-dSACs monosynaptically target PGCs, ETCs, and TCs, and additionally trigger a long-lasting inhibitory current in TCs that drives robust TC hyperpolarization (Figure 5.13H) and weak MC inhibition, potentially via GABA spillover.
Figure 5.13. GL-dSACs inhibit ETCs and TCs and can drive long-lasting TC inhibition
A-C: Morphology (A,B; scalebar: 100 μm) and whole-cell voltage-clamp recordings of mean postsynaptic currents (C) from a representative ETC (Ai,Bi,Ci), sTC (Aii,Bii,Cii), mTC (Aiii,Biii,Ciii), ETC/mTC pair (Aiv,Biv,Civ), and MC/MC pair (Av,Bv,Cv) following brief photostimulation (10 ms; blue line) of GL-dSACs expressing ChR2:mCherry. Results plotted as in Figure 5.6. Inset scalebar: 10 ms/50 pA. Postsynaptic currents in ETCs (n=4) and ETC-like sTCs (n=4) reversed at more hyperpolarized potentials than postsynaptic currents in PGCs (−53.3
D: Mean postsynaptic currents (recorded at $V_{\text{hold}}=+10$ mV) evoked by GL-dSAC photostimulation in ETCs (n=4), TCs (n=18), and MCs (n=7). Inset scalebar: 10 ms/20 pA. E-G: Amplitude (E), charge (F), and latency (G) of inhibitory synaptic input to ETCs, TCs (including sTCs [white triangles] and mTCs [black triangles]), and MCs evoked by GL-dSAC photostimulation. TCs exhibited significantly stronger synaptic input than MCs (E; 51.9 ± 36.3 [n=4] vs. 55.0 ± 21.9 [n=18] vs. 18.6 ± 9.7 pA [n=7], ETC vs. TC vs. MC; p=3.6×10⁻³, one-way ANOVA; TC vs. MC, p=2.8×10⁻³, post hoc Tukey-Kramer). Synaptic input to TCs transferred significantly more charge than synaptic input to ETCs or MCs (F; 0.9 ± 0.7 [n=4] vs. 8.5 ± 5.7 [n=18] vs. 2.1 ± 2.4 pC [n=7], ETC vs. TC vs. MC; p=3.3×10⁻³, one-way ANOVA; TC vs. ETC, p=0.020, TC vs. MC, p=0.015, post hoc Tukey-Kramer). The onset of synaptic input to MCs was significantly slower than the onset of synaptic input to ETCs and PGCs (G; 6.8 ± 0.5 [n=4] vs. 8.2 ± 0.9 [n=18] vs. 9.5 ± 1.4 [n=6] vs. 8.1 ± 1.1 ms [n=16], ETC vs. TC vs. MC vs. PGC; p=2.3×10⁻³, one-way ANOVA; MC vs. ETC, p=1.2×10⁻³, MC vs. PGC, p=0.031, post hoc Tukey-Kramer).

H: Mean hyperpolarization evoked by GL-dSAC photostimulation in TCs (n=26), compared to a representative TC with truncated apical dendrite (inset).

I: Whole-cell recording of a representative TC following GL-dSAC photostimulation (blue square) before (left), during (center), or after (right) glomerular activation by OSN stimulation (black triangle). Results plotted as in Figure 5.6. J: Mean change in sensory-evoked TC firing caused by GL-dSAC photostimulation at varying temporal lags. GL-dSAC photostimulation 50 ms following OSN stimulation significantly reduces sensory-evoked TC firing (p=0.012, t test with Bonferroni correction for multiple comparisons). Column numbers denote TCs recorded.

The long-lasting inhibitory currents observed in TCs exhibited several noteworthy features. First, these currents were remarkably consistent across trials and occurred in an all-or-none manner independent of the duration of GL-dSAC photostimulation (Figure 5.15). Second, these currents were recorded using a Cs⁺-based internal solution (see Experimental procedures), thereby excluding GABABR-mediated K⁺ channel modulation as a mechanism. Third, these currents occurred independent of glutamatergic transmission but were abolished by GBZ (n=3; Figure 5.13Ci-iii). Fourth, these currents were absent from TCs whose apical dendritic tuft was truncated during slice preparation (Figure 5.13H). Finally, these currents failed to reverse at negative holding potentials (Figure 5.13Cii-Civ). In total, the combination of these features outlines a novel GABAergic signaling mechanism within the glomerulus (see Discussion).

In our final experiment, we investigated how GL-dSAC-mediated inhibition influences sensory-evoked TC activity. Specifically, we photostimulated GL-dSACs before, during, or after the activation of individual glomeruli via extracellular OSN stimulation. GL-dSAC photostimulation before or during OSN stimulation surprisingly did not affect sensory-evoked TC firing (Figure 5.13I,J). However, GL-dSAC photostimulation following OSN stimulation by tens of milliseconds – times matching differences in glomerular activation latencies observed in vivo (Wachowiak, 2011) – robustly truncated all late sensory-evoked TC firing (Figure 5.13I,J). Synchronous GL-dSAC activation may thus be involved in regulating the sniff phase-coupling of TC activity in vivo, facilitating the temporal coding of sensory information by parallel TC and MC channels (Fukunaga et al., 2012).
Figure 5.14. GL-dSACs are necessary to evoke long-lasting TC inhibition

A: Parasagittal section from a representative adult Chrna2-Cre mouse injected with AAV1-DIO-ChR2:mCherry into the MOB at P2-3. Cre-dependent ChR2:mCherry expression is localized to neurons in the MOB IPL, as well as in a subset of medial septal neurons (red box). Infection of medial septal neurons results from diffusion of virus out of the neonatal MOB rather than retrograde infection, as diagonal band neurons – which heavily innervate the MOB – remain unlabeled, while medial septal neurons – which do not innervate the MOB – are labeled (Gaykema et al., 1990). Consistent with this, injection of virus into the adult MOB selectively labels GL-dSACs and not medial septal neurons (Figure 5.1D; data not shown). Scalebar: 500 μm. B: To test whether medial septal and other basal forebrain neurons drive the long-lasting TC inhibition observed following photostimulation in the GL (Figure 5.13), we injected AAV1-DIO-ChR2:mCherry into the forebrain of P2-3 Chrna2-Cre mice to enable selective optogenetic activation of forebrain neurons without GL-dSAC activation. A representative parasagittal acute slice (310 μm thick) is shown. Cre-dependent ChR2:mCherry expression is localized to neurons in the neocortex and forebrain, including the septal nucleus (Bii; red inset), and sparse dorsal TCs. No GL-dSACs expressed ChR2:mCherry following forebrain viral injection. Multiple TCs not expressing ChR2:mCherry exhibit strong fluorescence (Bi; yellow inset), reflecting intracellular fills with AF594 during whole-cell recordings. Scale is the same as in A. C:
Representative recordings from the boxed TCs in Bi. Scalebar: 50 μm. D: Whole-cell voltage-clamp recordings of mean postsynaptic currents from the cells in Bi,C following brief photostimulation (10 ms; blue line), equivalent to Figure 5.13. Inset scalebar: 10 ms/50 pA. Green and magenta traces correspond to the cells in Ci and Cii, respectively. E: Mean postsynaptic currents (recorded at V<sub>hold</sub>=+10 mV) evoked by photostimulation in TCs from mice with forebrain viral injection (n=8) or MOB viral injection (n=18; reproduced from Figure 5.13). Inset scalebar: 10 ms/20 pA. 0 of 8 TCs recorded in mice with forebrain viral injections exhibited a visible postsynaptic response to photostimulation, demonstrating that GL-dSAC activation is necessary to evoke the long-lasting TC response observed in Figure 5.13. F,G: Amplitude (F) and charge (G) of inhibitory synaptic input to TCs from mice with forebrain viral injection or MOB viral injection (including sTCs [white triangles] and mTCs [black triangles]). For TCs from mice with forebrain viral injection, charge was calculated by integrating the mean current waveform for 1 s following the onset of photostimulation. TCs from mice with MOB viral injection (n=18) exhibited significantly stronger amplitudes and charge than TCs from mice with forebrain viral injection (n=8) (F: 8.4 ± 2.7 vs. 55.0 ± 21.9 pA, forebrain vs. MOB; p=4.1×10<sup>−6</sup>, unpaired t test; G: −0.2 ± 1.4 vs. 8.5 ± 5.7 pC, forebrain vs. MOB; p=2.9×10<sup>−4</sup>, unpaired t test). Green and magenta points denote cells shown in Bi,C,D.

Figure 5.15. GL-dSAC-mediated long-lasting TC inhibition is all-or-none
A,B: Brief photostimulation (10 ms; blue line) evoked 1-2 action potentials in cell-attached recordings from GL-dSACs expressing ChR2:mCherry. Same cell as in Figure 5.11B. Results
plotted as in Figure 5.6. C,D: Same as A,B for prolonged photostimulation (1 s). E: Increasing photostimulation duration increases the number of evoked spikes in GL-dSACs expressing ChR2:mCherry. Red points denote cell shown in A-D. F,G: Neurobiotin fill (F) and reconstructed morphology (G) of a representative TC. Scalebar: 100 μm. H: Whole-cell voltage-clamp recording of the mean postsynaptic currents (recorded at +10 mV) evoked by varying duration photostimulation of GL-dSACs expressing ChR2:mCherry. I,J: Amplitude (I) and charge (J) of inhibitory synaptic input to TCs evoked by varying duration photostimulation of GL-dSACs. In contrast to the increasing number of spikes evoked in GL-dSACs with increasing photostimulation duration (E), inhibitory synaptic input to TCs occurred in an all-or-none manner. Red points denote cell shown in F-H.

5.5. Discussion

5.5.1. Summary
Understanding how inhibition shapes sensory processing in the olfactory system has been limited by the lack of selective markers for distinct MOB interneurons. Here, we have identified chrna2 as the first selective marker of GL-projecting dSACs, and used Chrna2-Cre mice to identify several structural and functional features by which GL-dSACs can critically regulate MOB sensory processing (Figure 5.16).

Structurally, GL-dSACs are prominently located in the IPL, where they are ideally positioned to integrate centrifugal cholinergic input with feedforward sensory input from ETC axons of several glomeruli. This highly convergent input is paralleled by highly divergent output, mediated by extensive GL-dSAC axonal arborization across multiple glomeruli. These structural features make GL-dSACs uniquely suited to regulate large expanses of the MOB circuit across distinct brain states and in response to diverse sensory input.

Functionally, GL-dSACs intrinsically fire at theta frequencies and reset their spontaneous firing phase in response to sensory input. GABA release from GL-dSACs mediates remarkably cell type-specific effects, including PGC inhibition and/or excitation, ETC inhibition, and long-lasting inhibition of TC apical dendrites. In turn, GL-dSACs do not directly target MCs, a striking omission of the largest class of MOB principal neurons. These functional features enable GL-dSACs to contribute to the temporal patterning of MOB activity while supporting parallel processing of sensory information by MCs and TCs. In particular, we propose that GL-dSAC activation can shift the balance of MC vs. TC activity to favor MC activity through a combination of direct TC inhibition and indirect MC disinhibition.
5.5.2. Emergent interneuron features

Our results demonstrate that PGCs, ETCs, and TCs all receive monosynaptic input from GL-dSACs. Of great interest, this finding identifies GL-dSACs as the first class of MOB interneurons known to target both glutamatergic and other GABAergic neuron classes. This divergent connectivity profile within the MOB circuit mirrors the divergent connectivity of Martinotti cells (MNCs) and neurogliaform cells (NGFCs) within neocortical circuits (Jiang et al., 2015), suggesting that GL-dSACs, MNCs, and NGFCs may fulfill common functional roles. Of note, GL-dSACs also share several other features with MNCs and NGFCs. Structurally, both GL-dSACs and MNCs are defined by their superficial axonal projections, while GL-dSACs, MNCs, and superficial NGFCs all arborizes their axons across superficial layers (Eyre et al., 2008; Kubota, 2014; Jiang et al., 2015). Functionally, these common axonal structures enable GL-dSACs, MNCs, and NGFCs to inhibit the distal apical dendrites of principal TCs and pyramidal cells (Kubota, 2014; Jiang et al., 2015) and thereby regulate the integration of sensory input in vivo (Murayama et al., 2009). In addition, NGFCs also express nAChRs and respond to endogenous ACh release, suggesting that both NGFCs and GL-dSACs rapidly modulate circuit activity across distinct brain states (Wester and McBain, 2014; Letzkus et al., 2015).

Of great interest, both GL-dSACs and NGFCs are also capable of mediating long-lasting inhibition of principal neurons, though likely via distinct mechanisms. Specifically, NGFCs mediate graded long-lasting inhibition of both principal neurons and interneurons by unitary volume transmission of GABA (Tamás et al., 2003). In contrast, synchronous GL-dSAC activation evokes all-or-none long-lasting inhibition selectively in TCs. We hypothesize that synchronous release of GABA from GL-dSACs excites at least a subset of PGCs with depolarized Cl⁻ reversal potentials (Smith and Jahr, 2002; Parsa et al., 2015) triggering regenerative GABA-induced GABA release (Parsa et al., 2015) that targets the TC apical dendritic tuft. Future work investigating functional PGC subclasses and M/TC–PGC connectivity (Najac et al., 2015) will be critical in evaluating this proposed mechanism.
GL-dSACs also share several features with somatostatin-expressing hippocampal lacunosum-moleculare-projecting oriens (O-LM) cells, which also express chna2 (Ishii et al., 2005; Leão et al., 2012). In particular, O-LM cells exhibit horizontal cell morphologies and arborize their axons across the most distal input layer of the CA1 circuit. Within this distal layer, O-LM cell axons inhibit the apical dendritic tufts of pyramidal cells to regulate the integration of sensory input in vivo (Lovett-Barron et al., 2014). Moreover, O-LM cells express nAChRs and respond to endogenous ACh release (Leão et al., 2012).

In total, this conservation of structure and function across GL-dSACs, MNCs, NGFCs, and O-LM cells suggests a core set of emergent interneuron features critical for regulating activity and sensory processing across distinct neocortical, hippocampal, and MOB circuits. Future investigations of other dSAC subclasses, as well as further dissection of horizontal vs. Golgi cell GL-dSACs, will be essential in exploring additional emergent interneuron features.

5.5.3. Temporal patterning of MOB activity
Theta frequency patterning of MOB activity emerges from both odor sampling via sniffing and the spontaneous bursting of ETCs (Wachowiak, 2011), and critically regulates olfactory processing. Changes in theta frequency sniffing rates can profoundly modulate the activation of both OSNs and M/TCs (Wachowiak, 2011). Moreover, theta frequency patterning provides a temporal reference for sensory encoding by M/TC activity (Fukunaga et al., 2012; 2014). Here, we now demonstrate that GL-dSACs spontaneously fire at theta frequencies and respond to sensory activation of the MOB circuit with a change in spike timing. These results suggest that GL-dSACs provide an additional source of theta frequency patterning intrinsic to the MOB. Moreover, the convergent ETC input onto GL-dSACs and divergent GL-dSAC output onto ETCs suggests that coherent ETC and GL-dSAC activity may be critical to long-range coordination of theta frequency activity (Kay and Lazzara, 2010).

Mechanistically, the highly precise spike timing response of GL-dSACs to sensory activation of the MOB circuit likely emerges from three functional features. First, sensory-evoked excitatory input to GL-dSACs is short in duration, in strong contrast to the LLDs observed in M/TCs (Carlson et al., 2000; De Saint Jan et al., 2009; Gire and Schoppa, 2009; Burton and Urban, 2014) and the asynchronous excitation observed in GCs (Burton and Urban, 2015). Second, sensory-evoked GL-dSAC firing is constrained to a window of opportunity by reliable feedforward inhibition (Pinto et al., 1996; Pouille and Scanziani, 2001) mediated by unknown interneurons. Third, dSACs express Kv3.1b (Eyre et al., 2009), a high voltage-activated potassium channel known to accelerate excitatory postsynaptic potential (EPSP) decay in other interneurons critically involved in precise temporal patterning of network activity (Hu et al., 2014).

5.5.4. Long-range coordination of glomerular activity
The axon collaterals of ETCs connected to mirror-symmetric glomeruli in each MOB reciprocally project to the IPL directly beneath the isofunctional glomerulus (Belluscio et al., 2002; Lodovichi et al., 2003) where they synapse exclusively onto GABAergic dendrites (Liu and Shipley, 1994). Of great interest, these ETC intrabulbar projections exhibit pronounced experience-dependent plasticity throughout life (Marks et al., 2006; Cummings and Belluscio,
Despite this remarkable anatomical specificity and plasticity, however, the cellular targets and overall function of ETC intrabulbar projections remain unknown. Given the unique positioning of GL-dSACs within the IPL and the highly convergent feedforward connectivity of ETCs with GL-dSACs, we hypothesize that ETC intrabulbar projections target GL-dSACs. Of note, this hypothesized circuitry provides an attractive mechanism for functionally coordinating the activation of entire mirror-symmetric glomerular microcircuits, rather than just a subset of local GCs. Moreover, the hypothesized circuitry — in addition to the strong inhibition observed in TCs following synchronous GL-dSAC activation — predicts that sequential activation of mirror-symmetric glomeruli will differentially regulate TC firing between the two glomeruli. In support of this hypothesis, sequential activation of mirror symmetric glomeruli by low concentration odors leads to differential principal neuron output (Zhou and Belluscio, 2012). Of note, an analogous role has been observed for NGFCs, which integrate callosal input to mediate interhemispheric inhibition of pyramidal cell apical dendrites across isofunctional regions of neocortex (Palmer et al., 2012). Long-range coordination of isofunctional local circuits may thus be a conserved function of interneurons in both neocortical and MOB circuits.

5.6. Appendix

5.6.1. Optogenetic confirmation of centrifugal cholinergic innervation of GL-dSACs
To more directly examine the origin of the nicotinic input to GL-dSACs observed above, we drove ChR2:EYFP expression in basal forebrain cholinergic neurons using either: 1) transgenic ChAT-ChR2:EYFP mice (n=1) (Zhao et al., 2011), or 2) AAV9-DIO-ChR2:EYFP injection into the horizontal limb of the diagonal band of Broca (HDB) of transgenic ChAT-IRES-Cre mice (n=4) (Rossi et al., 2011). Recordings were targeted to presumptive GL-dSACs (i.e., IPL-located dSACs) (Figure 5.17A,B), and axonal projections were classified following post-hoc morphological reconstruction (Figure 5.17C). Across 10 dSACs, 3 exhibited no response while 7 exhibited robust Mec-sensitive photostimulation-evoked EPSCs (Figure 5.17E-G) that could drive spiking (Figure 5.17D) and displayed kinetics matching the observed nicotinic currents in Chrna2-Cre-labeled GL-dSACs (Figure 5.10N). Strikingly, while all recorded dSACs were located in the IPL, centrifugal nicotinic input appeared highly selective for GL-dSACs, consistent with their selective expression of Chrna2 (Figure 5.1, Figure 5.3). Specifically, of the 3 non-responding dSACs, 2 were confirmed GCL-dSACs and 1 was unclassifiable, while of the 7 responding dSACs, 2 were confirmed GL-dSACs, 1 was a putative GL-dSAC (with axon truncated mid-EPL) and 4 were unclassifiable (data not shown). Further, 0 of 3 MCs and 0 of 7 GCs exhibited photostimulation-evoked currents (data not shown). Our results thus confirm that cholinergic neurons in the HDB provide robust and highly selective nicotinic input to GL-dSACs in the MOB, evincing the central role of GL-dSACs in the cholinergic regulation of olfaction.
Figure 5.17. GL-dSACs receive centrifugal cholinergic input
A: Presumptive GL-dSAC activity was recorded following photostimulation of cholinergic fibers in 4-7 week-old ChAT-IRES-Cre mice injected with AAV9-DIO-ChR2:EYFP in the horizontal diagonal band (HDB) ≥2 weeks prior. B,C: Whole-cell recording and post-hoc reconstruction of a representative dSAC with confirmed GL projection. Scalebar: 50 μm (B); 100 μm (C). D: Brief photostimulation (10 ms; blue line) evoked reliable firing in the cell shown in B,C. Results plotted as in Figure 5.6. E: Photostimulation evoked a robust Mec-sensitive EPSC in the cell shown in B,C. Mean response and individual trials shown with dark and light colors, respectively. F: Mean photostimulation-evoked EPSC recorded in presumptive GL-dSACs (n=2 with confirmed GL projections) before and after Mec (20-50 μM) application. G: Application of Mec significantly decreased the peak amplitude of the photostimulation-evoked EPSC (76.7 ± 12.9% reduction after Mec application; n=7; p=4.1×10^{-6}, paired t test).

5.7. Author contributions
Shawn D. Burton (S.D.B.) and Nathaniel N. Urban (N.N.U.) designed research; S.D.B., Greg LaRocca (G.L.), Annie Liu (A.L.), and Claire E. Cheetham (C.E.C.) performed research; S.D.B., G.L., and A.L. analyzed data; C.E.C. contributed unpublished reagents; S.D.B., G.L., A.L., C.E.C., and N.N.U. wrote the manuscript.

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for helpful discussions. Chapter 5 Appendix was completed in collaboration with Daniel T. Case and Rebecca P. Seal. The authors declare no conflict of interest.
6. General conclusions

6.1. Heterogeneity among oscillating neurons in the main olfactory bulb

In Chapter 2, we built directly upon three of our prior studies (Galán et al., 2006; Ermentrout et al., 2007; Padmanabhan and Urban, 2010) to investigate for the first time how intrinsic biophysical diversity influences the ability of real, periodically firing neurons to transform input correlations into synchronized spiking. Using acute slice electrophysiology, we found that periodically firing mitral cells (MCs), a class of principal neurons in the main olfactory bulb (MOB), exhibit considerable heterogeneity in their response to transient inputs and preferred periodic firing rate. Surprisingly, these two components of heterogeneity, both of which depend extensively on voltage-dependent channel expression, proved largely independent. In our main result, we revealed that the physiological level of heterogeneity in response to transient input imposed up to a 30% reduction in maximal output synchrony. In other words, a ~30% larger population of heterogeneous MCs is necessary to transform correlated input into the same number of synchronous spikes as a homogeneous population. Moreover, we found that moderate firing rate differences (~10 Hz) between MCs, similar to the differences observed between preferred periodic firing rates, drove a comparable reduction in output synchrony. These findings proved both robust and specific in simulations and also closely agreed with novel mathematical theory. Our study thus quantitatively established that intrinsic biophysical diversity among real neurons places significant constraints on correlation-induced synchronization of periodically firing neurons.

This study has direct implications in the field of oscillatory synchrony, where most studies have not accounted for the fact that real neurons exhibit considerable biophysical diversity. Moreover, the impact of MC diversity on output synchrony is highly relevant to the study of olfaction, where odors evoke (and are believed to be encoded by) distinct patterns of synchronized periodic firing of MCs (and the analogous invertebrate projection neurons). Our results also likely generalize to other brain areas, where differences in neuronal responses and periodic firing rates have been noted but not extensively examined.

6.2. Parallel processing in the main olfactory bulb

Parallel processing of complementary sensory information is an established neural coding motif in visual, auditory, and somatosensory regions of the mammalian brain. For example, parallel processing of visual input begins in the retina, where distinct classes of retinal ganglion cells transform convergent sensory input into complementary visual information. Whether similar parallel processing occurs in mammalian olfaction remains unknown but is currently an exciting focus of systems neuroscience. In Chapter 3, we described mechanisms by which MCs and tufted cells (TCs), the two classes of principal neurons in the MOB, may transform convergent sensory input into complementary olfactory information.

Using acute slice electrophysiology, we first demonstrated that physiological afferent stimulation drives TCs to fire with markedly higher rates and probabilities and shorter latencies than MCs, directly paralleling recent investigations of odor-evoked MC vs. TC activity in vivo (Table 1.1). We then used a combination of voltage- and current-clamp recordings in vitro to dissect the
synaptic and intrinsic biophysical properties contributing to the different MC vs. TC afferent-evoked activity. Critically, we found that TCs are intrinsically twice as excitable as MCs. This greater excitability, combined with stronger synaptic input, fully accounts for the stronger TC vs. MC response to afferent stimulation observed. These findings thus provide the first mechanistic explanation for the emerging hypothesis that TCs rapidly and reliably encode first order-sensory information (e.g., odor identity) while MCs slowly and more flexibly encode second order-sensory information (e.g., odor context and valence).

Surprisingly, we also found that M/TCs exhibit clear differences in activity patterns. Compared to MCs, TCs exhibited a greater propensity to “stutter”, or fire discrete clusters of high frequency action potentials separated by long pauses. This finding emphasizes the importance of action potential clusters to TC coding and further suggests that TCs transmit activity to downstream cortex differently than MCs do. We further found that this firing pattern regularity directly correlates with the amplitude of hyperpolarization-driven membrane potential sag in both MCs and TCs, motivating further investigation into the ionic and dynamical mechanisms underpinning M/TC activity.

6.3. Disinhibition in the main olfactory bulb

Tremendous progress has been made in understanding how interneurons shape sensory-evoked activity in principal neurons across the brain. In contrast, our understanding of the synaptic pathways regulating sensory-evoked activity in interneurons remains largely incomplete. In the MOB, inhibitory granule cells (GCs) are critically involved in decorrelating and temporally patterning odor-evoked principal neuron activity. In Chapter 4, we provided the first description of the synaptic basis of GC activity downstream of sensory input to the MOB. Using acute slice electrophysiology, we found that activation of sensory afferents evokes a barrage of asynchronous synaptic excitation as well as highly reliable, short-latency synaptic inhibition onto GCs. This short-latency inhibition emerged from a canonical disynaptic feedforward inhibitory circuit involving dendrite-targeting deep short-axon cells (dSACs), a largely uncharacterized class of MOB interneurons. We additionally used an optogenetic strategy to demonstrate that feedforward inhibition scales in strength with synaptic excitation onto GCs as more sensory afferents are activated, thus identifying a potential circuit mechanism supporting stimulus intensity-invariant activity in GCs. Using biophysical multicompartmental modeling, we further demonstrated that feedforward inhibition interacts with the asynchronous timecourse of synaptic excitation to suppress spike time precision in GCs. Moreover, feedforward inhibition onto GCs significantly attenuates subcellular depolarization and calcium influx within local release-competent spines, fostering parallel processing between different dendritic compartments. In total, this study thus identified feedforward inhibition onto GCs as a core feature of MOB circuitry and established asynchronous excitation and feedforward inhibition as critical regulators of sensory-evoked GC activity.

The findings presented in Chapter 4 motivate a reappraisal of current models of sensory processing in the MOB, which do not include feedforward inhibition onto GCs and largely neglect subcellular GC activity, and thus represent a significant advance in the field of olfaction. Moreover, this study provides – to our knowledge – the first functional consideration of the influence of dendritic inhibition on release-competent dendrites, and is thus highly relevant to the
study of dendrite-targeting interneurons, compartmentalized dendritic inhibition, dendritic neurotransmitter release, and synaptic integration in general. Further, the demonstration that feedforward inhibition can paradoxically suppress the temporal fidelity of GC firing introduces a novel computational role of feedforward inhibition with broad implications in neural processing throughout the rest of the brain. Additionally, in Chapter 4 Appendix, we quantitatively established the morphological subdivision of GCs into superficial and deep GCs (sGCs and dGCs, respectively), and provided functional evidence for the preferential connectivity of TCs and MCs with sGCs and dGCs, respectively. These results thus address classical hypotheses raised more than three decades ago (Orona et al., 1983; Mori et al., 1983) and extend the findings presented in Chapter 3 by identifying a novel synaptic mechanism supporting parallel processing of sensory information by MCs and TCs.

6.4. Molecular identification of a novel class of interneurons in the main olfactory bulb

The mammalian brain contains a vast diversity of GABAergic inhibitory interneurons. The identification of cell type-selective molecular markers has fostered tremendous insight into how distinct cortical interneurons shape sensory processing and behavior. In the MOB, inhibitory circuits precisely regulate the activity of M/TCs to drive olfactory-guided behavior. In contrast to cortical circuits, however, selective markers for distinct MOB interneurons remain largely unknown. This gap in knowledge has greatly limited cell type-selective study of interneuron function and mechanistic understanding of sensory processing in the MOB.

Recent studies have begun to address this gap in knowledge and identified novel interneuron circuitry in the superficial layers of the MOB (Kiyokage et al., 2010; Liu et al., 2013; Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013; Garcia et al., 2014; 2016; Banerjee et al., 2015). However, the deep layers of the MOB contain a large and diverse population of GABAergic dSACs whose functional roles remain almost entirely unknown.

In Chapter 5, we identified nicotinic acetylcholine receptor subunit α2 (chrna2) as the first selective marker of a novel subclass of glomerular layer-projecting dSACs (GL-dSACs). Using Chrna2-Cre mice together with immunohistochemistry, acute slice electrophysiology, and optogenetic circuit mapping, we examined the structure, intrinsic physiology, sensory recruitment, neuromodulation, synaptic output, and functional role of GL-dSACs in the MOB circuit. We found that GL-dSACs integrate centrifugal cholinergic input with highly convergent and broadly tuned sensory input and provide highly divergent output to both interneurons and TCs across large expanses of the MOB. The unique selectivity of GL-dSAC inputs and outputs suggests that these interneurons play a key role in regulating the balance in activity between MCs and TCs.

This study provided several significant new insights to the study of olfaction and neural circuits. Foremost, we identified a novel MOB interneuron and demonstrated that GL-dSACs share numerous features with neocortical Martinotti and neurogliaform cells and hippocampal oriens lacunosum-moleculare cells, suggesting common functional principles across distinct brain circuits. Additionally, we demonstrated that GL-dSACs form a lateral inhibitory circuit that targets TCs but strikingly excludes MCs. This surprisingly selective connectivity introduces a novel mechanism by which MCs and TCs process sensory information in parallel. Further, in
Chapter 5 Appendix, we demonstrated that GL-dSACs exhibit the strongest response to endogenous acetylcholine release from centrifugal fibers of any MOB cell type thus far examined, providing exciting new data to a contentious field (e.g., see Ma and Luo, 2012; Rothermel et al., 2014; Bendahmane et al., 2016).
7. Inhibitory circuits of the mammalian main olfactory bulb

7.1. Abstract

Synaptic inhibition is central to sensory processing throughout the mammalian brain, including the main olfactory bulb (MOB), the first station of sensory processing in the olfactory system. In the MOB, GABAergic signaling precisely regulates principal mitral and tufted cell (M/TC) activity through three main modes: 1) odor-evoked inhibition, 2) recurrent inhibition, and 3) lateral inhibition. Decades of research across numerous laboratories have established the central role of granule cells (GCs), the most abundant GABAergic MOB interneurons, in each of these modes of GABAergic signaling. In addition to GCs, however, the MOB contains a vast diversity of other GABAergic interneurons, and recent findings suggest that, while fewer in number, these oft-ignored interneurons are just as important as GCs in shaping M/TC activity. Here, I challenge the dogma of GC centrality. In this review, I first outline the specific properties of each GABAergic MOB interneuron, with particular emphasis placed on direct interneuron recordings and cell-type selective manipulations. On the basis of these properties, I then critically re-evaluate the evidence for GC involvement in each of the above modes of GABAergic signaling. This analysis yields a strikingly disparate model of inhibitory circuits in the MOB that stresses not only the abundance, but also the connectivity and physiology of each interneuron class.

7.2. Introduction

The inhibitory circuitry of the MOB is heavily structured by the exquisite odorant receptor (OR) and laminar organization of the circuit. Sensory information enters the main olfactory system when odors bind to specific but overlapping sets of ORs expressed on the dendrites of peripheral olfactory sensory neurons (OSNs). Each mature OSN in the mouse expresses 1 of 1,000-1,200 ORs, and all OSNs expressing the same OR project axons to precisely converge within ~2 glomeruli on the surface of the ipsilateral MOB (for review, see Mombaerts, 2004; Schoenfeld and Cleland, 2005; Mori and Sakano, 2011).

OR-specific glomeruli are located in the glomerular layer (GL) and represent the primary odor coding modules of the main olfactory system (for review, see Wachowiak and Shipley, 2006; Cleland, 2010). Each glomerulus is a large microcircuit harboring a complex array of interconnections. Within each glomerulus, OSN terminals excite the apical dendritic tufts of M/TCs (Najac et al., 2011; Gire et al., 2012). Each mature M/TC connects to a single glomerulus, with a few tens of homotypic M/TCs connected to each glomerulus (Royer et al., 1998; Sosulski et al., 2011; Ke et al., 2013). Further linked to each glomerulus is a diverse array of juxtaglomerular cells (JGCs) – including glutamatergic external tufted cells (ETCs) and GABAergic superficial short-axon cells (sSACs) and periglomerular cells (PGCs) – that collectively modulate the transfer of sensory input to M/TCs.

MC somata are arrayed in the compact MC layer (MCL) deep to the GL and external plexiform layer (EPL), while TC somata reside throughout the EPL (for review, see Macrides et al., 1985). Each M/TC emits 2-5 lateral dendrites that extend up to ~1 mm radially within the EPL (Mori et al., 1983; Orona et al., 1984). Both apical and lateral M/TC dendrites actively propagate action potentials.
potentials (Chen et al., 1997; Bischofberger and Jonas, 1997; Margrie et al., 2001; Charpak et al., 2001; Xiong and Chen, 2002) and form reciprocal dendrodendritic synapses with a diverse collection of GABAergic EPL-interneurons (EPL-INs) and the apical dendrites of GCs (Rall et al., 1966; Price and Powell, 1970b; Toida et al., 1994). At these unusual synapses, M/TC glutamate release drives interneuron depolarization and consequent GABA release back onto the M/TC. Activation of a single M/TC can thus yield recurrent self-inhibition as well as lateral inhibition of other M/TCs innervating the same interneurons (for review, see Schoppa and Urban, 2003).

Beneath the MCL is the compact internal plexiform layer (IPL), which is densely packed with ETC and TC axon collaterals (Liu and Shipley, 1994; for review, see Macrides et al., 1985), glutamatergic and GABAergic centrifugal fibers (Boyd et al., 2012; Markopoulos et al., 2012; Nunez-Parra et al., 2013), a variety of centrifugal neuromodulatory fibers (Linster and Fontanini, 2014), and the radially-oriented apical dendrites of GCs. Within this dense plexus resides a subset of GABAergic deep short-axon cells (dSACs) (Eyre et al., 2008; Chapter 5). Deep to the IPL is the granule cell layer (GCL), harboring an additional population of dSACs interspersed within a sea of GC somata and basal dendrites. M/TC axons course through the IPL and GCL and collect within the lateral olfactory tract (LOT) to project to various cortical and subcortical targets (for review, see Mori and Sakano, 2011), which, together with various neuromodulatory centers, provide massive centrifugal innervation to the MOB (Luskin and Price, 1983; Shipley and Adamek, 1984).

### 7.3. GABAergic interneurons of the main olfactory bulb

From superficial to deep, the main GABAergic MOB interneurons are thus: sSACs, PGCs, EPL-INs, dSACs, and GCs. While further complexity certainly exists (for an extensive review of molecular complexity, see Nagayama et al., 2014), here I focus only on those interneurons for which sufficient functional data exists to gauge their contribution to the main modes of GABAergic signaling listed above. The identification of cell type-selective molecular markers has fostered tremendous insight into how distinct cortical interneurons shape sensory processing and behavior (for review, see Kubota, 2014; Wester and McBain, 2014; Letzkus et al., 2015). In contrast to cortical circuits, however, selective markers for distinct MOB interneurons have remained elusive, strongly impeding investigation of cell type-selective interneuron function with modern molecular tools. Special emphasis is thus given to a handful of recent studies identifying novel selective markers for distinct MOB interneurons, while additional discussion is given to where identification of other markers might crucially advance the field.

Early order-of-magnitude estimates of principal and interneuron abundances (Shepherd, 1972) have proven critical in guiding research on GABAergic MOB interneurons (for review, see Schoppa and Urban, 2003; Egger and Urban, 2006). More recently, however, histological accounts quantifying absolute and relative abundances of different neuron types have emerged (Table 7.1). Of note, these accounts reveal that GC abundances were previously overestimated. This, together with the specific connectivity and physiology of the different GABAergic interneurons described below, strongly challenges GC centrality in MOB GABAergic signaling.
Table 7.1. Abundance of cell classes in the MOB

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<th>Class</th>
<th># (×10^3)</th>
<th>Quantification method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(GABAergic interneurons)</td>
<td></td>
</tr>
<tr>
<td>sSAC</td>
<td>87.2</td>
<td>TH-labeled cells in GL</td>
<td>Parrish-Aungst et al., 2007</td>
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<tr>
<td></td>
<td>125.0</td>
<td>TH-labeled cells in GL average</td>
<td>McLean and Shipley, 1988</td>
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<tr>
<td></td>
<td>106.1</td>
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<tr>
<td>PGC</td>
<td>297.5</td>
<td>GAD65-GFP-labeled cells in GL</td>
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<tr>
<td>EPL-IN</td>
<td>55.7</td>
<td>CRH-Cre-labeled cell density in EPL × EPL volume</td>
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<tr>
<td></td>
<td>53.4</td>
<td>CRH-Cre-labeled cell density in EPL × EPL volume</td>
<td>Garcia et al., 2016</td>
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<tr>
<td></td>
<td>54.6</td>
<td>average</td>
<td></td>
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<tr>
<td>dSAC</td>
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<td></td>
<td>1000.0</td>
<td>NeuN-labeled cells in GCL average</td>
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<td>820.6</td>
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<td>(Glutamatergic neurons and glomeruli)</td>
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<td>Benson et al., 1984</td>
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<td>38.4</td>
<td>Large cells in MCL</td>
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<td>33.0</td>
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<td>TC</td>
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<td>Estimated ~2.4:1 TC:MC ratio</td>
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<td>VGLUT1/VGLUT2-labeled cell density in GL × GL volume</td>
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<td></td>
<td>280.6</td>
<td>average</td>
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<td>Glomeruli</td>
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<td>Large cresyl violet-enclosed neuropil structures in GL</td>
<td>Royet et al., 1988</td>
</tr>
<tr>
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<td>1.7</td>
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<td>Pomeroy et al., 1990</td>
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<td>3.8</td>
<td>Large vGluT2-labeled structures in GL</td>
<td>Richard et al., 2010</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>average</td>
<td></td>
</tr>
</tbody>
</table>


7.3.1. Superficial short-axon cells

sSACs are located in the GL and are both GABAergic (GAD65-negative, GAD67-positive) and dopaminergic, and are selectively marked by either tyrosine hydroxylase (TH) or dopamine
transporter (DAT) (Aungst et al., 2003; Kosaka and Kosaka, 2008a; Kiyokage et al., 2010; Banerjee et al., 2015). sSACs have relatively large, ovoid somata (~11 μm diameter) with 3-5 sparsely branched dendrites extending within and around 2-4 glomeruli (Pinching and Powell, 1971a; Aungst et al., 2003; Kosaka and Kosaka, 2008a; Kiyokage et al., 2010; Banerjee et al., 2015), endowing sSACs with broad odor tuning from multiple neighboring glomeruli (Banerjee et al., 2015). Single cell reconstructions and focal retrograde tracer injections have strikingly revealed that sSACs can extend multiple presumptive axon branches up to ~1 mm throughout the GL (Aungst et al., 2003; Kosaka and Kosaka, 2008a; Kiyokage et al., 2010; Banerjee et al., 2015).

The majority (~70%) of sSACs (i.e., “ETd sSACs”) receive indirect sensory input via glutamatergic EPSC bursts mediated by ETCs (and potentially M/TCs), while the remaining sSACs (i.e., “ONd sSACs”) appear to receive rapid, reliable direct sensory input from OSNs (Hayar et al., 2004a; Kiyokage et al., 2010). These patterns of synaptic input likely culminate in a rapid, relatively synchronous response to glomerular activation, though the spiking response of sSACs has not been directly examined. Consistent with this prediction, however, Ca²⁺ imaging from sSACs in vivo has demonstrated unilaterally excitatory odor-responses that monotonically increase in strength with odor concentration and typically exhibit simple temporal dynamics that track the duration of odor sampling (Wachowiak et al., 2013; Banerjee et al., 2015). Interestingly, a subset of ETd sSACs extend their presumptive axons across several tens of glomeruli, while the remaining sSACs contact <10 glomeruli, suggesting that sSACs may support distinct scales of interglomerular interactions (Kiyokage et al., 2010). Within the GL, sSACs receive the strongest amount of excitatory centrifugal feedback (Markopoulos et al., 2012; Boyd et al., 2012).

Several conflicting results have emerged from studies examining the synaptic output of sSACs using extracellular stimulation together with micro-surgical disruption of sub-GL layers (Puopolo and Belluzzi, 1998a; Aungst et al., 2003; Shirley et al., 2010; Whitesell et al., 2013). These discrepancies have likely emerged from multiple confounds, including methodological differences, tremendous synaptic complexity within the glomerulus, and the presence of multiple cell types mediating interglomerular interactions (Eyre et al., 2008; Chapter 5; see below). However, two recent studies employing opto- and chemogenetic perturbations in TH-Cre and DAT-Cre mice have minimized these confounds to provide important insight into the synaptic output of sSACs. Both Liu et al. (2013) and Banerjee et al. (2015) showed that brief optogenetic activation of sSACs evokes monosynaptic inhibition in ETCs. Liu et al. (2013) further demonstrated that the corelease of DA from sSACs boosts ETC Iₙₙ currents, enhancing ETC rebound bursts, while Banerjee et al. (2015) uncovered direct electrical coupling between sSACs and ETCs. Remarkably, sSACs (and GL-dSACs) robustly innervate ETCs while largely avoiding MCs, despite the extensive anatomical overlap of ETC and MC apical dendritic tufts within the glomerulus (Whitesell et al., 2013; Banerjee et al., 2015; Chapter 5). Nevertheless, optogenetic activation of sSACs in vivo strongly suppresses spontaneous and odor-evoked M/TC activity (Banerjee et al., 2015), underscoring the critical role of ETCs in coordinating glomerular activity (Gire and Schoppa, 2009; De Saint Jan et al., 2009; Najac et al., 2011; Gire et al., 2012) and identifying sSACs as surprisingly powerful regulators of M/TC activity.
Given sSAC morphology, the majority of GABA- and DAergic output onto an ETC presumably arises from interglomerular projections of distant sSACs, while electrical coupling presumably arises from somatodendritic contacts of intraglomerular sSACs. Neither Liu et al. (2013) nor Banerjee et al. (2015) examined the glomerular specificity of sSAC output, however, owing to the inability of direct stimulation (either extracellularly or optogenetically) to differentiate orthodromic activation of distant cells from antidromic activation of nearby cells. In a related study, however, Schoppa and colleagues used a clever application of glutamate uncaging together with micro-surgical dissection of sub-GL layers to demonstrate that ETC inhibition arises from interglomerular projections (of either sSACs or GL-dSACs) (Whitesell et al., 2013). No quantitative estimates of sSAC output connectivity currently exist.

7.3.2. Periglomerular cells
PGCs exhibit pronounced morphological, neurochemical, and functional heterogeneity (for review, see Wachowiak and Shipley, 2006; Kosaka and Kosaka, 2011; 2016; Crespo et al., 2013; Nagayama et al., 2014), but almost unilaterally exhibit small somata (~7 μm diameter) in the periglomerular space with a single, small dendritic tuft occupying ~25% of a single glomerulus (Hayar et al., 2004b; Shao et al., 2009). Of note, an unknown fraction of PGCs also extend an axonal projection, the functional role of which is entirely unknown (for review, see Kosaka and Kosaka, 2011; 2016). However, it appears that all PGCs form dendrodendritic synapses and are capable of dendritic neurotransmitter release. Here, I therefore review the functional capacity of PGCs with respect to their dendritic physiology, while allowing that PGCs likely also contribute in other unknown capacities via their axonal projections.

PGCs can be broadly subdivided into 3 main functional subclasses. Approximately one-third of PGCs (i.e., “type 1” or “ONd” PGCs) receive direct sensory-evoked input from OSNs and exhibit broad immunohistochemical overlap with OMP-positive, MAP2-negative glomerular compartments, while the remaining PGCs (i.e., “type 2” or “ETd” PGCs) overlap with OMP-negative, MAP2-positive glomerular compartments and receive indirect sensory-evoked excitatory input from ETCs (Kosaka et al., 1997; 2001; Toida et al., 1998; 2000; Kosaka and Kosaka, 2007a,b; Hayar et al., 2004a; 2005; Murphy et al., 2004; 2005; Shao et al., 2009; Kiyokage et al., 2010). Type 2 PGCs can be further subdivided into non-overlapping and functionally distinct populations of calbindin/Kv3.1- and calretinin-positive PGCs (“CB-PGCs” and “CR-PGCs”, respectively) (Kosaka et al., 1995; 1997; Kosaka and Kosaka, 2007a; Panzanelli et al., 2007; Parrish-Aungst et al., 2007; Whitman and Greer, 2007; Najac et al., 2015). An undetermined subset of dopaminergic JGCs are likely to be type 1 PGCs rather than sSACs (for review, see Kosaka and Kosaka, 2011; 2016). Therefore, the above cell counts (Table 7.1) represent upper- and lower-bound estimates for sSAC and PGC abundances, respectively.

Similar to GCs (see below), excitatory postsynaptic input to PGCs (from OSNs, ETCs, and centrifugal input alike) is mediated by both AMPARs and NMDARs (Murphy et al., 2004; De Saint Jan and Westbrook, 2007; Grubb et al., 2008; Markopoulous et al., 2012), consistent with the equal reduction of spontaneous IPSC frequencies in ETCs upon application of either NMDAR or AMPAR antagonists (Hayar et al., 2005).

GABA release from PGCs can drive self-signaling (Smith and Jahr, 2002; Murphy et al., 2005) as well as lateral signaling onto homotypic PGCs (Murphy et al., 2005; Parsa et al., 2015),
postsynaptic inhibition of ETCs (Hayar et al., 2004a; 2005), and presynaptic inhibition of OSNs (for review, see McGann, 2013). The nature of GABAergic input to PGCs has been the matter of some debate, though PGCs in general (even mature PGCs) exhibit unusually high intracellular Cl\(^{-}\) concentrations (Smith and Jahr, 2002; Parsa et al., 2015). Strikingly, Parsa et al. (2015) have recently confirmed that both exogenous and endogenous GABA release within a glomerulus, even from a single PGC, is sufficient to trigger excitatory and regenerative GABA-induced GABA release from at least some PGCs, identifying a potentially critical mechanism for mediating glomerulus-wide GABAergic signaling (e.g., see Economo et al., 2016; Chapter 5). Direct electrical coupling between homotypic PGCs further supports coordination of PGC activity (Schoppa, 2006b).

The excitatory role of GABA notwithstanding, PGCs have long been known to exhibit heterogeneous and peculiar physiology, even for GABAergic MOB interneurons. Indeed, while some PGCs fire repetitively to somatic current injection or glomerular activation, many exhibit limited spiking capacity and pronounced L-type Ca\(^{2+}\)-mediated plateau potentials (Puopolo and Belluzzi, 1998b; Hayar et al., 2004a,b; Murphy et al., 2005; Shao et al., 2009; Masurkar and Chen, 2011; Sethupathy et al., 2013). Critically, these plateau potentials drive highly asynchronous GABA release and support action potential-independent release of GABA (Smith and Jahr, 2002; Murphy et al., 2005), attributes of GABAergic signaling long attributed exclusively to GCs.

Of great importance, De Saint Jan and colleagues have recently begun untangling PGC heterogeneity by systematically linking PGC physiology to molecular identity (Najac et al., 2015). Specifically, this study demonstrated that CB-PGCs (included within the larger population of Kv3.1-labeled PGCs) are strongly connected to the glomerular microcircuit, receiving pronounced indirect sensory-evoked input that drives reliable firing of multiple action potentials both in vitro and in vivo. In contrast, CR-PGCs are surprisingly weakly connected to the glomerular microcircuit and do not exhibit sensory-evoked firing. It is important to note, however, that CR-PGCs may nevertheless generate plateau potentials and thereby significantly contribute to GABAergic signaling. Najac et al. (2015) further provided the first definitive demonstration that CB-PGCs not only receive input from ETCs, but also from TCs and MCs alike, and further provide output onto all three glutamatergic cell types. Indeed, firing of a single MC is sufficient to activate CB-PGCs to drive both recurrent inhibition and “lateral” inhibition of homotypic ETCs, TCs, and MCs – a finding of outstanding relevance to the evaluation of MC recurrent inhibition (see below).

While not explicitly addressed in this review, PGCs are also continuously generated throughout life, and thus constitute an important channel for MOB plasticity and olfactory learning (for review, see Adam and Mizrahi, 2010).

7.3.3. External plexiform layer interneurons
The EPL is populated by a morphologically diverse array of axonless and axon-bearing interneurons variously described as Van Gehuchten, multipolar, satellite, horizontal, and short-axon cells (for review, see Kosaka and Kosaka, 2011; Crespo et al., 2013; Nagayama et al., 2014). While a clear distinction can be drawn between the axonless and larger axon-bearing interneurons in this layer (e.g., see Kosaka and Kosaka, 2008b), it remains unclear whether other
morphological subclasses represent distinct cell classes or variations within a continuum. As the few functional investigations of interneurons in the EPL thus far do not support further subdivision beyond axonless and axon-bearing subsets, I refer to the axonless subset as EPL-INs and the axon-bearing subset as intermediate short-axon cells (iSACs), to avoid confusion with the dopaminergic sSACs and the inframitral dSACs.

Investigation of EPL-INs has heavily relied on their inclusive expression of calretinin (CR), parvalbumin (PV), and vasoactive intestinal peptide (VIP), as well as the restricted expression of somatostatin (SST) in ~50% of deep EPL-INs. However, each of these markers is variously expressed in other interneuron classes, including iSACs (PV, CR), PGCs (PV, CR, VIP, SST), and dSACs (PV, CR, VIP, SST) (for review, see Kosaka and Kosaka, 2011; Crespo et al., 2013; Nagayama et al., 2014), thus limiting the utility of CR, PV, VIP, and SST in supporting cell type-selective labeling and manipulation of EPL-INs. In a pivotal study, however, Arenkiel and colleagues revealed that corticotropin-releasing hormone (CRH) is selectively expressed only in EPL-INs within the MOB (Huang et al., 2013), laying the groundwork for future investigations into the functional roles of EPL-INs (e.g., see Garcia et al., 2014; 2016).

EPL-INs have medium sized somata (~9 μm diameter) and extend one or a few sparsely branched primary dendrites that collectively extend up to ~80 μm and exhibit beaded, sparsely spiny profiles (e.g., see Kosaka and Kosaka, 2008b; Lepousez et al., 2010; Huang et al., 2013; Kato et al., 2013). EPL-INs exhibit functional phenotypes comparable to PV-positive interneurons throughout the rest of the brain, including fast-spiking behavior (≤200 Hz), prodigious spontaneous EPSC rates (~20 Hz), and large contributions from Ca2+-permeable, rectifying AMPARs (Hamilton et al., 2005; Huang et al., 2013; Kato et al., 2013).

EPL-INs form both reciprocal and non-reciprocal synapses exclusively with M/TCs (Toida et al., 1994; 1996; Lepousez et al., 2010; Huang et al., 2013; Kato et al., 2013), including basket-like innervation of some M/TC somata (Crespo et al., 2002; Kosaka and Kosaka, 2008b; for review, see Crespo et al., 2013), which likely functions similarly to the powerful perisomatic inhibition of pyramidal cells by neocortical basket cells. Given their release competent dendritic nature, EPL-INs can likely mediate action potential-independent GABA release, similar to PGCs, though this remains to be explicitly tested. Glomerular activation evokes a long-lasting barrage of excitatory input to EPL-INs (Hamilton et al., 2005; Kato et al., 2013), similar to the pattern of excitatory input observed in GCs (Burton and Urban, 2015). Unlike GCs, however (see below), this excitatory input evokes short-latency, rapid firing of a few action potentials in EPL-INs.

In vivo, EPL-INs spontaneously fire intermittent, high frequency action potentials and exhibit large and frequently persistent increases in firing rate in response to odors (Huang et al., 2013; Miyamichi et al., 2013). EPL-INs further exhibit uniquely broad odor tuning and high sensitivity to respiration rates (Miyamichi et al., 2013; Kato et al., 2013), reflecting an acute sensitivity to both chemo- and mechanosensory activation of OSNs (Grosmaitre et al., 2007; Carey et al., 2009). These features not only reveal a critical role for EPL-INs in redistributing global levels of activity within the MOB (Uchida et al., 2013), but also further suggest high connectivity with M/TCs. Indeed, three independent measures of connectivity employing paired in vitro recordings (Huang et al., 2013; Kato et al., 2013) and transsynaptic viral tracing (Miyamichi et al., 2013) recently demonstrated remarkably higher EPL-IN–MC than GC–MC reciprocal connectivity.
Specifically, up to ~50% of nearby (≤200 μm) EPL-IN–MC pairs are reciprocally connected \textit{in vitro}, compared to only ~4% of GC–MC pairs (Huang et al., 2013; Kato et al., 2013). Likewise, transsynaptic viral tracing demonstrated a ~12-fold greater rate of EPL-IN–MC than GC–MC reciprocal connectivity (Miyamichi et al., 2013). Thus, while EPL-INs are an order of magnitude less abundant than GCs (Table 7.1), they are also an order of magnitude more connected than GCs. EPL-INs are therefore on equal functional standing with GCs.

7.3.4. Granule cells

GCs have a relatively uniform morphology, though less abundant variations exist (e.g., see Naritsuka et al., 2009; Merkle et al., 2014). GCs have small somata (~6 μm diameter), one to a few sparsely branched and spiny basal dendrites, and rather extensive apical dendritic trees that extend into the EPL and bear ~225 large spines, or “gemmales” – the site of reciprocal dendrodendritic synapse formation (Rall et al., 1966; Price and Powell, 1970a; Mori et al., 1983; Orona et al., 1983; Woolf et al., 1991b; Geramita et al., 2016). GCs have long been hypothesized to form two distinct subpopulations, based on their remarkably selective apical dendritic innervation of the superficial vs. deep EPL, putatively supporting differential connectivity with TCs vs. MCs, respectively (Mori et al., 1983; Orona et al., 1984). Quantitative evidence supporting this hypothesis has recently emerged, as well as surprising functional differences between sGCs and dGCs that collectively yield greater sGC excitability (Geramita et al., 2016).

While GCs are widely known to densely populate the GCL, they also form the most abundant cell population within the MCL (Parrish-Aungst et al., 2007), where sGC recordings can be readily obtained (e.g., see Burton and Urban, 2015; Geramita et al., 2016). This critical but largely ignored fact has critical ramifications for GCL-targeted perturbations (e.g., viral injections, micro-surgical dissections, and extracellular stimulation), which not only non-selectively affect GCs, dSACs, and centrifugal fibers within the GCL, but also minimally influence a large population of sGCs within the MCL. For this reason, the development of a selective molecular marker for GCs would substantially advance the field by enabling the first cell type-selective manipulations of GCs. While no such marker currently exists, three molecules are of particular note. First, leucine-rich repeat membrane protein 5T4 exhibits strikingly selective expression within MCL-located sGCs (Imamura et al., 2006), suggesting that the development of a 5T4-Cre mouseline may enable highly selective perturbation of a subset of sGCs. Second, several studies have demonstrated that Emx1-IRES-Cre-driven constitutive reporter expression may preferentially label sGCs (both within the MCL and GCL), as well as a small population of JGCs (Gorski et al., 2002; Kohwi et al., 2007; Rebello et al., 2014). Finally, strong evidence exists for the specific and potentially selective expression of CaMKIIα in GCs (Zou et al., 2002), a fact that shockingly few studies have capitalized on (Shimshek et al., 2005). Note, however, that further testing is needed to determine whether dSACs also express CaMKIIα.

Of note, GC–M/TC reciprocal synapses consist of only one or a few contacts (Woolf et al., 1991a). Assuming a single reciprocal synapse per gemmule, each GC thus reciprocally connects with ~225 M/TCs. For EPL-IN–M/TC connectivity rates to exceed GC–M/TC connectivity rates by ~12-fold (see above), each EPL-IN must reciprocally connect with on the order of 10^3 M/TCs.
Excitatory synaptic input to GCs occurs in two spatially and functionally distinct modes. First, dendrodendritic synapses formed by M/TC lateral dendrites provide kinetically slow, depressing, AMPAR- and NMDAR-mediated inputs to the apical dendrites of GCs in the EPL (Isaacson and Strowbridge, 1998; Isaacson, 2001; Dietz and Murthy, 2005; Schoppa, 2006a; Balu et al., 2007). Second, axodendritic synapses formed by both M/TC axon collaterals and centrifugal fibers provide kinetically fast, facilitating, predominantly AMPAR-mediated inputs to the proximal somatodendritic domains of GCs in the GCL (and MCL) (Halabisky and Strowbridge, 2003; Schoppa, 2006a; Balu et al., 2007; Boyd et al., 2012; Markopoulos et al., 2012). Interestingly, this proximal input is far more effective in driving GC firing than dendrodendritic M/TC input (Halabisky and Strowbridge, 2003), identifying a critical role for centrifugal input (and M/TC axon collateral input) in regulating GC activity. Indeed, GCs receive robust excitatory centrifugal input on their proximal somatodendritic domains from both anterior olfactory nucleus and anterior piriform cortex (Boyd et al., 2012; Markopoulos et al., 2012).

Inhibitory synaptic input to GCs is equally as prominent as excitatory input to GCs and targets GCs throughout their entire somatodendritic axis, including their gemmules (Price and Powell, 1970c; Panzanelli et al., 2009; Nunez-Parra et al., 2013; Burton and Urban, 2015). Inhibitory input to GCs arises from dSACs (see below) as well as from centrifugal GABAergic innervation originating in the basal forebrain (Gracia-Llanes et al., 2010; Nunez-Parra et al., 2013).

Sensory input to the MOB evokes long-latency and sparse GC firing both in vivo (Wellis and Scott, 1990; Luo and Katz, 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Labarrera et al., 2013; Fukunaga et al., 2014) and in vitro (Burton and Urban, 2015). Interestingly, GCs exhibit stronger excitatory and inhibitory responses to odors in awake mice (though see Fukunaga et al., 2014), with far weaker sniff-phase coupling than in the anesthetized mouse (Cazakoff et al., 2014). These results suggest that GC activity may become more heavily regulated by centrifugal and dSAC-mediated inputs than by sniff-coupled M/TC inputs in the awake state.

The typically sparse, long-latency sensory-evoked activity of GCs partially arises from GC intrinsic properties, including the expression of A-type potassium channels (Schoppa and Westbrook, 1999; Schoppa, 2006a) and canonical transient receptor potential (TRPC) channels, which mediate long-lasting NMDAR-dependent plateau potentials (Egger, 2008; Stroh et al., 2012). Extrinsic synaptic properties, including highly asynchronous excitatory input and reliable feedforward inhibition, also strongly contribute to the sparse, long-latency firing of GCs (Burton and Urban, 2015).

Given their release competent dendritic nature, GCs can likely mediate action potential-independent GABA release, similar to PGCs (and likely similar to EPL-INs). Note, however, that due to the practical difficulty of finding connected M/TC–GC pairs, all investigations of action potential-independent GABA release from GCs (Isaacson and Strowbridge, 1998; Halabisky et al., 2000; Isaacson, 2001; Isaacson and Vitten, 2003; Dietz and Murthy, 2005; Dietz et al., 2011) have thus far relied on measures of recurrent and lateral inhibition in MCs, which are not necessarily specific to GCs (see below). Identical arguments also necessarily apply to the existence of asynchronous GABA release from GCs.
While the existence of action potential-independent GABA release thus remains to be directly demonstrated, several mechanisms exist within GCs that may support such signaling on both local (i.e., gemmule- and dendritic branch-specific) and global (i.e., cell-wide) spatial scales. First, sensory input evokes robust Ca\(^{2+}\) transients within individual GC gemmules (Egger et al., 2005), which are supported by the local expression of Nav (Bywalez et al., 2015). Second, sensory input frequently evokes spikelets in GCs both in vivo (Mori and Takagi, 1978b; Wellis and Scott, 1990; Luo and Katz, 2001; Labarrera et al., 2013) and in vitro (Burton and Urban, 2015), strongly suggesting the existence of dendritic branch-specific Nav-mediated spikes. GABA release mediated by either of these forms of local activity would necessarily mediate rather restricted recurrent inhibition of M/TCs. Third, both TRPC-mediated plateau potentials and T-type Ca\(^{2+}\)-mediated low-threshold spikes lead to strong global Ca\(^{2+}\) increases (Isaacson and Vitten, 2003; Egger et al., 2003; 2005; Egger, 2008; Stroh et al., 2012) – key candidate mechanisms for lateral, asynchronous inhibition of multiple M/TCs.

While not explicitly addressed in this review, GCs are also continuously generated throughout life, and thus constitute an important channel for MOB plasticity and olfactory learning (for review, see Lazarini and Lledo, 2011).

7.3.5. Deep short-axon cells
dSACs have large cell bodies and display ~4 distinct somatodendritic morphologies (Price and Powell, 1970b; Schneider and Macrides, 1978; López-Mascaraque et al., 1986; Eyre et al., 2008), and without contest, constitute both the least abundant (Table 7.1) and least understood GABAergic interneuron class in the MOB. Nevertheless, results from a handful of recent studies compelling argue that these interneurons are pivotally involved in sensory processing in the MOB.

Foremost, in a pioneering study, Nusser and colleagues systematically examined the full axodendritic morphologies of a large collection of dSACs and revealed that, similar to neocortical interneurons, dSACs can be classified into largely non-overlapping subclasses based on their axonal projections, yielding GCL-projecting, EPL-projecting, and GL-projecting dSACs (Eyre et al., 2008). Of great interest, a single GL-dSAC extends its axon across multiple glomeruli, and thus may significantly regulate sensory-evoked MOB activity (Eyre et al., 2008; Chapter 5). Critically, this redefinition of dSACs from previous classification schema based on somatodendritic morphology or neurochemical content afforded the first intuitive hypotheses on dSAC function in the MOB, including multi-gglomerular coordination by GL-dSACs and M/TC disinhibition by EPL- and GCL-dSACs. Further, Eyre et al. (2008) provided critical functional data on dSAC output, which together with Pressler and Strowbridge (2006), revealed that dSACs can strongly inhibit GCs.

In a follow-up study, Nusser and colleagues capitalized on the specific (but non-selective) expression of GABA\(_{\alpha1}\) and specific Kv channels to provide the first quantification of total dSAC abundance, revealing far greater numbers than previously guessed (Eyre et al., 2009; Table 7.1). Strikingly, this abundance, combined with the extensive axonal arbors and synaptic output of dSACs, led Eyre et al. (2009) to estimate that each EPL-and/or GCL-dSAC innervates on the order of \(10^3\) GCs, with up to 30 EPL-and/or GCL-dSACs converging onto each GC. Of note, these calculations used the early order-of-magnitude estimates of principal neuron to
interneuron abundances, which substantially overestimated GC abundance. Performing the same calculations with recent histological cell counts (Table 7.1) yields a convergence of 50 EPL- and/or GCL-dSACs onto each GC. Thus, while comparatively few in number, dSACs can powerfully regulate GCs. Indeed, consistent with this prediction, sensory input to the MOB evokes rapid, reliable, and highly convergent dSAC-mediated feedforward inhibition onto all GCs (Burton and Urban, 2015).

Given that the major (though not exclusive – see Gracia-Llanes et al., 2003) target of EPL- and GCL-dSACs is GCs, and that the contribution of GCs to multiple modes of GABAergic signaling in the MOB is unclear (see below), how dSACs in general contribute to sensory processing is likewise unclear. Recent research has begun to resolve this uncertainty however. Specifically, by identifying nicotinic acetylcholine receptor subunit α2 (chrna2) as the first selective molecular marker of GL-dSACs, Urban and colleagues enabled the first systematic functional investigation of GL-dSACs and surprisingly revealed a critical role for GL-dSACs in regulating the balance of sensory-evoked activity between MCs and TCs (Chapter 5). Strikingly, in contrast to ultrastructural predictions that GL-dSACs exclusively innervate other GABAergic interneurons (Eyre et al., 2008), optogenetic activation of GL-dSACs evoked reliable, short-latency, apparently monosynaptic input to PGCs, ETCs, and TCs (Chapter 5). GL-dSACs likewise exhibit highly regular theta frequency spontaneous activity (Chapter 5), suggesting a critical role in the temporal patterning of MOB activity comparable to the role of theta frequency bursting in ETCs (Hayar et al., 2004a,b; 2005). Further investigation has additionally demonstrated that GL-dSACs receive the strongest level of centrifugal nicotinic cholinergic input of any neuron type in the MOB (Chapter 5 Appendix), identifying GL-dSACs as a central component in the cholinergic modulation of MOB activity.

Other dSAC subclasses are likely to play similar roles in the centrifugal control of MOB activity, as dSACs are more heavily innervated by centrifugal piriform projections than any other cell type in the MOB (Boyd et al., 2012). Further, of outstanding interest, a fraction of GCL-dSACs send long-range inhibitory projections out of the MOB to innervate multiple downstream cortical areas, including piriform cortex and the olfactory tubercle (Kosaka and Kosaka, 2007b; 2010; Eyre et al., 2008). While low in abundance, these long-range GCL-dSAC projections may critically gate downstream cortical activity and plasticity, as recently demonstrated by the low abundance long-range inhibitory projections from entorhinal cortex to hippocampus (Melzer et al., 2012; Basu et al., 2016). The capacity of dSACs to support unique firing modes, including state-dependent burst and persistent firing (Pressler and Strowbridge, 2006, Pressler et al., 2013) further suggests that dSACs may mediate transitions in network states within the MOB.

7.4. Re-evaluating granule cell-mediated inhibition

7.4.1. Odor-evoked inhibition
In both awake and anesthetized preparations, odors frequently suppress spontaneous M/TC unit firing (e.g., see Buonviso and Chaput, 1990; Yokoi et al., 1995; Rinberg et al., 2006; Davison and Katz, 2007; Fantana et al., 2008; Bathellier et al., 2008; Cury and Uchida, 2010; Tan et al., 2010; Dhwale et al., 2010; Shusterman et al., 2011; Gschwend et al., 2015), likely via synaptic inhibition that hyperpolarizes the membrane potentials of MCs in particular (Margrie et al., 2001; Luo and Katz, 2001; Cang and Isaacson, 2003; Fukunaga et al., 2014; Kollo et al., 2014).
Odor-evoked inhibition has long been thought to arise via GC-mediated lateral inhibition driven by strongly activated heterotypic M/TCs. In this manner, “surround” M/TCs tuned to distinct but similar odors can narrow the tuning of a “center” M/TC (Mori and Shepherd, 1994; Yokoi et al., 1995). Supporting this hypothesis, application of GABA<sub>AR</sub> antagonists <i>in vivo</i> suppresses odor-evoked M/TC inhibition (Yokoi et al., 1995; Margrie et al., 2001; Tan et al., 2010) and broadens M/TC tuning (Yokoi et al., 1995; Tan et al., 2010). Indeed, GCs exhibit broader tuning than MCs (Tan et al., 2010), thus supporting the notion of an inhibitory surround, whether topographically organized (Yokoi et al., 1995) or not (Fantana et al., 2008).

Moreover, Yokoi et al. (1995) found that iontophoretic application of either bicuculline or CNQX to the EPL achieved comparable suppression of odor-evoked inhibition, consistent with the involvement of reciprocal dendrodendritic synapses in inhibiting M/TC firing. Of the GABAergic interneurons forming reciprocal dendrodendritic synapses, GCs are the most abundant (Table 7.1) and therefore constitute the most likely driver of odor-evoked inhibition.

However, as discussed elsewhere (e.g., see Cleland and Sethupathy, 2006), GABA<sub>AR</sub> antagonists are in no way specific to GCs. Further, as often applied to the surface of the MOB during <i>in vivo</i> experiments, GABA<sub>AR</sub> antagonists are far more likely to influence PGC- and sSAC-mediated inhibition. Moreover, while Yokoi et al. (1995) observed similar relief of odor-evoked inhibition with bicuculline or CNQX, they observed no effect with AP5—a result inconsistent with the known synaptic properties of GCs (see above). Additionally, odors frequently evoke MC inhibition even at low concentrations, which is seemingly incompatible with the higher odor concentration thresholds observed in GCs than in MCs (Tan et al., 2010).

Finally, GCs are highly abundant but exhibit relatively low output divergence (Kato et al., 2013; Miyamichi et al., 2013; see above), refuting any arguments based on numerical superiority. Evidence for GC-mediated odor evoked M/TC inhibition is thus currently lacking.

Recently, Schaefer and colleagues strikingly demonstrated that optogenetic inhibition of inframitral GABAergic interneurons (including GCs and dSACs) does not significantly influence spontaneous M/TC or odor-evoked TC firing rates (Fukunaga et al., 2014; though see Gschwend et al., 2015), further arguing against GC-mediated odor-evoked inhibition. Consistent with this finding, Arenkiel et al. (2007) found that odor-evoked M/TC unit firing rates are not significantly affected by broad optogenetic activation of surrounding MCs using transgenic Thy1-ChR2 mice (in which ChR2 expression is heavily enriched in MCs). These <i>in vivo</i> findings are further supported by <i>in vitro</i> studies demonstrating that: 1) lateral dendrite-targeting inhibition (such as at most M/TC–GC synapses) is ineffective in modulating somatic MC firing (Lowe, 2002; McIntyre and Cleland, 2016), 2) micro-surgical dissection through the superficial GCL does not enhance OSN stimulation-evoked M/TC firing (Lagier et al., 2004), and 3) lateral inhibition under even the most conducive conditions of M/TC co-activation mediates only moderate firing rate changes that are computationally more likely to effect M/TC decorrelation than strict odor tuning (Arebian et al., 2008; Giridhar et al., 2011; Geramita et al., 2016).

If GCs do not mediate odor-evoked inhibition, then which interneurons do? Cleland and colleagues insightfully advanced PGCs as likely candidates (Cleland and Sethupathy, 2006; for review, see Cleland, 2010), and PGC-mediated odor-evoked inhibition has since emerged in other models accurately reproducing M/TC activity (Cleland and Linster, 2012; Fukunaga et al., 2014).
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2012). Supporting this hypothesis, PGCs are recruited at lower sensory input levels than both MCs and GCs (Gire and Schoppa, 2009; Tan et al., 2010; Kikuta et al., 2013), and can thereby powerfully regulate glomerular activation (Gire and Schoppa, 2009). Moreover, Shipley and colleagues have importantly provided the first definitive demonstration that JGCs mediate powerful and rapid feedforward inhibition onto MCs (Shao et al., 2012; 2013), which, together with the minimal direct influence of sSACs on MCs (Banerjee et al., 2015), confirms classical ultrastructural predictions of PGC-mediated feedforward inhibition onto MCs (Pinching and Powell, 1971b). Finally, Schaefler and colleagues have recently provided direct evidence for PGC-mediated odor-evoked inhibition of MCs, demonstrating that optogenetic inhibition of GAD65-expressing PGCs, but not inframitral interneurons, suppresses odor-evoked MC inhibition (Fukunaga et al., 2014).

What does PGC- vs. GC-mediated odor-evoked inhibition mean for sensory processing in the MOB? The most striking corollary of this debate emerges from the distinct glomerular organization of PGCs and GCs. Specifically, the limited overlap of homotypic MC lateral dendrites (Kikuta et al., 2013; Ke et al., 2013) strongly suggests that homotypic MCs primarily interact with distinct populations of GCs and therefore distinct interglomerular circuits. GC-mediated odor-evoked inhibition would thus predict that an odor would only inhibit a subset of the MCs connected to a given glomerulus. In contrast, while connectivity within a glomerulus is heterogeneous and not all-to-all (in particular, see above), the recurrent nature of glomerular synaptic interactions typically produces glomerulus-wide excitatory (Carlson et al., 2000; Puopolo and Belluzzi, 2001; Schoppa and Westbrook, 2001; Gire and Schoppa, 2009; De Saint Jan et al., 2009) and inhibitory (Parisa et al., 2015) events. Therefore, PGC-mediated odor-evoked inhibition would predict that all MCs connected to a given glomerulus are inhibited by an odor. Of outstanding interest, Wachowiak and colleagues have recently shown with in vivo Ca^{2+} imaging that the polarity of odor-evoked activity (i.e., excitation vs. inhibition) is indeed uniform across homotypic MCs (Economou et al., 2016). While this uniform polarity may arise from multiple mechanisms – including PGC, sSAC, and GL-dSAC circuitry – it very likely does not arise from GC-mediated inhibition.

Overwhelming evidence thus now points to a GL-based mechanism for odor-evoked inhibition of MC firing, likely involving PGCs and not GCs. Future investigation into how rapid PGC-mediated feedforward inhibition interacts with polysynaptic sSAC- and GL-dSAC mediated glomerular inhibition (Liu et al., 2013; Banerjee et al., 2015; Chapter 5) will be critical in dissecting more complex odor-evoked M/TC firing patterns, such as sequential excitation-inhibition and inhibition-excitation (e.g., see Shusterman et al., 2011).

7.4.2. Lateral inhibition
The reciprocal dendrodendritic synapse provides a clear and highly attractive framework for lateral inhibition (Rall et al., 1966), and lateral inhibition is central to almost all current models of sensory processing in the MOB (though see Cleland and Sethupathy, 2006). Surprisingly, however, very few studies have directly evinced EPL-based lateral inhibition (as opposed to interglomerular interactions within the GL), and consequently little is explicitly known about lateral inhibition. Lateral inhibitory currents or potentials have been observed between 10-20% of heterotypic MC pairs in vitro (Isacson and Strowbridge, 1998; Urban and Sakmann, 2002), though moderate lateral inhibition-mediated decreases in firing rate (without observable
membrane potential hyperpolarization) are observed in ~50% of heterotypic MC pairs (Arevian et al., 2008; Giridhar et al., 2011). Lateral inhibition can also occur independent of action potentials and shows strong NMDAR-dependence in the absence of extracellular Mg$^{2+}$ (Isaacson and Strowbridge, 1998).

Given this limited knowledgebase, either GCs or EPL-INs (or both) may drive lateral inhibition between heterotypic M/TCs, while other GABAergic interneurons are unlikely to contribute, given the intraglomerular nature of PGCs and the lack of strong connectivity between MCs and sSACs and GL-dSACs. Indeed, both GCs and EPL-INs are likely to exhibit action potential-independent GABA release and strong NMDAR-dependence in the absence of extracellular Mg$^{2+}$. Further, the probability of any two nearby MCs to connect to the same EPL-IN (0.52 = 0.25) is ~150-fold greater than the probability that they connect to the same GC (0.042 = 0.0016). This disparity is only partially mitigated by the ~15-fold greater abundance of GCs than EPL-INs (Table 7.1). Given that the low MC–GC connectivity rates were observed in vitro, where many long-range inputs are likely to be severed during slice preparation, it is possible that the majority of excitatory inputs to GCs originate from distantly located M/TCs. Strikingly, however, MC–GC connectivity was limited to intersomatic distances of <100 μm using transsynaptic viral tracing in vivo, while MC–EPL-IN connectivity well exceeded these distances (Miyamichi et al., 2013). In addition, any geometric arguments regarding the likelihood of detecting GC-mediated lateral inhibition (Egger and Urban, 2006) must necessarily also apply to EPL-INs, which exhibit a similar dendritic spread within the EPL.

Further investigation is thus needed to evaluate the role of GCs in lateral inhibition. At present, however, high connectivity rates make a strong argument for the involvement of EPL-INs in M/TC lateral inhibition. Reproducing the experimental paradigms of Isaacson and Strowbridge (1998) or Urban and Sakmann (2002) with judicious application of cell type-selective opto- or chemogenetic inhibition of GCs and/or EPL-INs may help resolve this uncertainty.

7.4.3. Recurrent inhibition

Both in vitro and in vivo studies have extensively characterized the properties of recurrent inhibition in MCs, including its: 1) voltage dependence and firing rate relationship (Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Margrie et al., 2001; Xiong and Chen, 2002; Halabisky and Strowbridge, 2003), 2) N- and P/Q-type CaV dependence (Isaacson and Strowbridge, 1998; Isaacson, 2001; Isaacson and Vitten, 2003), 3) combined AMPAR and NMDAR dependence in physiological Mg$^{2+}$ (Isaacson and Strowbridge, 1998; Halabisky and Strowbridge, 2003; Maher and Westbrook, 2005), 4) slow time course, including relative to MC lateral dendrite Ca$^{2+}$ transients (Isaacson and Strowbridge, 1998; Schoppa and Westbrook, 1999; Margrie et al., 2001; Dietz et al., 2011), 5) action potential independence in physiological Mg$^{2+}$ (Isaacson and Strowbridge, 1998; Halabisky et al., 2000; Isaacson, 2001; Isaacson and Vitten, 2003; Dietz and Murthy, 2005; Dietz et al., 2011), 6) acceleration with A-type K$^+$ channel blockade (Schoppa and Westbrook, 1999; Kapoor and Urban, 2006), 7) modulation by GABAA$\beta$R regulation of GABA release (Isaacson and Vitten, 2003), 8) shunting effect (Halabisky and Strowbridge, 2003), 9) dependence on NMDAR-mediated Ca$^{2+}$ flux (Halabisky et al., 2000; Chen et al., 2000), 10) role in regulating action potential attenuation within lateral dendrites (Margrie et al., 2001; Xiong and Chen, 2002; Lowe, 2002), 11) short-term depression (Dietz and Murthy, 2005), and 12) postnatal maturation (Dietz et al., 2011). As with odor-evoked inhibition and lateral inhibition, however,
there is strikingly little direct evidence from these studies that GCs are the major interneurons mediating recurrent inhibition onto MCs. Indeed, as summarized by Schoppa and Urban (2003): “Because granule cells outnumber periglomerular cells, it is generally assumed that most of the inhibition is derived from the former”. As discussed throughout the present review, however, the greater abundance of GCs has not only been overestimated, but assessments of connectivity and physiology are just as critical to consider.

M/TC recurrent inhibition may be mediated by GCs, EPL-INs, and/or PGCs. The strongest mechanistic evidence supporting the contribution of GCs to recurrent inhibition comes from two studies that manipulated the excitatory or inhibitory synaptic input onto GCs. First, Abraham et al. (2010) used a viral strategy to enhance synaptic Ca\(^{2+}\) flux into GCs and observed a significant increase in the strength of recurrent inhibition. While thus suggesting a direct link between GC excitation and recurrent MC inhibition, multiple caveats must be considered. First, the viral strategy used was not selective to GCs, and thus likely influenced the activity of many surrounding dSACs – the functional consequences of which are difficult to predict. Second, this manipulation added to the basal level of recurrent inhibition, and thus does not directly show that GCs are sufficiently active under basal conditions to contribute to recurrent inhibition. Indeed, attenuating synaptic Ca\(^{2+}\) flux into GCs did not significantly decrease the strength of recurrent inhibition. Likewise, Fukunaga et al. (2014) demonstrated that optogenetic suppression of GCs reduced the frequency of observing large recurrent IPSPs, but did not grossly impact the overall strength of MC recurrent inhibition in vitro. In the second study, Nunes and Kuner (2015) used a viral strategy to ablate inhibitory synaptic input selectively to GCs via deletion of GABA\(_A\)R\(\beta3\) – which is not expressed in dSACs (Eyre et al., 2012) – and observed a significant increase in the strength of recurrent inhibition. As above, however, this finding does not directly show that GCs are sufficiently active under basal conditions to contribute to recurrent inhibition. Moreover, the timecourse of recurrent inhibition observed by Nunes and Kuner (2015) was markedly faster than recurrent inhibition observed elsewhere, complicating the interpretation of their results. Nevertheless, these studies strongly suggest that GCs can contribute to recurrent inhibition. Of note, however, these results also suggest that other interneurons likewise contribute to recurrent inhibition.

The remarkably high rate of reciprocal EPL-IN–MC connectivity observed in vitro (Huang et al., 2013; Kato et al., 2013) and in vivo (Miyamichi et al., 2013) confirms a clear role for EPL-INs in recurrent inhibition. Of note, however, the dependence of recurrent inhibition in physiological Mg\(^{2+}\) on both AMPARs and NMDARs (Isaacson and Strowbridge, 1998; Halabisky and Strowbridge, 2003) argues against recurrent inhibition being mediated exclusively by EPL-INs, as these cells exhibit strong Ca\(^{2+}\)-permeable AMPAR dependence with little contribution from NMDARs (Kato et al., 2013). Thus, EPL-INs are likely to substantially contribute to the AMPAR-dependent portion of recurrent inhibition.

The contribution of PGCs to recurrent inhibition, while long predicted on ultrastructural grounds (Pinching and Powell, 1971b), has nevertheless only been considered as an afterthought at best. Two recent studies strongly argue for a contribution of PGCs to recurrent inhibition, however. First, as discussed above, Shipley and colleagues have provided the first definitive demonstration that JGCs (later confirmed to be PGCs) mediate powerful and rapid feedforward inhibition onto MCs (Shao et al., 2012; Banerjee et al., 2015). Second, De Saint Jan and colleagues have
recently demonstrated that individual MCs, TCs, and ETCs alike can activate PGCs to evoke “lateral” inhibition of homotypic MCs, TCs, and ETCs and likely also recurrent inhibition (Najac et al., 2015), though this remains to be directly shown. These two studies were also recently complimented (albeit indirectly) by Schaefer and colleagues, who demonstrated a pronounced reduction in MC recurrent inhibition in vivo upon surface application of gabazine and muscimol, a perturbation that likely influences superficially located PGCs more strongly than the deeper GC circuitry (Fukunaga et al., 2012).

Therefore, while GCs almost certainly contribute to recurrent inhibition, the prevailing view that they are the only (or even predominant) interneuron doing so is thus far unsubstantiated. Of particular note, however, this issue may be readily resolved in vitro with single MC recordings and either: 1) judicious use of cell type-selective opto- or chemogenetic inhibition, or 2) careful sectioning of MC apical dendrites together with application of Ca$^{2+}$-permeable AMPAR antagonists.

7.5. Concluding remarks and future directions

Here, I have defined the main GABAergic interneurons of the MOB and reviewed several recent findings that collectively challenge the dogma of GC centrality. Importantly, the goal of this review was not to discount the importance of GCs to olfaction, but rather, to motivate further investigation (particularly using cell type-selective manipulations) into how GCs and other GABAergic interneurons influence sensory processing in the MOB.

The arguments of this review may be concisely summarized as follows: 1) odor-evoked inhibition is mediated primarily by PGCs, and secondarily by sSACs and possibly GL-dSACs, 2) lateral inhibition is far more likely to be mediated by EPL-INs than GCs, and 3) PGCs, EPL-INs, and GCs all contribute to recurrent inhibition. Of note, however, there are of course other aspects of GABAergic signaling in the MOB not considered here in which GCs are expected to take a lead role. In particular, substantial evidence suggests that GCs (and particularly adult-born GCs) are centrally involved in MOB plasticity and learning (for review, see Lazarini and Lledo, 2011). However, even here it is important to note that other GABAergic interneurons of the MOB are also critically involved, as PGCs likewise exhibit continuous neurogenesis (for review, see Adam and Mizrahi, 2010), sSACs exhibit pronounced experience-dependent changes in TH expression (for review, see Cave and Baker, 2009), and EPL-INs and dSACs regulate the activity-dependent integration of adult born GCs (Arenkiel et al., 2011; Pallotto et al., 2012; Garcia et al., 2014; 2016). An integrative approach that not only considers each of the interneuron types in unison, but also factors in connectivity and physiology together with cell abundances, will thus be crucial in advancing our understanding GABAergic signaling in the MOB going forward.
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