OLFACOTORY BULB SYNCHRONY: SPATIALLY LOCALIZED COINCIDENT INHIBITION OF MITRAL CELLS BY GABAERGIC MICROCIRCUITS

By

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*We also certify that written approval has been obtained for any proprietary material contained herein.
For my first mentor Rom Harré who instilled in me a passion for understanding the mind, the world, and our place in them.
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# List of Abbreviations

2-P – two-photon microscopy  
5-HT – serotonin (5-hydroxytryptamine)  
ACh – acetylcholine  
ACSF – artificial cerebral spinal fluid  
AMAPR – AMPA receptor  
AMPA – alpha-amino-3-hydroxy-5-methyl-isoxazolepropionic acid  
AP – action potential  
cAMP – 3’-5’cyclic adenosine monophosphate  
Ch2R – channel rhodopsin  
CIF – cumulative intensity function  
CNO – clozapine-N-oxide  
CREB – cAMP response element-binding protein  
DREADD – designer receptor exclusive activated by designer drug  
EPL – external plexiform layer  
EPSC – excitatory post synaptic current  
EPSP – excitatory post synaptic potential  
ERK – extracellular-signal-regulated-kinase  
GABA – gamma-Aminobutyric acid  
GABAR – GABA receptor  
GC – granule cell  
GCL – granule cell layer  
iGluR – ionotropic glutamate receptor  
IN – interneuron  
ING – interneuron-network gamma  
IPL – inner plexiform layer  
IPSC – inhibitory post synaptic current  
IPSP – inhibitory post synaptic potential  
LFP – local field potential  
LLD – long lasting depolarization  
LOT – lateral olfactory tract  
MC – mitral cell  
MCL – mitral cell layer  
mGluR – metabotropic glutamate receptor  
MOB – main olfactory bulb  
NMDA – N-methyl-D-aspartate  
NMDAR – NMDA receptor  
OB – olfactory bulb  
OGB-1 – oregon green bapta – 1
List of Abbreviations (cont.)

ON – olfactory nerve
OSN – olfactory sensory neuron
PG – periglomerular
PING - pyramidal-interneuron-network gamma
PV – parvalbumin
TC – tufted cell
TPH – tryptophan hydroxylase
Olfactory Bulb Synchrony: Spatially Localized Coincident Inhibition of Mitral Cells by GABAergic Microcircuits

Abstract

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Activity in the mammalian olfactory bulb (OB) has been long known to oscillate in response to olfactory stimulus. Oscillations in neural substrates are thought to represent synchronous activity of underlying neurons. Due to the circuitry and cytoarchitecture of the OB, the primary neurons, mitral cells (MCs), have been long thought to participate in lateral inhibition that would improve contrast during odor sensing. Activating large ensembles of MCs in an acute slice of the OB with serotonin (5-HT) recruited an increase in inhibitory events onto MCs, surprisingly without a concomitant increase in inhibitory cell action potential rates. The increase in MC inhibitory rate was used to probe the lateral extent to which synchronous inhibition can be found amongst MCs. Contrary to what might be expected due to cell morphology, only nearby MCs share coincident inhibition above levels expected by chance. Directly activating MCs’ inhibitory partners, granule cells (GCs), using electrical stimulation evokes synchronous inhibition above chance levels in MC pairs that did not have not have significant synchronous inhibition in 5-HT. Simulating the temporal jitter associated with the cellular processes of neurotransmitter release indicated that both 5-HT and electrical stimulation recruit divergent inhibition from spiking interneurons. Due to differences in the amplitude distribution of inhibitory events between 5-HT and electrical stimulation it is possible that these two conditions recruit distinct populations of
inhibitory synapses or interneurons. Driving GCs through NMDA receptors by removing the tonic blockade of Mg$^{2+}$ caused an increase in inhibitory events on MCs similar to that found with 5-HT. Under this experimental condition synchronous inhibition between MCs was not detectable.
Chapter 1 – Introduction

The olfactory bulb (OB) functions as the first-order processing center of olfactory information. Its accessibility has made it a structure of study over many years. A description of the cytoarchitecture and circuitry of the OB is a necessary first step to understanding the effects that synchrony shared between principle cells of the bulb would have. A majority of the findings presented herein depend upon the use of a neuromodulator, serotonin. A thorough discussion of the known effects of all neuromodulators, their known receptor locations, afferents, and cellular effects will be presented to give context to the findings of this work. The link between network oscillations and cellular synchrony has often been at the center of theoretical work concerning neuronal function. Present during odor sensing, oscillations typify the behavior of the OB. An examination of their known and theorized properties and experimental findings on OB synchrony are a critical component of an introduction to the present work.

§ 1 – Cytoarchitecture and circuitry of the mammalian olfactory bulb

The mammalian main olfactory bulb (MOB or OB) is a paleocortical laminated structure (Fig. 1-1) composed of three cellular layers: the glomerular layer, the mitral cell layer (MCL), and the granule cell layer (GCL) and two molecular layers, the external plexiform layer (EPL) and the internal plexiform layer (IPL) (Shepherd, 2004).
**The glomerular layer**

The glomerular layer is the outermost layer of the OB and is composed of many hundreds of spherical glomeruli, each receiving innervation from an unique subtype of olfactory sensory neuron (OSN) from the nasal epithelium having passed through the cribriform plate (Vassar et al., 1994; Mombaerts et al., 1996). Within the glomerular layer exist the somata of populations of juxtaglomerular cells: periglomerular and short axon cells (Pinching and Powell, 1971). Both of the two cell types are inhibitory cells (Shepherd, 2004; Shipley et al., 2008). Periglomerular cells typically project a dendrite arborization into a single glomerulus where they synapse with multiple partners, receiving excitation from external tufted cells in addition to OSNs. They in turn inhibit mitral cells (MCs), external tufted cells, and the OSN axons through dendritic release of vesicles filled with the neurotransmitter gamma-aminobutyric acid (GABA). The axon of inhibitory juxtaglomerular cells remains within the glomerular layer and projects to a glomerulus dissimilar from its dendritic arbor, up to five glomeruli away. (Pinching and Powell, 1971, 1972).

**The external plexiform layer**

The next deepest layer of the OB is the external plexiform layer (EPL). Within the EPL reside several cell types including both excitatory projection neurons the middle tufted cells (TC) and inhibitory interneurons known as Van Gehuchten cells (Ramón y Cajal, 1909; Shepherd, 2004). Middle tufted cells have been found to synapse onto more superficial granule cells (Mori et al., 1983; Orona et al., 1983) than MCs albeit in a similar dendrodendritic fashion as MCs (Ezeh et al., 1993; Christie et al., 2001). Their cortical projection patterns are similar to those of MCs but restricted to the rostral
structures of piriform cortex (Haberly and Price, 1977; Scott et al., 1980; Nagayama et al., 2010), however it is thought that their information content may be different (Mori et al., 2013) as only tufted cells are known to form associational connections within the bulb that traverse from one side of the bulb to the other, e.g. dorsal tufted cells sending axon collaterals to the ventral side (Schoenfeld et al., 1985). The degree to which middle tufted cells and MCs inhibit each other laterally through a granule cell (GC) intermediary has been poorly studied, but some reports have indicated that such interactions are possible (Mori et al., 1983). Parvalbumin positive (PV+) interneurons, which resemble Van Gehuchten cell drawings, have wide dendritic processes that appear to tile the EPL (Kato et al., 2013; Miyamichi et al., 2013). The cells are excited by MC activity and receive numerous spontaneous excitatory post synaptic potentials (EPSPs) when recorded in the patch-clamp configuration in a slice. They have been proposed to linearly regulate the population activity of MCs in a given bulbar region (Kato et al., 2013). A population of corticotrophin releasing hormone positive (CRH+) cells have also been reported in the EPL. These interneurons appear to resemble PV+ interneurons both morphologically and electrophysiologically (Huang et al., 2013), but a definitive double stain has not been performed.

**The mitral cell layer**

The mitral cell layer (MCL) contains the somata of the primary cell of the OB, the mitral cell (MC). Mitral cells project a large apical dendrite into the glomerular layer where they ramify within a single glomerulus (Ramón y Cajal, 1909; Shepherd, 2004). In addition to their apical dendrites MCs send out lateral dendrites into the deep external plexiform layer. Mitral cells project axons to the subependymal zone where they
fasciculate to form the lateral olfactory tract. It is unknown if any interneurons make axo-
somatic inhibitory connections with MCs, but some have reported that axons of GCL
Golgi, but particularly Cajal, and horizontal cells will project axons through and to the
MCL (Schneider and Macrides, 1978).

The granule cell layer

The granule cell layer is composed of somata of granule cells (Ramón y Cajal,
1909; Shepherd, 2004), Blanes cells (Pressler and Strowbridge, 2006), Golgi cells
(Pressler et al., 2013), horizontal cells, verticle cells of Cajal, bi-tufted neurons, deep
stellate cells, and deep short axon interneurons (Schneider and Macrides, 1978; López-
Mascaraque et al., 1986; Eyre et al., 2008; Kosaka and Kosaka, 2011). Most of these cells
were identified by Cajal in his seminal studies of the nervous system (Ramón y Cajal,
1909; Shepherd, 2004). Granule cells are axonless interneurons that project a short basal
dendrite deeper into the granule cell layer and an apical dendrite into the external
plexiform layer where it arborizes and forms dendro-dendritic synapses with the lateral
dendrites of MCs. Superficial GCs and deeper GCs have been observed forming distinct
patterns of apical dendrite projections. Superficial GCs project to the superficial portion
of the EPL, while deeper GCs have larger dendrite trees and occupy the deeper areas of
the EPL (Orona et al., 1983). Blanes cells are known to inhibit GCs (Pressler and
Strowbridge, 2006), but it is unknown if they target other neurons of the bulb. Golgi cells
have only been recently recorded from and their function is unknown (Pressler et al.,
2013).
**Centrifugal inputs of the olfactory bulb**

Several brain regions project centrifugally into the OB, including the olfactory tubercle, piriform cortex, accessory olfactory nucleus, locus coeruleus, and the raphe nucleus (Shepherd, 2004). Projections of the piriform cortex synapse onto the apical dendrite of granule cells in two distinct locations, one proximal to the granule cell soma, and one distal. These two locations appear to have different post-synaptic receptor complements, where the proximal sites are the only known synapse capable of long term potentiation within the OB (Gao and Strowbridge, 2009). Additional axon collaterals from piriform cortex target periglomerular cells in the glomerular layer. Noradrenergic projections from locus coeruleus are known to project to the external plexiform layer where they synapse onto GC apical dendrites in addition to MC lateral dendrites, as well as project to the glomerular layer where they form synapses with the juxtaglomerular cells. Serotonergic projections of the raphe nucleus have been found within the granule cell layer and within the glomerular layer (Shepherd, 2004).

**Inhibition and bulbar input and output**

The canonical circuit of the bulb, that between GCs and MCs, has been extensively studied due to its unique nature, and its proposed role in refining the olfactory sensory space. The synapse between MCs and GCs is formed at the lateral dendrite of MCs and the distal end of the GC apical dendrite in the external plexiform layer (Fig. 1-2A). It is reciprocal in nature, where MCs release glutamate into the synaptic cleft and activate a complement of AMPA and NMDA ionotropic receptors on GCs, in addition to metabotropic glutamate receptors (mGluRs) (Sahara et al., 2001; Dong et al., 2007; Heinbockel et al., 2007). Ca$^{2+}$ enters through both NMDA receptors and t-type calcium
channels and elicits GABA release into the synaptic cleft inhibiting the partner MC (Nicoll, 1971; Isaacson and Strowbridge, 1998; Chen et al., 2000). The phenomena of lateral inhibition where GCs release GABA into neighboring synapses from that which was directly activated was first hypothesized based upon the anatomy of the OB (Rall et al., 1966; Woolf et al., 1991; Scott et al., 1993) and has since been noted experimentally (Yokoi et al., 1995; Margrie et al., 2001; Arevian et al., 2008). It is thought to serve to refine the sensory space by reducing noise and refine the tuning map of MC activation profiles (Yokoi et al., 1995; Laurent, 1999). Some have suggested that the cortical projections onto GCs serve as a prediction of the cortex and that the bulb acts as an interferometer by comparing the real inputs to the predicted inputs from cortex (Schoenbaum and Eichenbaum, 1995; Haberly, 2001).

Inhibition of OB inputs in the glomerular layer have been seen as a means of modulating the gain on the chemo-sensory input the brain receives through the olfactory system. When multiple OSNs are activated, the strongest will inhibit their neighbors through inter-glomerular inhibition mediated by periglomerular cells (Urban and Sakmann, 2002; Aungst et al., 2003). In this way the system does not become saturated with a cacophony of inputs, however a test in vivo reported only weak interglomerular inhibition, but robust intraglomerular inhibition (McGann et al., 2005). Interestingly, some periglomerular cells appear capable of co-transmitting dopamine with GABA, which has been hypothesized to enhance their inhibition of their targets through D2 receptors present on the post-synaptic targets (Kosaka et al., 1985; Maher and Westbrook, 2008). External tufted cells circuit properties have only recently been studied in earnest. It has been reported that TCs respond to ON stimulation with larger
depolarizations than MCs (Gire et al., 2012). Axon collaterals of external tufted cells project to the IPL and with some, but not all cells projecting axon collaterals to the lateral olfactory tract. Recent work has identified external tufted cells as the target of periglomerular cell inhibition during long-lasting depolarizations (LLD) (Carlson et al., 2000; Hayar et al., 2004a, 2005; Jan et al., 2009; Whitesell et al., 2013).

§ 2 – Neuromodulation of olfactory bulb circuits

Several neuromodulators of bulbar activity have been identified including dopamine, noradrenaline, acetylcholine, and serotonin (Halász and Shepherd, 1983). Their activity within the OB has been linked to varying extents to memory formation, odor discrimination, odor habituation, altered odor coding, and input gain modulation. Often the cessation of their activity within the bulb results in anosmic or hyposmic animals highlighting the critical role that neuromodulators play in maintaining homeostasis within the olfactory system’s first processing point.

Serotonin

Early work in the OB of mammals that examined the presence of neuromodulatory innervation found that serotonergic fibers of the dorsal raphe were present in great numbers throughout all layers of the OB, most densely in the glomerular layer (de Olmos et al., 1978; Macrides et al., 1981; Shipley and Adamek, 1984; McLean and Shipley, 1987a, 1987b).

The serotonergic system of the mammalian central nervous system is composed of tryptophan hydroxylase (TPH) positive cells of the raphe nucleus. These TPH cells
project widely throughout the central nervous and enteric systems and have been reduced to two broad classes based upon their location within the raphe. The caudal raphe regions compose the serotonergic cells that project to the lower portion of the body, including the midbrain, hindbrain and spinal cord. The telencephalon and portions of the mesencephalon is innervated by the dorsal and medial raphe.

Serotonin, or 5-hydroxytryptamine (5-HT), is a neurotransmitter formed by tryptophane hydroxylase and amino acid decarboxylase as a modified form of the amine tryptophan. The neurotransmitter binds to transmembrane receptors, of which there are four identified classes with subtypes. 5-HT1 receptors are G-protein coupled receptors to the G\textsubscript{i} pathway and have been detected widely throughout the central nervous system (Hoyer et al., 1994; Nichols and Nichols, 2008). At the terminals of serotonergic cells they act as auto-inhibitors of release. On postsynaptic targets the receptors decrease firing probabilities when activated. 5-HT2 receptors are g-protein coupled receptors that utilize the G\textsubscript{q} pathway(Hoyer et al., 1994). They serve to enhance excitation of postsynaptic cells by raising cAMP levels and intracellular calcium through IP3 mediated release mechanisms from internal stores. Through this mechanism when activated they potentiate glutamatergic signaling (Nichols and Nichols, 2008). 5-HT2 receptor family members are found widely throughout the telencephalon. 5-HT3 receptors are ionotropic receptors that are cation non-specific (Nichols and Nichols, 2008). The final class of serotonin receptors 5-HT4 are G\textsubscript{s} coupled receptors (Hoyer et al., 1994; Nichols and Nichols, 2008) and are closely linked to 5-HT2Rs with similar, though weaker, staining patterns.
Serotonergic terminals are not found at tight synaptic junctions, but in general have a more pronounced distance (>20 nm) in the synaptic cleft allowing for more spillover into the parenchyma. Evidence for asymmetric synapses have additionally been observed (Descarries et al., 1975). Functionally, the serotonin system has been linked to several classes of behaviors, systems and disorders, including feeding, mood, attention, motivation, maternal instinct, depression, anxiety, obsessive compulsive disorder, disorders of energy balance, autism, and schizophrenia (Müller and Jacobs, 2010).

Autoradiographic studies of 5-HT1 and 2 receptors identified binding sites for both receptor subtypes in the external plexiform and interior granule cell layers of the OB (Pazos and Palacios, 1985; Pazos et al., 1985). In situ hybridization studies of the 5-HT2A and 2C receptors found that their mRNA was being transcribed in the glomerular, external plexiform, internal granular and the mitral cell layers. The 5-HT2A receptor staining was strongest in the MC layer and was infrequent in the granule cell layer (Mengod et al., 1990; Pompeiano et al., 1992, 1994; Wright et al., 1995). Later immunohistochemical staining confirmed that MCs strongly expressed 5-HT2A receptors and that expression was seen tufted cells (Hamada et al., 1998; Wu et al., 1998; Yuan et al., 2003). Immunohistochemical staining of GCs was sparse and infrequent.

Selective deafferentation of 5-HT in adult rats results in anosmia within 4 weeks (Moriiizumi et al., 1994). One day following either 5-HT depletion or antagonism of 5-HT2 receptors in neonates odor preference learning is abolished (McLean et al., 1993, 1996). Agonism of 5-HT2 receptors by global DOI administration, as a replacement for an unconditioned stimulus, was not sufficient to induce odor preference learning (Price et al., 1998). The role of serotonin in odor preference learning appears to be permissive of
noradrenergic mediated mechanisms (Langdon et al., 1997); this permissivity was linked to regulation of cAMP in MCs by 5-HT2 receptors (Yuan et al., 2003). Studies of serotonin’s role in adult mammalian olfaction has been limited to 5-HT4 receptor agonism and global antagonism, reflecting an enhancement of social recognition and an impairment in a go/no-go olfactory task (Letty et al., 1997; Marchetti et al., 2000). One study in bees demonstrated that ectopic application of serotonin reduced memory storage and retrieval of an odor (Menzel et al., 1990). A recent study in fruit bats demonstrated a role for bulbar serotonin in olfactory memory formation, whereby a dose dependent effect of serotonin depletion decreased recognition of a novel odor associated with positive reward. This change in memory formation was accompanied by decreases in ERK and CREB signaling locally within the OB (Ganesh et al., 2010).

Cellular changes in both extracellular units and intracellular recordings have been sparsely reported in the literature. In a series of papers in the mid 1960’s electrophoretic application of a saturated serotonin solution was reported to reduce MC unit spiking rates (Baumgarten et al., 1963; Bloom et al., 1964; Salmoiraghi et al., 1964). Whole-cell intracellular recordings of MCs have shown a direct depolarizing and indirect hyperpolarizing effect of serotonin bath application in an acute slice (Hardy et al., 2005). Periglomerular and juxtaglomerular interneurons, as well as external tufted cells have also shown to be directly depolarized by serotonin bath application (Hardy et al., 2005; Petzold et al., 2009; Liu et al., 2012). Stimulation of the raphe diminishes the excitation of glomerular activity by increasing periglomerular cell generated inhibition of OSNs (Petzold et al., 2009). The effects of serotonin on OB function in the processing of odorant information and the MC-GC circuit remain poorly understood.
**Dopamine**

Both endogenous dopamine sources and exogenous projections from the substantia nigra to the OB have been characterized (Kosaka et al., 1985; Lévy et al., 1999). Juxtaglomerular cells contain a subpopulation that is tyrosine hydroxylase positive and co-release dopamine with GABA onto their target mitral/tufted cells and OSN terminals (Kosaka et al., 1985; Borisovska et al., 2013; Liu et al., 2013) that contain pace making properties and persistent sodium currents (Pignatelli et al., 2005; Puopolo et al., 2005). Unlike other neuromodulatory systems isolating the OB from other CNS structures does not abolish the content of dopamine within the OB (Macrides et al., 1981).

Both D1 and D2 receptors have been detected within the OB. D1 receptors have been detected within the granule cell layer and in the glomerular layer (Boyson et al., 1986; Mijnster et al., 1999), though in higher mammals not within the glomerular layer (Camps et al., 1990). D2 receptors have been found moderately in the glomerular layer and weakly in the granule cell layer of most mammals (Boyson et al., 1986; Charuchinda et al., 1987; Camps et al., 1990). D2 receptors have been localized to OSN terminals within the glomerular layer (Nickell et al., 1991; Koster et al., 1999), as well as MCs and periglomerular cells (Gutièrrez-Mecinas et al., 2005).

Studies have shown that this dopamine acts on D2 receptors and dampens the excitability the OSNs reducing the excitation of MCs to olfactory nerve stimulation (Hsia et al., 1999; Berkowicz and Trombley, 2000). Dopamine has also been shown to act on GCs by reducing their GABA channel Cl⁻ conductance, while increasing MC GABA channel Cl⁻ conductance (Brünig et al., 1999). Similar findings in the frog extended this
work: a decrease in GC excitatory response to LOT stimulation that was D2 dependent, as well as a decrease in MC Ca\(^{2+}\) influx in lateral dendrites with D2 activation (Davison et al., 2004). Recent studies have elucidated some of the effects of juxtaglomerular cell’s dopamine co-transmission with GABA on glomerular circuits. GABA hyperpolarization activates \(I_h\) currents that are enhanced by D1 receptor activation on external tufted cells (Liu et al., 2013).

**Noradrenaline**

Noradrenergic terminals’ greatest density were found in the external plexiform layer and MC with only weak signal within the granule cell layer and sparse to absent in the glomerular layer, though other studies have identified greater staining in the granule cell layer (Jaffé and Cuello, 1980; Nadi et al., 1981; Shipley et al., 1985; McLean et al., 1989; McLean and Shipley, 1991). Using in situ hybridization \(\alpha1A\) was found within throughout the MC and granule cell layer and only sparsely on the deeper cells surrounding the glomerulus. \(\alpha1B\) receptor mRNA was found in the MC, cell bodies of the external plexiform layer and within the glomerular layer. \(\alpha1D\) receptor was found densely in the granule cell layer and only weakly in the mitral cell and glomerular layer (Day et al., 1997). Receptor staining has identified extensive staining of the \(\alpha2C\) receptor in the granule cell layer and moderate staining in the glomerular layer (Nicholas et al., 1993a). \(\beta\) receptor has been identified most strongly on MCs, some juxtaglomerular cells, within the granule cell layer and external plexiform layers (Wanaka et al., 1989; Nicholas et al., 1993b; Yuan et al., 2003).

Behavioral studies of the role of noradrenaline in the OB have found that antagonism of \(\beta\) receptors affected maternal identification of newborns (Lévy et al.,
Blockade of either \( \alpha_1 \) or \( \beta_1 \) receptors has been shown to interfere with fine odor discrimination (Doucette et al., 2007). Cell culture experiments recording from putative GCs have found that glutamatergic signaling is negatively attenuated by application of norepinephrine reducing inward currents, likely through \( \alpha_2 \) receptors. This same study demonstrated an \( \alpha_2 \) receptor mediated reduction spontaneous IPSCs in MCs with the addition of norepinephrine or clonidine. Co-applying GABA and norepinephrine did not antagonize GABA alone. Authors suggested that this reflected at a presynaptic site of action for the reduction in spontaneous IPSCs (Trombley and Shepherd, 1992). This model was further refined when one of the original authors identified an \( \alpha_2 \) mediated reduction in MC calcium currents, supporting the idea that reduced glutamatergic currents in GCs arose from a reduced probability of release in MCs (Trombley, 1992).

Acute slice recordings have identified a distinct role for noradrenaline signaling at the dendro-dendritic synapse between MCs and GCs. There it acts to enhance the inhibition onto MCs by increasing GC excitability and increasing chloride conductance through GABAA receptors (Gire and Schoppa, 2008). This is complicated, as GCs have both \( \alpha \)-receptor subtypes which have opposing roles the precise effects of noradrenaline upon GC physiology depends upon the concentration of noradrenaline (Nai et al., 2009, 2010). At higher concentrations of noradrenaline, \( \alpha_1 \) receptors on MCs are activated causing increased MC excitability (Nai et al., 2009).

Taken as a whole noradrenaline functions within the OB to increase network excitability, potentially leading to increased synchronization of MC spiking and oscillatory behavior (Escanilla et al., 2010).
**Acetylcholine**

Cholinergic projections into the OB from the horizontal limb of the diagonal band of Broca and to a lesser extent the lateral preoptic area and vertical limb of the diagonal band, innervate primarily the MC and the glomerular layer, though other studies later showed a greater number of projections to the granule cell layer (Shute and Lewis, 1967, 1975; Macrides et al., 1981). Early studies reported cholinergic cells in the olfactory tubercle that project primarily to MCs and distal glomeruli as part of the intermediate olfactory tract (Shute and Lewis, 1967), later studies did not report this innervation (Macrides et al., 1981; Záborszky et al., 1986). Receptor staining using radiographic binding within the OB first identified heavy staining for muscarinic receptors m1 and m2 in all layers (Le Jeune et al., 1995) and in situ hybridization for nicotinic receptors (β2) primarily on MCs, but also weak and intermittent staining in the external plexiform (α2, α3, α4, β2), glomerular (α4), and internal plexiform (α2, α3), granule cell layers (α4) (Wada et al., 1989; Moser et al., 1996).

Studies blocking or abolishing acetylcholine (ACh) signaling have identified a functional role for acetylcholine in short term memory (Ravel et al., 1994; Devore et al., 2012), odor discrimination (Linster and Cleland, 2002; Mandairon et al., 2006), and specificity of an odor to a task (Hunter and Murray, 1989; Mandairon et al., 2006). In vivo recordings using ionmicroelectrophoretic methods have been mixed. In the first study of its effects on unit activity of bulbar neurons Bloom et al. described only suppression of unit firing following iontophoretic application of ACh (Bloom et al., 1964). One early study using unit recordings showed both inhibitory and excitatory responses from cells exposed to acetylcholine. Localization of the units saw a
predominance of inhibitory actions in juxtaglomerular responses, and a mixture of
excitatory and inhibitory responses near the MC (Ravel et al., 1987). Another study
describes increases in excitatory voltages likely mediated by MCs and decreased
inhibition (Elaagouby et al., 1991). Mitral cells depolarize through a nicotinic receptor
mediated process in the presence of an acetylcholine receptor agonist, carbachol, and in
the presence of nicotine. Of the local interneurons, only the subclass of bipolar
periglomerular cells respond to nicotine bath application (Castillo et al., 1999). Granule
cells exhibit inhibitory responses to muscarinic receptor activation (Castillo et al., 1999),
but alters the after-hyperpolarization to an after-depolarization (Pressler et al., 2007)

Taken as a whole the effects of acetylcholine appear to increase bulbar output by
increasing excitability of MCs and decreasing excitability of inhibitory neurons (Ravel et
al., 1987). A recent study concluded that cholinergic inputs introduce an excitatory bias
to odor coding in the OB (Rothermel et al., 2014). Perceptually this may increase
detection thresholds or salience of odors as signals are boosted in the output cells with
diminished reciprocal and lateral inhibition.

§ 3 –Synchrony and oscillations of the mammalian olfactory

bulb

During odor sampling in vivo local field potential recordings, a mixture of the
summed local activity of spiking, synaptic and dendritic electrical processes, and volume
conducted potentials from distant locales (Berens et al., 2010; Kajikawa and Schroeder,
2011), of the mammalian OB contain at least three distinct populations of oscillatory
behaviors: the theta, beta, gamma rhythms (Kay et al., 2009). Oscillations are generally
thought of as synchronous activity of local circuits capable of transmitting data throughout the brain. They are thus closely linked to synchronous input and output of neuronal structures.

**Theta-band oscillations**

Theta frequency band activity, 6-12 Hz, has been linked to the breathing rhythm of the animal (Buonviso et al., 2003; Martin et al., 2004). The source of the oscillation is generally believed to be a combination of synchronized ORN activation through mechanical stimulation during inhalation/exhalation (Hayar et al., 2004b) and central inputs from other brain regions (Ravel and Pager, 1990). The oscillations are tightly synchronized with breathing, however occlusion of the nares has been shown to decouple this synchronization but not completely abolish the rhythm (Ravel et al., 1987). The role of theta band oscillations in the OB can be thought of as being akin to that of theta oscillations of the hippocampus, capable of serving as a timing mechanism to relate the temporal structure of inputs. In this fashion it has been hypothesized that a single breath cycle forms the fundamental unit of information during odor sensing, as represented by a single theta cycle (Kepecs et al., 2006).

**Beta-band oscillations**

Beta band activity, 15-30 Hz, was first identified in the OB by Boeijinga and da Silva in 1989 (Boeijinga and Lopes da Silva, 1989). It has since been often studied alongside gamma band activity, in behavioral task contexts and in anesthetized animals (Chapman et al., 1998; Neville and Haberly, 2003; Beshel et al., 2007). One study has pointed to beta-band activity being associated with fine discrimination tasks (Kay and
Beshel, 2010). This has led some speculation that cortical feed-back projections dictate beta band oscillation (Bressler, 1984; Martin et al., 2007). There is some evidence that gamma and beta-band oscillations may utilize the same circuit with a rebalancing of power from gamma to beta depending odorant concentration (Neville and Haberly, 2003). This idea is supported by the mutual exclusivity between gamma-band oscillations and beta-band oscillations as they never occur together (Cenier et al., 2009; Fourcaud-Trocmé et al., 2011), however there is some evidence that MCs generate spikes preferentially during one type of oscillation and not the other giving rise to the idea that two separate circuits generate their respective oscillations (Cenier et al., 2009; Kay et al., 2009). The source of beta band oscillations remains controversial, though it is somewhat accepted that is not intrinsic to the OB circuitry. When measuring the local field potential (LFP) from multiple locations, beta oscillations are synchronous at neighboring locations, unlike gamma oscillations (Neville and Haberly, 2003).

_Gamma-band oscillations_

The nature of gamma band, 35-80 Hz, activity has been widely studied. First described by Adrian (Adrian, 1942) and extensively studied by Freeman and colleagues (Freeman, 1972, 1975; Bressler and Freeman, 1980; di Prisco and Freeman, 1985; Eeckman and Freeman, 1990; Kay and Freeman, 1998), they have been shown to be intrinsic to the MOB (Gray and Skinner, 1988; Friedman and Strowbridge, 2003; Lagier et al., 2004). In vivo, the oscillations always occur with the same phase relationship to the theta-band oscillations occurring during the falling phase of the theta oscillation at the end of inspiration (Fourcaud-Trocmé et al., 2011).
One of the first computational models of the brain proposed that gamma-band oscillations were the result of inhibitory dynamics of GCs acting upon MCs in a feedback loop (Rall and Shepherd, 1968), though experimental evidence for the source of gamma oscillations within the bulb is limited. While numerous studies have recorded gamma oscillations within the bulb (see above), few have attempted to determine the cellular basis of gamma. One of the first empirical studies to attempt to identify the basis of the oscillations examined the temporal relationship between the local field potential and action potential firing by cellular units. Through this study it was concluded that gamma oscillations of the OB could not be the result of an interaction between two oscillating populations of cells (Eeckman and Freeman, 1990).

Several investigators have attempted to generate gamma-oscillations in an OB slice with some success (Friedman and Strowbridge, 2003; Lagier et al., 2004; Gire and Schoppa, 2008; Pandipati et al., 2010). The earliest attempts utilized stimulation of either the glomerular layer (Friedman and Strowbridge, 2003) or the olfactory nerve (Lagier et al., 2004) giving a tetanic stimulation in both cases. For a period of time after the cessation of stimulation a field recording would oscillate in the low gamma-band, suggesting that a network within the OB itself resonates in the gamma band before losing the energy to synchronize as a population. In a pair of papers a research group utilized bath applied NMDA to increase basal excitability of all cells with NMDA receptors (NMDARs) and then applied norepinephrine, known to enhance network excitability, to the slice (Gire and Schoppa, 2008; Pandipati et al., 2010). In the first paper of the pair LFP data was filtered at a frequency near to the oscillation frequency of peak power making it difficult to assess the validity of the findings. In their second paper a less
aggressive high-pass filter was used and the resulting power spectrum contained a relatively enhanced gamma frequency band with increased power, but the integrated power of the entire spectrum showed that the activity levels are significantly enhanced at all frequencies forcing speculation that the experimental conditions may be pathological.

Intriguing work has demonstrated that MCs exhibit subthreshold oscillations that vary with the level of their depolarization. The peak frequency of these oscillations falling within the gamma-band (Desmaisons et al., 1999) This finding was extended in two computational models of the MOB. In the first model Galan et al. explored MC synchrony exclusively in the context of MC’s subthreshold oscillations and the inhibition they receive from GCs. Galan et al. (Galán et al., 2006) demonstrated that stochastic inhibition was a sufficient condition of synchronizing MCs spiking. The cross-spectra of their inhibitory events looking exceptionally similar to LFPs exhibiting gamma oscillations (Galán et al., 2006). The second model by Kopell, Kay and colleague explicitly sought to understand gamma oscillations as a function of MC’s subthreshold oscillations. Like Galan et al. they demonstrated that MC spiking would synchronize at gamma frequencies, but they went on to critically show that GC spiking was not necessary for this network level synchrony (Brea et al., 2009). Taken together the combined predictions were that gamma oscillations would be apparent in the MOB as the result of aperiodic graded inhibition. In this context it has been hypothesized that gamma oscillations are representative of the increased probability of MCs to fire action potentials in phase with the gamma oscillation, and that synchrony with one another is not by definition entrained to the gamma rhythm. Instead, in many instances two MCs are not synchronous with each other (Rojas-Líbano and Kay, 2008). The prediction that the
gamma oscillation can be used to infer the probability of MC firing was confirmed with
in vivo recordings from an anesthetized animal (David et al., 2009). To date, only the
Brea-Koppel-Kay model can account for the beta-gamma exclusivity in a model of OB
oscillations. The model claims that cortical feedback projections onto the GCs causing
them to spike leads to widespread inhibition that slows faster gamma-band oscillations
into the beta range; though, the authors does not explicitly state this.

The Brea-Koppel-Kay model of OB gamma oscillations contrast with the
predominant models of hippocampal and cortical gamma-band oscillations, interneuron-
network gamma (ING), and pyramidal-interneuron-network gamma (PING) respectively
(Traub et al., 1996; Cunningham et al., 2004), by arguing that interneuron
synchronization is unnecessary. Instead intrinsic properties of the principle cells dictate
the oscillation through a process that could be described as population-level resonance.
In this model, inhibition’s role in shaping the oscillations is only to reset the phase curve
of the resonating principle cells, or with greater excitation of inhibitory cells from an
exogenous source, such as piriform cortex, to govern the frequency of resonance and in
doing so slow the oscillations into the beta band. The Brea-Koppel-Kay model is similar
in some respects to the PING model, namely that in the PING model a subpopulation of
principle cells are “chattering” at high frequencies onto the interneuron network to
synchronize network activity. This closely mirrors the ideas of the Brea-Koppel-Kay
model where active MCs interact with inhibitory GCs to synchronize the network. The
critical difference between them is that synchrony occurs in the PING model through
connections between the inhibitory cells, such that the inhibition is coherent onto the
pyramidal cells, whereas in the Kopell-Kay model the population of MCs can be thought of as a noisy resonator that must be reset aperiodically by graded inhibition.

A recent study by Leopousez and Lledo (Leopousez and Lledo, 2013) described two populations of gamma oscillations, a high and a low gamma oscillation during in vivo recordings. By infusing the OB bulb with the GABAR blocker picrotoxin they increased the power of a lower frequency gamma oscillation and decreased a higher frequency oscillation. Utilizing a glutamate reuptake inhibitor, TBOA, they found they could mimic this finding suggesting that increased MC synchrony mediated through glutamate spillover (Isaacson, 1999) alters oscillatory behavior. Supporting the computation model of subthreshold oscillation dependent gamma (Brea et al., 2009), Lepousez and Lledo found now oscillations within the cross correlation of MC spiking during normal odor sensing. With mild disinhibition by picrotoxin significant oscillations in MC spike correlations were observed.

The most simplistic explanation for the data reported by Leopousez and Lledo is that their low dose of picrotoxin is disinhibiting and laterally exciting local MCs which otherwise would not have participated in the oscillation thus increasing the power of the oscillation, and entraining MC spiking to a population rhythm through positive feed-back mechanisms. What is unclear is how this would decrease the average frequency of the oscillation, though author’s data suggesting that the resonant frequency of the circuit is lowered may serve as an explanation.

*Role of bulbar oscillations in odor sensing*

Gamma-band oscillations within the OB have been hypothesized to carry odorant information through the spatial amplitude of the oscillation (di Prisco and Freeman, 1985;
Freeman and Schneider, 2007). It is not clear how this might be accomplished. Mitral cell projections to piriform cortex only sparsely excite pyramidal cells of the region alongside activation of region wide global inhibition (Poo and Isaacson, 2009; Stettler and Axel, 2009). Several experimenters have noted that gamma-band oscillations appear to be global across the whole of the OB leading to speculation that the gamma oscillation reflects the population coding of the MCs and is an emergent property of the olfactory circuit. More recent examinations of this global entrainment of the bulb to a particular gamma rhythm were not conclusive (Kay and Lazzara, 2010). This contrasts with other studies which showed that downstream limbic and hippocampal regions have oscillations weakly coherent with the MOB during odor presentation (Kay and Freeman, 1998; Vanderwolf, 2001; Csicsvari et al., 2003; Kay, 2003; Martin et al., 2007). Notably, bulbar oscillations have been shown to be modulated by stimulus properties (Cenier et al., 2008), experience (Ravel et al., 2003), and state (Freeman and Schneider, 1982).

Beta-band oscillations are highly correlated between piriform cortex and the OB (Bressler, 1984), and likely reflect top-down processing of odor information after it has been received (Kay et al., 2009). Beta-band oscillations are present during low odor concentration or fine odor discrimination tasks involving novel odorant mixtures (Neville and Haberly, 2003; Ravel et al., 2003; Kay and Beshel, 2010). As the animal is habituated to an odorant mixture beta-band oscillations grew as gamma-oscillations decreased (Ravel et al., 2003); demonstrating that oscillatory behavior can reflect odorant recognition or familiarity.
Experimental studies of synchrony in the bulb

Several groups have looked at synchrony in the bulb with the majority of studies focusing on excitatory and spiking synchrony of MCs (Isaacson, 1999; Carlson et al., 2000; Schoppa and Westbrook, 2001, 2002; Urban and Sakmann, 2002; Christie et al., 2005; Hayar et al., 2005; Ma and Lowe, 2010; Borisovska et al., 2011), and only a small handful who have examined inhibitory synchrony (Hayar et al., 2005; Schoppa, 2006a). Gap junction proteins and mRNA were found in the glomerular layer alongside robust expression in mitral cell soma (Miragall et al., 1996; Condorelli et al., 1998; Rash et al., 2000, 2005; Zhang and Restrepo, 2003; Kosaka and Kosaka, 2004; Christie et al., 2005). Shortly thereafter it was shown that electrical coupling between MCs took place in the absence of spiking and would synchronize MC spiking (Christie et al., 2005). With the loss of a cell adhesion molecule that plays a role in gap junction formation MC spike synchrony was reduced (Borisovska et al., 2011). Other studies found lateral excitation between MCs as the consequence of glutamate spillover in both the EPL and in the glomerulus (Isaacson, 1999; Carlson et al., 2000).

Excitatory synchrony, as can be mediated through gap junctions, has been observed to occur intra-glomerularly, i.e. only between MCs who share a glomerulus (Carlson et al., 2000; Urban and Sakmann, 2002; Christie et al., 2005; Hayar et al., 2005). Some of this excitatory synchrony may be from glutamate spillover (Schoppa and Westbrook, 2001; Jan et al., 2009; Gire et al., 2012). Spike synchrony and correlations in slices have also been primarily observed occurring intra-glomerularly (Hayar et al., 2004a; Christie et al., 2005; Ma and Lowe, 2010; Borisovska et al., 2011). Excitatory synchrony mediated through glutamate spillover from MC lateral dendrites is not
dependent upon MC glomerular co-projection (Isaacson, 1999). Lateral inhibition and synchronous inhibition has been observed occurring regardless of the location of a MC or TC’s apical tuft (Urban and Sakmann, 2002; Schoppa, 2006a; Arevian et al., 2008; Giridhar et al., 2011; Whitesell et al., 2013).

Synchrony between neighboring MCs’ inhibitory input was the focus of a pair of studies (Schoppa, 2006a, 2006b). In the studies olfactory nerve stimulation evoked long lasting depolarizations of MCs. With this paradigm spike synchrony within 5 ms bins was found above that in the 5-10ms bin, as well as in some of the pairs he recorded oscillations in the spike synchrony. Concurrent with the spike synchrony he reported synchronous spiking in GCs who received synchronous excitation. Schoppa argued that rebound spikes from observed synchronous inhibition precisely timed MC spiking. It is possible that the initial stimulation and slow depolarization of MCs had an effect upon measured synchrony, as correlated inputs have been shown to synchronize MCs (Galán et al., 2006).

This correlation-induced synchronization (Galán et al., 2006) was dependent upon MCs subthreshold oscillations (Desmaisons et al., 1999). Correlated inputs given to MCs would see them reset their membrane potential oscillation such that they were more likely to spike in unison (Galán et al., 2006). Because these oscillations resonate near the gamma frequency (Desmaisons et al., 1999), the power spectra of simulated MC activity began to appear like those of field potential recordings during odor sensing (Galán et al., 2006). This property of phase resetting was used to construct the Brea-Kay-Kopell model (Brea et al., 2009), and was a feature of the oscillation around a limit-cycle described by Freeman (Freeman, 1975)
Recent work recording MCs in vivo using multi-unit arrays during odor presentation found that pairs of MCs within 200 μm possessed dependent activity that could not be accounted for by odor tuning, the breathing rhythm, or the activity of other MCs (Gerkin et al., 2013). This dependent activity was not reported beyond 200 μm. Within the dataset MCs were not found to coherently spike with one another during odor presentation at frequencies above 20 Hz, well below the field potential oscillation, coherent spiking was not found in MC pairs thought to be greater than 200 μm apart. Excess MC spike coincidence above chance did not increase in the presence of an odor.
The layers of the olfactory bulb form a laminar structure with the glomerular layer (Glom, white with grey dashed circles) forming the outer shell of the bulb. Spherical structures called glomeruli (denoted by the dashed grey circles) contain the ramifications of apical dendrites of mitral and tufted cells. Periglomerular (PG, purple) cells are inhibitory interneurons that coordinate the activity of mitral and granule cells that share a glomerulus. The external plexiform layer (EPL, dark grey shading) is the next deepest layer of the bulb. It contains the transversing apical dendrites of mitral and tufted cells, as well as lateral dendrites of the same. The lateral dendrites of tufted cells are more superficial than those of MCs and both of whose contact the apical dendrite ramifications of within the EPL. These contacts form dendrodendritic reciprocal synapses. The somas of tufted cells (TC, peach) are located throughout the EPL. Depending on location they are designated either external (superficial) or middle TCs. A population of parvalbumin (PV, fuchsia) positive interneurons (IN) form inhibitory axotic connections with MCs and potentially tufted cells within the EPL. The location of this inhibition, whether on apical or lateral dendrites, is unclear. The mitral cell layer (MCL, orange shading) contains the somas of mitral cells (MC, black/red). Mitral cells project to, typically, only one glomeruli within the glomerular layer. Their axons project through the granule cell layer (GCL, light blue shading) and form the lateral olfactory tract (LOT, light grey shading). The GCL contains the soma of a diverse population of interneurons. The most numerous of which is the granule cell (GC, green). Granule cells have a long apical dendrite and a shorter basal dendrite and are axonless. Facilitating excitatory connections from centrifugal inputs synapse onto these dendrites within the GCL. Other non-GC granule
cell layer interneurons (Non-GC GCL IN, brown) within the GCL are not known to regularly project their axons or dendrites into other layers. They are primarily GABAergic and include Blanes, Golgi, Horizontal, and deep short-axon cells. Their circuitry is not well understood. However, Blanes cells are understood to inhibit GCs, though if this inhibition is targeted solely onto GCs is unknown.
Figure 1-2 Schematic drawing of the dendro-dendritic synapse

(A) Schematic drawing of the primary circuit of the olfactory bulb. The dendrodendritic synapse between mitral cells (MC, red/black) and granule cells (GC, green), which form dendrodendritic synapses in the external plexiform layer of the olfactory bulb. (B) A blow up of the synapse formed between GCs and MCs. Mitral cells release glutamate (black arrow) onto iGluRs orange) AMPA and NMDA on GCs which in turn release GABA (red capped line) onto GABA receptors (purple) on mitral cells. As denoted in the diagram lateral inhibition between MCs can go through a GC intermediary at the dendrodendritic synapse. Excitation of one MC->GC synapse can release GABA at a secondary GC->MC synapse.
Chapter 2 – Modulation of olfactory bulb network activity by serotonin

Abstract

While inhibition has often been proposed as a central mechanism for coordinating activity in the olfactory system, relatively little is known about how activation of different inhibitory local circuit pathways can generate coincident inhibition of principal cells. We used serotonin (5-HT) as a pharmacological tool to induce spiking in ensembles of mitral cells (MCs), a primary output neuron in the olfactory bulb, and recorded intra-cellularly from pairs of MCs to directly assay coincident inhibitory input. We find that 5-HT di-synaptically depolarized granule cells (GCs) only slightly but robustly increased the frequency of inhibitory postsynaptic inhibitory currents in MCs. Serotonin also triggered more coincident IPSCs in pairs of nearby MCs than expected by chance, including in MCs with truncated apical dendrites that lack glomerular synapses. That serotonin-triggered coincident inhibition in the absence of elevated GC somatic firing rates suggested that synchronized MC inhibition arose from glutamate receptor-mediated depolarization of GC dendrites or other (non-GC) interneurons outside the glomerular layer. Tetanic stimulation of GCL afferents to GCs triggered robust GC spiking, coincident inhibition in pairs of MCs and recruited large-amplitude IPSCs in MCs. Enhancing neurotransmission through NMDARs by lowering the external Mg^{2+} concentration also increased inhibitory tone onto MCs but failed to promote synchronized inhibition. These results demonstrate that coincident MC inhibition can occur through
multiple circuit pathways and suggests that the functional coordination between different GABAergic synapses in individual GCs can be dynamically regulated.

**Introduction**

Rhythmic sensory input to the olfactory bulb (OB), the second-order brain region in the olfactory system, recruits complex inhibitory responses (Hamilton and Kauer, 1989) that help shape spike patterns of mitral and tufted neurons during basal respiration and sniffing (Abraham et al., 2010). Divergent inhibitory input from granule cells (GCs), the most numerous interneuron subtype within the OB, onto principal cells have been proposed to mediate spike synchronization among subpopulations of principal cells (Galán et al., 2006). However, the ability of GCs, and other bulbar interneurons, to generate coincident inhibition on groups of principal neurons has been studied directly (using paired intracellular recordings) only infrequently.

Coincident inhibitory responses onto mitral cells (MCs)—synaptic input that could potentially synchronize firing patterns in output neurons by either resetting intrinsic membrane potential oscillations (Desmaisons et al., 1999) or triggering rebound activity (Desmaisons et al., 1999; Galán et al., 2006; Balu and Strowbridge, 2007)—have only been reported in the literature infrequently, including directly following current steps to MCs (Isaacson and Strowbridge, 1998) and in short-duration epochs following tetanic sensory afferent stimulation (Schoppa, 2006a). Complicating the interpretation of responses to tetanic stimulation in a resonant brain region such as the OB (Rall and Shepherd, 1968; Freeman, 1975; Desmaisons et al., 1999; Galán et al., 2006) is the possibility that coincident spiking or synaptic activity following the stimulus might
reflect the triggering stimulus rather than synchronization emerging *de novo* through the intrinsic properties of OB neurons or their synaptic connections.

In the present study, we find that the neuromodulator serotonin (5-HT) directly depolarizes MCs, increasing their spontaneous firing rates in rodent brain slices, while appearing to have little direct action on inhibitory GCs. We then used 5-HT to determine whether a gradual increase in spiking activity induced over a large population of principal cells promotes coincident inhibition in MCs. We find a selective increase in synchronized inhibitory input among nearby, but not distant, pairs of MCs with 5-HT. The ability of 5-HT to promote coincident inhibition required non-NMDAR-mediated excitation of GABAergic interneurons and was not mimicked by treatment with low Mg²⁺ extracellular solutions that directly enhanced dendrodendritic inhibition (DDI). These results demonstrate that AMPAR-triggered depolarization of OB interneurons can generate coincident inhibition in the absence of synchronized excitatory drive from MCs.

**Results**

*Direct and indirect effects of serotonin in olfactory bulb neurons*

While coherent inhibitory inputs have been proposed to underlie spike synchrony in principal cells in the OB during sensory responses (Freeman, 1972; Kashiwadani et al., 1999; Galán et al., 2006; Kay and Stopfer, 2006), relatively few studies have directly assayed the inhibitory local circuits responsible for synchronous inhibition. Typically, electrical stimulation methods are employed to either activate glomerular inputs (Aungst et al., 2003; Schoppa, 2006a, 2006b) or focally activate circuits within the plexiform layers (Nakashima et al., 1978; Isaacson and Strowbridge, 1998; Friedman and
Strowbridge, 2003). Utilizing in vitro OB slices, we used a pharmacological approach to activate large populations of MCs to probe circuits responsible for inhibitory synchronization.

In rat horizontal OB brain slices, bath application of 200 µM 5-HT significantly increased the frequency of spontaneous MC spiking assayed in cell-attached recordings (from 3.0 ± 1 to 20.3 ± 4.7 Hz; P < 0.001; paired t-test; n = 8; (Fig. 2-1A-C). This depolarizing effect appeared to be mediated by postsynaptic 5-HT receptors on MCs as we found a similar increase in spontaneous spiking when 5-HT was tested after ionotropic glutamate and GABA<sub>A</sub> receptors were blocked using an antagonist cocktail containing APV, NBQX and gabazine (from 4.3 ± 2.3 to 20.6 ± 5.9 Hz; P < 0.01; n =11; (Fig. 2-1C). While previous studies have identified effects of 5-HT on neurons within the glomerular layer (Hardy et al., 2005; Petzold et al., 2009), the depolarization of MCs we observed by 5-HT persisted in dissected OB slices that lack a glomerular layer (Fig. 2-1D; as in Friedman and Strowbridge, 2003), suggesting a direct effect on postsynaptic receptors located on the MC somata or dendritic processes contained in the external plexiform layer. These results are consistent with Hardy et al. (2005) who showed intracellular depolarization following of activation of 5-HT2Rs on MCs, though the location of the postsynaptic 5-HT receptors was not determined in that study. In similar experiments recording from tufted cells (TCs; fast synaptic transmission blocked), we found heterogeneous results with 3 of 4 cell-attached recordings from TCs remaining silent before and after application of 200 µM 5-HT. The one spontaneously-active TC increased its firing rate in 5-HT from 4.1 to 13.1 Hz. One previous study (Liu et al., 2012) found direct depolarization of external TCs following activation of 5-HT2ARs.
Given the possible heterogeneous effects of 5-HT on different subclasses of TCs, we focused the present study on 5-HT effects on MCs.

In cell-attached MC recordings, bath application of 200 µM 5-HT elicited a rapid increase in spontaneous firing that subsided with a time constant of 44 ± 9 sec (Fig. 2-1E). We also found rapid onset kinetics in intracellular recordings under whole cell current-clamp conditions (from -69.2 ± 0.9 to -58.7 ± 2.2 mV; P < 0.001; n = 9; 200 µM 5-HT tested in APV, NBQX and gabazine; Fig. 2-1F). We took advantage of the rapid activation and desensitization kinetics following 5-HT applications in this study to determine the network consequences of brief (2-3 min) periods of induced spiking in populations of MCs.

We first asked if 5-HT also activated GABAergic GCs that form reciprocal dendrodendritic synapses with mitral and tufted cells (Fig. 2-2A-B; (Rall et al., 1966; Isaacson and Strowbridge, 1998)). In 12 out of 14 GCs tested, 200 µM 5-HT depolarized the membrane potential. The mean membrane potential change following 5-HT application in GCs was 3.4 ± 0.7 mV; n = 14. Serotonin also increased the apparent frequency of spontaneous EPSPs (Fig. 2-2C) though many of these synaptic events were small amplitude and therefore difficult to analyze. Blocking ionotropic glutamate and GABA\textsubscript{A} receptors with APV, NBQX and gabazine abolished most spontaneous synaptic activity in GCs and occluded the depolarizing effect of 5-HT (Fig. 2-2D-F; significantly different than the response to 5-HT without synaptic blockers; P< 0.002; unpaired t-test). These results suggest that 5-HT indirectly activated GCs, presumably primarily through the numerous dendrodendritic synapses formed between MCs and distal GC dendrites in the EPL. We also measured the sustained synaptic depolarization evoked by 200 µM 5-
HT in 4 GCs visualized using live 2-photon microscopy. All 4 visualized GCs had dendritic arbors that ramified within the EPL. Serotonin triggered a comparable steady-state depolarization in visualized GCs with intact dendritic arbors (Fig. 2-2F), suggesting that the relatively small amplitude of the synaptic depolarization recorded in GCs was not attributable to recordings from interneurons with truncated dendrites (and therefore few dendrodendritic synapses).

Consistent with the small amplitude of the steady-state depolarization 5-HT evoked in GCs and the relatively hyperpolarized resting potentials (-67.7 ± 1.1 mV; n = 14; Fig. 2-2G), we did not observe action potentials in whole-cell GC recordings within 5 min of 5-HT application (0 out of 14 GCs tested). We confirmed the low incidence of 5-HT induced spiking in GCs by performing 22 cell-attached recordings under the same conditions where 5-HT reliably induced MC spiking (as in Fig. 2-1B-C). The same small fraction of GCs (3/22) were spontaneously spiking before and after application of 200 µM 5-HT (Fig. 2-2E). Of the 3 out of 22 GCs that were spontaneously active before 5-HT, two increased their firing rates and one decreased firing following 5-HT treatment. We confirmed that we could detect spiking in cell-attached GC recordings by electrically stimulating in the granule cell layer following 5-HT application (Fig. 2-2E). Results from the GC cell-attached recording are summarized in Fig. 2-2H and suggest that 5-HT-driven depolarization of OB principal cells synaptically excites GCs, resulting in a relatively small amplitude sustained somatic depolarization that remained subthreshold in nearly all GCs tested.
**Inhibitory feedback to mitral cells evoked by serotonin**

Despite only rarely triggering somatic spiking activity in GCs, 5-HT effectively increased the frequency of spontaneous inhibitory postsynaptic responses recorded in MCs (Fig. 2-3A1). The average frequency of spontaneous IPSCs recorded in MCs increased from 19.8 ± 1.9 to 47.0 ± 3.6 Hz following 3 min treatment with 200 µM 5-HT (137.4 % increase; \( P < 0.0001; n = 61\); IPSCs recorded as inward currents as a consequence of reversed Cl⁻ gradient). The kinetics of 5-HT-evoked increase in inhibitory tone paralleled closely the time course of elevated MC spiking recorded in cell-attached recordings, including the rapid desensitization phase after ~ 3 min exposure to 200 µM 5-HT (Fig. 2-3A-B).

Blockage of GABA_A receptors with gabazine (GBZ, 10 µM) occluded the effect of 5-HT on spontaneous synaptic currents in MCs (n = 3 MCs; Fig. 3A2).

Serotonin appeared to increase GABAergic inhibition of MCs via AMPA receptor-mediated depolarization of GCs. Blockade of NMDA receptors with D-APV (25 µM) lowered the basal IPSC frequency recorded in MCs (\( P < 0.05; n = 7\) cells) but failed to occlude the transient increase in IPSC frequency following 5-HT (Fig. 2-3C).

Blockade of AMPARs with NBQX (5 µM) lowered the spontaneous IPSC frequency in MCs (\( P < 0.005; n = 7\) cells) and also prevented 5-HT from increasing inhibitory tone (Fig. 2-3D). Serotonin triggered a similar increase in spontaneous inhibitory tone (160%) in dissected OB slices lacking glomerular layers (Fig. 2-3E-F; \( P < 0.0001\)), suggesting that this response was independent of previously described effects of 5-HT on periglomerular circuits (Petzold et al., 2009). Bath application of 5-HT had no effect on the rate of miniature IPSCs recorded in 1 µM TTX, suggesting that the primary action of this transmitter was not related to mechanisms regulating GABA release from
interneurons (Fig. 2-3E). The results summarized in Figs. 2-3E-G are consistent with the hypothesis that the primary effect of 5-HT in these experiments is on principal neurons and that the increase in inhibitory tone following bolus applications of 5-HT arises from AMPAR-mediated excitation of GABAergic interneurons outside the glomerular layer.

Serotonin appeared to depolarize MCs by binding to postsynaptic 5-HT2 receptors. The 5-HT2R antagonist cinanserin (20 µM; Peroutka and Snyder, 1981) completely occluded the effect of bath application of 200 µM 5-HT (IPSC frequency 14.2 ± 4.5 Hz before 5-HT vs. 10.2 ± 3.1 Hz in 5-HT; \( P > 0.05 \); paired t-test; \( n = 5 \)). By contrast, the 5-HT1R antagonist pindolol (25 µM; Tricklebank et al., 1984) failed to block 5-HT-mediated increase in IPSC frequency (12.1 ± 3.1 Hz before 5-HT vs. 67.5 ± 14 Hz in 5-HT; \( P < 0.001 \); n = 5). Similarly, the 5-HT3/4R antagonist SDZ 205-557 (10 µM; Buchheit et al., 1991; Eglen et al., 1993) did not prevent the 5-HT-mediated increase in MC IPSC frequency (9.4 ± 2.9 before 5-HT vs. 31.7 ± 8.2 in 5-HT; \( P < 0.02 \); n = 6).

Also consistent with an action on 5-HT2Rs, we found the 5-HT2AR-selective agonist TCB2 (10 µM; Fox et al., 2010) triggered a statistically-significant increase in IPSC frequency (16.5 ± 2.8 Hz in control vs. 21.0 ± 3.0 Hz in TCB2; \( P < 0.02 \); n = 5).

Synchronous inhibition onto subpopulations of mitral cells

In addition to increasing the overall frequency of spontaneous IPSCs, 5-HT triggered synchronous inhibition in pairs of closely-spaced MCs but only infrequently in more distant pairs of MCs (Fig. 2-4A). Synchronous IPSCs following 5-HT treatment did not result from activity in inhibitory local circuits in the glomerular layer because we observed similar synchronous inhibitory responses in a near pair of MCs in which both cells had apical dendrites truncated before the glomerular layer (Fig. 2-4B). We used the
clipped cumulative intensity function (CIF; Hahnloser, 2007) to quantify the frequency of synchronous IPSCs within a given onset latency window (2 ms duration) and the frequency of coincident events expected by chance ("Shuf"). In the paired recording between visualized MCs without apical dendritic tufts, 5-HT triggered a statistically significant increase in CIF over the CIF expected by chance during 5-HT treatment ($P < 0.0002$; Fig. 2-4C). The synchronous MC inhibition assayed by CIF only persisted for ~1 min, paralleling the rapid desensitization of the 5-HT evoked increase in IPSC frequency (see Fig. 2-3B). We also found a statistically significant increase in MC IPSC CIF in a different visualized pair of MCs in which each neuron contacted a different glomerulus (70 µm between cell bodies; 0.22 IPSC CIF; $P < 0.001$; not shown). Over a set of 13 paired recordings between nearby MCs (cell bodies separated by less than 200 µm; mean separation $= 83.2 \pm 13$ µm), 5-HT reliably triggered more synchronous IPSCs than expected by chance (actual CIF was significantly larger than CIF computed following shuffling inter-IPSC intervals; $P < 0.005$; Fig. 2-4D). We also observed a smaller, but still statistically significant, increase in IPSC synchrony in 8 pairs of MCs separated by between 200 and 1000 µm and no increase in IPSC synchrony above chance in 5 MC pairs separated by > 1000 µm (Fig. 2-4D). The lack of IPSC synchrony in distant pairs of MCs was not due to variation in effectiveness of 5-HT in driving inhibitory local circuits since 5-HT elevated mean IPSC frequencies similarly in three paired recording configurations (Fig. 2-4E; Kruskal-Wallis ANOVA, $P > 0.05$).

When plotted directly against MC separation (Fig. 2-4F), 5-HT driven “excess” IPSC synchrony (actual CIF – expected CIF estimated by shuffling inter-event intervals) decreased sharply with distance; most paired recordings separated by less than 150 µm
had excess IPSC synchrony. By contrast, few paired recordings separated by more than 150 µm had more IPSC synchrony than expected by chance (near 0 excess CIF). These results suggest that despite the long length of MC dendritic processes (100s of microns; Shepherd et al., 2004), coordinated inhibition only reliably occurred in pairs of closely-spaced MCs under our experimental conditions. We also observed a large variation in degree of inhibitory coupling even between pairs of nearby MCs, as illustrated by the range of excess CIF estimates shown in Fig. 2-4F. Serotonin triggered a statistically significant, but transient, increase in excess IPSC CIF that was extinguished rapidly (~1 min after the IPSC frequency was maximal; Fig. 2-4G). Mitral cell excess IPSC CIF was not correlated with the mean IPSC frequency in that episode (R^2 = 0.20; n = 26 episodes from 13 MC paired recordings with < 200 µm separation.) Together, these results suggest that the spatial extent of inhibitory local circuits revealed by 5-HT is largely restricted to pairs of MCs separated by < 150 µm and is most prominent in pairs separated by < 80 µm.

Next, we tested whether other experimental treatments that increased the frequency of spontaneous IPSCs also promoted synchronous inhibition in nearby pairs of MCs. Reducing extracellular Mg\(^{2+}\) concentration eliminated the tonic blockade of NMDARs (Ascher and Nowak, 1987) and enhanced GC-mediated reciprocal dendrodendritic inhibition (Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Chen et al., 2000). Treatment with Mg\(^{2+}\)-free ACSF increased the frequency of spontaneous IPSCs in MCs (Fig. 2-5A-D; P < 0.005; n = 8 experiments). Despite the robust increase in inhibitory tone evoked by low Mg\(^{2+}\) ACSF, we observed no increase in frequency of synchronous IPSCs assayed through cross-correlation analysis in two example
experiments (Fig. 2-5B) and when IPSC CIF was assayed in 4 experiments (Fig. 2-5D; \( P > 0.05 \)). Even when the CIF analysis was restricted to large amplitude IPSCs (>200 pA), that might reflect GABA release events triggered by dendritic spikes in GCs, we did not observe increased IPSC synchronization in low \( \text{Mg}^{2+} \) ACSF (\( P > 0.05 \) actual CIF vs. shuffled interval controls; paired t-test.) These results demonstrate that the synchronous inhibition of MCs triggered by 5-HT was not simply a result of enhanced inhibitory tone. Enhancing excitatory synaptic drive onto interneurons using low \( \text{Mg}^{2+} \) ACSF elevated the frequency of spontaneous IPSCs, and also triggered many more large-amplitude IPSCs than 5-HT, without promoting synchronous IPSCs in nearby pairs of MCs. Figure 5E summarizes the effects of two different experimental manipulations that increased IPSC frequency (5-HT and low \( \text{Mg}^{2+} \) ACSF) on synchronous MC inhibition. Only 5-HT increased inhibitory tone and also promoted synchronous inhibition, an effect that was robust over wide range of CIF analysis window durations from 0.5 to 10 ms. The excess CIF evoked by 5-HT increased as the analysis window was lengthened from 0.5 to 4 ms and then was asymptotic, suggesting that the jitter associated with mechanisms underlying coincident inhibition of MCs was less than 4 ms. This estimate includes the jitter associated with transmitter release at both inhibitory synapses and the temporal variance associated the processes that link the two release sites.

Finally, we asked if tetanic stimulation in the GCL (Fig. 2-6A-B) evoked synchronous inhibition in MCs. GCL stimulation often triggered spiking in GCs (e.g., Fig. 2-2E; spiking triggered in 12 of 13 cell-attached GC recordings); the mean firing rate was 7.8 Hz ± 2.2 Hz in 5 GCs that responded to GCL stimulation throughout the analysis window employed below (100 – 1100 ms following the last stimulus). We assayed
coincident inhibition in pairs of MCs separated by ~100 µm (range 60 – 250 µm), a separation distance in which 5-HT only infrequently triggered synchronous inhibition (example traces presented in Fig. 2-6C). IPSC synchrony was significantly elevated in 6 of 8 paired recordings following a tetanic stimulation (100 – 1100 ms following the last GCL stimulation; \( P < 0.05 \)); the group mean CIF also was significantly greater following GCL stimulation than CIF computed from shuffled IPSC times (Fig. 2-6D; 160% increase over bootstrap; \( P < 0.0005 \)). In the same set of 8 paired recording experiments, 5-HT triggered only modest IPSC synchrony and a 15% increase in IPSC CIF \( (P < 0.05) \). When analyzed individually, only 2 of 8 paired recordings had a significant elevation in IPSC CIF beyond the level expected by chance in 5-HT. Four of the 6 paired recordings that showed no increase in IPSC synchrony in 5-HT had statistically significant increases in IPSC CIF following tetanic stimulation. Excess IPSC CIF also was significantly increased over control conditions following GCL stimulation \( (P < 0.0002) \) but not following 5-HT (Fig. 2-6E; \( P > 0.05 \); \( n = 8 \) MC pairs). There was no statistically significant difference in mean IPSC frequency triggered by GCL stimulation and bath application of 5-HT (Fig. 2-6F; \( P > 0.05 \)).

One advantage of assaying inhibitory synchrony using the CIF method is the ability to quantitatively determine the minimum test window duration required to capture coincident IPSCs. Simply expanding the duration of the sliding analysis window will always yield more detected coincident events. However, subtracting the bootstrap estimate of the frequency expected by chance for that window generated a metric (excess IPSC CIF) that can be compared across different conditions. As shown in Fig. 2-6G, this metric increased in parallel as the window duration was increased in both 5-HT and GCL.
stimulation conditions (open and filled symbols, respectively). Both curves were well fit by a model with each pair of near-coincident IPSCs originating from the same interneuron that had a synaptic release jitter of $2 \pm 0.5$ ms (mean $\pm$ SD; solid line in Fig. 2-6G; Kay and Wong, 1987; Sabatini and Regehr, 1999). More complex models of coincident inhibition arising from near-synchronous excitation of multiple interneurons included the jitter associated with EPSC responses on granule cells (1.1 – 2.3 ms; Balu et al., 2007) and the jitter associated with EPSP-spike coupling (1.4 – 4.7 ms; (Schoppa, 2006b; Rodriguez-Molina et al., 2007)). Even using the EPSP and spike coupling mechanisms described in prior studies with relatively low jitter required significantly longer sliding analysis windows to capture coincident events than we found in our measurements (dashed line in Fig. 2-6G; time constant significantly greater than either 5-HT or GCL stimulation conditions; $P < 10^{-4}$). Using the estimate of the jitter associated with EPSP-spike coupling in OB GCs (4.7 ms; Schoppa, 2006b) required even longer duration analysis windows (dotted line in Fig. 2-6G). These results suggest that following 5-HT and GCL stimulation, the coincident inhibition we observe in MCs likely arises from divergent output from individual interneurons.

Finally, we asked if we could differentiate the spontaneous IPSCs triggered by 5-HT, 0 Mg$^{2+}$ ACSF and GCL stimulation. As shown in the plots in Fig. 2-6H, 5-HT triggered IPSCs with a very similar distribution of amplitudes as control conditions ($P > 0.05$; K-S test). Reducing extracellular Mg$^{2+}$ ions results in a uniform shift toward larger amplitude IPSCs (different from control, $P < 10^{-8}$; K-S test) while GCL stimulation skewed the amplitude distribution toward large-amplitude IPSCs (different from control, $P < 10^{-8}$; different from 0 Mg$^{2+}$, $P < 10^{-8}$; K-S test). While the uniform enhancement of
IPSC amplitude in 0 Mg\(^{2+}\) ACSF could arise from multiple mechanisms, the selective increase in spontaneous large-amplitude IPSCs following GCL stimulation is consistent with the preferential recruitment of a population of IPSCs that was not strongly active under control conditions.

**Discussion**

We make three principal conclusions in this study. First, activation of large ensembles of MCs with 5-HT recruits inhibitory local circuits through a 5-HT2R- and AMPAR-dependent mechanism that form divergent synapses on nearby but not distant pairs of principal neurons. Serotonin-driven inhibitory circuit activation functioned without frequent somatic spiking in GCs. Second, enhancement of dendrodendritic inhibition by removing extracellular Mg\(^{2+}\) ions increases inhibitory tone on MCs but fails to recruit coincident IPSCs on nearby pairs of MCs beyond the frequency expected by chance. Finally, tetanic stimulation of afferents in the GCL triggers robust synchronous inhibition of MCs, even in MC pairs where 5-HT triggered only modest coincident inhibition. These results suggest that different local circuits may regulate inhibition onto nearby MCs than clusters of principal cells that span multiple glomerular modules.

While a variety of stimulation methods have been used to increase inhibitory tone onto MCs (Rall and Shepherd, 1968; Nakashima et al., 1978; Jahr and Nicoll, 1982), our results demonstrate that they can do so through very different local circuit pathways. Enhancing currents through NMDARs by applying Mg\(^{2+}\) free ACSF increased the frequency of spontaneous IPSCs recorded in MCs but failed to recruit coincident inhibition even in closely-spaced pairs of principal cells. This treatment likely facilitated
inhibition by directly activating reciprocal dendrodendritic synapses, an inhibitory pathway effectively driven by currents through NMDARs (Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Isaacson, 2001). The absence of coincident inhibition (beyond the frequency expected by chance) suggests that the activation of granule cell dendrites during low Mg²⁺ treatment was localized and did not trigger synchronous GABA release at multiple dendrodendritic synapses at different positions along the dendritic arborization of individual GCs. The uniform shift towards larger amplitude IPSCs in low Mg²⁺ ACSF may reflect a higher probability of GABA release from dendrodendritic synapses onto MCs due to the larger Ca²⁺ influx under this experimental condition (Isaacson and Strowbridge, 1998; Halabisky et al., 2000; Isaacson, 2001).

Electrical stimulation of afferents in the GCL reliably triggered coincident inhibition in pairs of MCs and action currents in cell-attached recordings from GC somata, consistent with synchronous inhibition arising from spiking in GCs. Tetanic stimulation also skewed the MC IPSC amplitude distribution towards large amplitude events, consistent with recruitment of an inhibitory circuit that was not active spontaneously in control conditions. Coincident IPSCs recorded in MCs had very low onset latency jitter, indicative of near-synchronous release of GABA from multiple synapses formed by the same presynaptic interneuron. Significantly greater jitter would be expected for synchronous inhibition arising from clusters of interneurons that spiked synchronously because they received coincident excitation. By assaying coincident inhibition starting 100 ms after the last electrical shock, we sought to minimize IPSC synchronization mediated by synaptic inputs directly activated by the tetanic stimulus itself.
Activation of MCs with bath 5-HT triggered widespread spiking in MCs and also recruited coincident inhibition in groups of nearby principal cells, primarily in MC pairs separated by less than 80 µm. The di-synaptic depolarization we report in GCs following 5-HT is consistent with an increase in inhibitory tone in MCs mediated by GABA release at dendrodendritic synapses formed between granule and MC dendrites (Isaacson and Strowbridge, 1998; Schoppa et al., 1998). However, coincident IPSCs could potentially arise from inhibitory cell types in the glomerular layer. Petzold (Petzold et al., 2009) described an action of 5-HT on glomerular-layer inhibitory circuits mediated by 5-HT2C receptors on periglomerular interneurons that could contribute to inhibitory tone in MCs despite the electrotonically distant location of these synaptic inputs. Presumably divergent connections formed by GABAergic periglomerular cells underlie coincident IPSCs recorded in the subclass of TCs located near the glomerular layer (external tufted cells; Hayar et al., 2005). Several lines of evidence suggest that the modulation of inhibitory tone we observe in MCs is distinct from this effect in the glomerular layer. First, we observe similar increases in MC IPSC frequency in slices in which the glomerular layer was surgically removed. Second, we find a similar, statistically significant increase in IPSC synchronization in MCs with apical dendrites that do not arborize in the same glomerulus. Finally, modulation of periglomerular inhibitory circuits appears to rely primarily on 5-HT2C receptors while modulation of inhibitory tone can be evoked by the selective 5-HT2AR agonist TCB2. Petzold (Petzold et al., 2009) found no effect on glomerular-layer circuit function when they tested the same 5-HT2AR agonist, a result that is consistent with the high density of 5-HT2ARs found primarily in the MC layer using immunohistochemistry (Hamada et al., 1998) and in situ
hybridization (Pompeiano et al., 1994). While there is a consensus in the literature regarding 5-HT2CR expression in the glomerular layer (Pompeiano et al., 1994; Abramowski et al., 1995; Clemett et al., 2000; Petzold et al., 2009), several of these studies also found 5-HT2CR expression in deeper OB layers.

Unlike inhibitory responses triggered by tetanic GCL stimulation and low Mg\(^{2+}\) ACSF, the IPSC amplitude distribution was not significantly affected by 5-HT. This result suggests that 5-HT affected primarily inhibitory local circuits that were already active spontaneously (or triggered IPSCs that had similar amplitudes to spontaneous IPSCs). Surprisingly, the widespread excitatory synaptic drive elicited by 5-HT did not lead to frequent somatic spikes in GCs. Instead, intracellular recordings revealed these interneurons were typically were only modestly depolarized by glutamatergic EPSPs during 5-HT and did not spike spontaneously. However, the low jitter we find associated with coincident MC inhibition driven by both 5-HT and GCL stimulation likely results from spiking in common presynaptic interneurons. While the blockade of 5-HT stimulated inhibition on MCs by AMPAR antagonists argues that the primary site of 5-HT action was not on interneurons, our results cannot exclude additional modulatory actions of metabotropic receptors that affect interneuron function.

There are at least two possible mechanisms that could explain 5-HT stimulated synchronous inhibition in the absence of frequent GC somatic spiking. Serotonin-triggered coincident inhibition could arise from dendritic spikes in GCs that failed to trigger somatic action potentials or from spiking in an as-yet-unidentified inhibitory local interneuron that targets MC cell bodies and/or proximal dendrites. Several studies have reported dendritically-localized spiking in GCs, though often involving primarily low-
threshold Ca\(^{2+}\) spikes (Egger et al., 2003, 2005). Spontaneous fast Na\(^{+}\)-based dendritic spikes have been reported in frog GCs under basal (unstimulated) conditions (Zelles et al., 2006). If also present in mammals, spontaneous “D-spikes” could function to synchronize multiple dendrodendritic release sites in one GC, enabling coincident inhibition of nearby pairs of principal cells. The sensitivity of coincident inhibition in 5-HT to AMPAR antagonists suggests that these responses were likely recruited selectively by excitatory inputs with fast kinetics. While we rarely observed spikelets in somatic intracellular recordings from GCs under basal or 5-HT-stimulated conditions that might correspond to dendritic D-spikes (Zelles et al., 2006), these events might not be easily detectable given the frequent synaptic inputs mammalian GCs normally receive in 5-HT.

Coincident inhibition of MCs also could arise from other (non-GC) interneurons that target MC somata or proximal dendrites, explaining the bias toward synchronous inhibition in near but not far MC pairs. At present, there is little evidence for axo-dendritic or axo-somatic inhibition of MCs apart from periglomerular circuits. A wide variety of morphologically-defined interneuron subtypes have been identified in the EPL and GCL that could potentially form axonal connections with MCs (Schneider and Macrides, 1978; Pressler and Strowbridge, 2006; Kosaka and Kosaka, 2011; Pressler et al., 2013). Recent studies (Kato et al., 2013; Miyamichi et al., 2013) described a new class of parvalbumin (PV)-immunoreactive interneuron located in the external plexiform layer that could potentially mediate the synchronous IPSCs we observe in MCs in 5-HT. Alternatively, one or more subtypes of large “short-axon” interneurons in GCL, such as Blanes cells (Pressler and Strowbridge, 2006) and Golgi cells (Pressler et al., 2013), may contact MCs in addition to GCs.
While our experimental results cannot definitively determine whether coincident inhibition in 5-HT arises from dendritic D-spikes that couple multiple dendrodendritic GABA release sites within individual GCs or from divergent axonal input from another interneuron class, several lines of evidence are suggestive of the latter hypothesis. First, GCs are known to synapse along the length of MC secondary dendrites and, therefore, could potentially provide synchronous inhibition to widely separated pairs of MCs—which we did not observe. In principal, cable attenuation could explain the bias we found toward synchronous inhibition only in closely-spaced MC pairs. However, using focal uncaging of GABA, Lowe (Lowe, 2002) recorded IPSCs originating from dendrodendritic synapses on lateral dendrites ~200 µm from the cell body that were often larger than spontaneously occurring IPSPs. Based on this prior uncaging study, we expected that divergent GC inputs should be detectable in our experimental conditions in MC pairs separated by 300–400 µm. The ability of GCL stimulation to recruit coincident inhibition in a set of paired MC recordings that showed little synchronous inhibition in 5-HT (Fig. 2-6D-E) also is suggestive of a role for two distinct inhibitory interneuron subtypes outside the glomerular layer. Inhibitory circuits recruited during 5-HT treatment typically linked clusters of MCs separated by less than ~100 µm (likely only one or a few glomerular modules) while local circuits activated by GCL stimulation extended over larger clusters of MCs and included more glomerular modules. The significantly different IPSC amplitude distributions following 5-HT treatment and GCL stimulation also are suggestive of a role for non-GC interneurons.

Multiple investigators have hypothesized that the inhibitory local circuits formed between MCs and GCL interneurons operate on different spatial scales relative to the size
of glomerular columns (Egger and Urban, 2006; Balu et al., 2007). The smallest spatial scale is likely to be the reciprocal dendrodendritic synapse formed between MC and GC dendrites (Isaacson and Strowbridge, 1998; Halabisky et al., 2000) which may function to gate AP propagation along the MC secondary dendrite (Chen et al., 2000). Unexpectedly, we found that widespread activation of these synapses following low Mg\textsuperscript{2+} ACSF triggered robust MC inhibition but little inhibitory synchrony, suggesting that dendrodendritic synapses may function to coordinate MCs on smaller spatial scales than assayed in this study. The other unexpected finding from this study was that activation of large ensembles of MCs did not generate inhibitory synchrony on the same spatial scale as MC dendritic processes, many of which span millimeters (Shepherd, 2004). Instead, we find coincident inhibition was restricted to a spatial scale similar to the size of a glomerular column. Given previous work demonstrating the ability of correlated inhibitory synaptic input to drive synchronous firing in MCs (Galán et al., 2006; Schoppa, 2006a), a primary prediction from our study is that activation of inhibitory local circuits driven di-synaptically by 5-HT should promote spike synchrony in MCs with same or neighboring glomerular modules. Unfortunately, it is not possible to directly test this prediction using the experimental paradigm used in this study because of the direct depolarizing effects of 5-HT on MCs. In principle, optogenetic methods that selectively depolarize the same interneuron population that was di-synaptically excited by 5-HT could test this hypothesis though additional studies will be required to identify the appropriate interneuron subclass.
Materials and Methods

Slice preparation: Horizontal OB slices (300 µm thick) were prepared from P14-P19 Sprague-Dawley rats, either sex, as previously described (Halabisky et al., 2000; Friedman and Strowbridge, 2003; Balu and Strowbridge, 2007). Slices were incubated in ACSF at 35 °C for 30 minutes and then maintained at room temperature. All experiments were carried out in accordance with the guidelines approved by the Case Western Reserve University Animal Care and Use Committee.

Electrophysiology: All recordings were performed in a submerged recording chamber. Most recordings were performed at 34-35 °C; experiments visualizing GC dendritic arbors using 2-photon imaging were performed at 30 °C. Slices were continuously perfused with an extracellular solution containing (in mM): 124 NaCl, 5 KCl, 1.23 NaH₂PO₄, 1.2 MgSO₄, 26 NaHCO₃, 10 dextrose, 2.5 CaCl₂, equilibrated with 95% O₂/5% CO₂ (pH 7.3) at a flow rate of 1.5-2.0 ml/min. Whole-cell patch-clamp electrophysiological recordings were made using AxoPatch 1B or 1D amplifiers (Axon instruments) and borosilicate glass pipettes (2-8 MΩ). All data was digitized at 5 or 10 kHz by an ITC-18 computer interface (Instrutech) and a Windows 7 personal computer. Slices were imaged using IR/DIC optics on an upright fixed stage Zeiss Axioskop 1FS microscope and cells were identified based upon morphology, soma location within a slice, and intrinsic membrane properties. For current-clamp whole cell and cell-attached recordings, the internal solution contained (in mM): 140 K methylsulfate, 4 NaCl, 10 HEPES, 0.2 EGTA, 4 MgATP, 0.3 Na₃GTP, 10 phosphocreatine-Tris. A CsCl based internal was used for all MC voltage-clamp recordings that reversed the Cl⁻ gradient; it contained (in mM): 115 CsCl, 4 NaCl, 10 HEPES, 1 EGTA, 25 TEA-OH, 5 QX-314, 4
MgATP, 0.3 Na\(_3\)GTP, 10 phosphocreatine-Tris. All internal solutions were equilibrated to a pH of 7.3 and an osmolarity of ~290 mmol/Kg. Serotonin phosphocreatine was prepared for use daily from powder (Sigma Aldrich). All other drugs were purchased from Tocris and were prepared in aliquots and frozen at -20 °C until use. Drugs were added to the bath by changing the source of external solution. Evoked responses were obtained using a constant current stimulus isolation unit (A-360; World Precision Instruments, FL) and a monopolar tungsten electrode (FHC) placed in the GC layer. Neurons filled with Alexa594 through the patch pipette were imaged using a custom two-photon laser scanning system (Balu et al., 2007).

**OB slice micro-dissection:** Horizontal OB slices lacking a glomerular layer were prepared by first making two cuts perpendicular to the mitral cell layer (MCL) that extended through the entire slice, leaving a dissected slice ~ 2mm wide. The glomerular layer was then separated from the remaining tissue by making a third cut, parallel to the MCL, through the EPL (Friedman and Strowbridge, 2003). All micro-dissection cuts were performed in a submerged recording chamber while visualizing the slice through a 5x objective. Dissected slices were allowed to recover for at least 20 min before attempting recordings. Dissected slices were stained with methylene blue dissolved in ACSF at the conclusion of each experiment to visualize the OB laminar structure. Only recordings from slices in which the glomerular layer was completely removed were analyzed.

**Data analysis:** Data analysis was performed with custom Matlab 2012b (Mathworks) scripts. Inhibitory and excitatory postsynaptic currents (IPSCs, EPSCs), and action potentials were automatically detected as previously described (Larimer and Strowbridge,
Briefly, traces were smoothed via a Savitsky-Golay filter and the instantaneous derivative was calculated. Events were defined by a cumulative dV/dt or dI/dt that exceeded a set threshold, and event times were defined by the first deviation towards threshold. Average frequencies of spikes or synaptic events were determined by taking the reciprocal of the average inter-event interval. All data are presented as mean ± S.E.M. unless otherwise noted. The paired Students t-test was used to determine statistical significance, unless otherwise specified. Autocorrelation and cross-correlation were performed by detecting events and convolving a 2 ms Gaussian kernel with the binary event times (Galán et al., 2006).

The clipped cross-intensity function (CIF; Hahnloser, 2007) was computed based on detected IPSC event times and used to determine whether the incidence of synchronous events was greater than expected by chance. To calculate the CIF for two response channels, we computed the total number of coincident (event onset latencies that differed by less than the synchronous window cutoff of 2 ms, except for the analyzes in Figs. 5-6 that used varying duration windows) events and divided by the the total number of events on the channel with the fewest events. For example, one paired MC recording episode that contained 200 detected IPSCs in MC1 and 250 IPSCs in MC2, with 30 IPSCs that each occurred within 2 ms on both channels, would have an IPSC CIF of 0.15 (30/200). Two advantages of the CIF method, compared with correlation-based methods for detecting synchrony, are the ability to quantitatively define event synchrony over variable coincidence stringencies and the ability to directly compute estimates of synchrony expected by chance. Unless noted, all analyzes were based on the average CIF computed over three control (pre 5-HT) and two consecutive 30 sec episodes acquired...
between 140 and 210 sec after switchover to ACSF containing 5-HT. We computed CIF expected by chance for each episode by permuting the inter-event intervals contained in each channel; bootstrap CIF estimates were computed over $10^4$ to $10^6$ iterations. Bar plots labeled shuffled CIF reflect the mean of the bootstrap runs computed from each episode. “Excess” CIF was computed by subtracting the mean bootstrap CIF from the CIF computed from the actual event timing. Probability estimates obtained from individual episodes were computed from cumulative density functions based on the bootstrap estimates.

Estimates of excess IPSC CIF from interneurons that form divergent synaptic connections with MCs and clusters of interneurons synchronized by common excitatory input (Fig. 2-6G) were generated by a common template of 100 exactly synchronous events across two MCs and distributed with Poisson inter-event intervals. For the divergent interneuron model (solid line in Fig. 2-6G), a separate IPSC release jitter values drawn from a Gaussian distribution with a mean of 2.0 ms and a SD or 0.5 ms (Kay and Wong, 1987; Sabatini and Regehr, 1999) was added to each simulated MC channel. We constructed two models of non-divergent interneurons coupled by common excitatory inputs. The non-divergent model with lowest jitter (dashed line in Fig. 2-6G) was made by including glutamate release jitters of $1.1 \pm 0.2$ ms (Balu et al., 2007) on each interneuron channel as well as the jitter associated with EPSP-spike coupling in neocortical neurons ($1.4 \pm 0.9$ ms; Rodriguez-Molina et al., 2007). Using the less reliable EPSP-spike coupling reported in OB GCs ($4.7 \pm 1.5$ ms; Schoppa, 2006b) and the jitter associated with dendrodendritic EPSPs onto GCs ($2.3 \pm 3.2$ ms; (Balu et al., 2007))
required much longer sliding windows to capture synchronous IPSCs (dotted line in Fig. 2-6G).
Figure 2-1 Serotonin directly depolarizes olfactory bulb mitral cells.

(A) Cartoon of intracellular mitral cell (MC) recording configuration with synaptically-connected granule cells (GCs). (B) Example cell-attached MC recording with fast synaptic transmission blocked (5 µM NBQX, 25 µM d-APV, 10 µM gabazine; GBZ). Bath application of serotonin (5-HT; 200 µM) increased the frequency of spontaneous MC action currents from 4.3 ± 2.3 to 20.6 ± 5.9 Hz. (C) Plot of increase in MC firing frequency following 5-HT application in control conditions (left, n=8 experiments, *** $P < 0.001$, paired t-test) and when fast synaptic transmission was blocked with NBQX+APV+GBZ (Blockers) prior to 5-HT application (right, n=11 cell-attached recording experiments; *** $P < 0.001$, paired t-test). (D) Plot of increase in MC firing following 5-HT application in dissected OB slices without glomerular layers. Same conditions as indicated in C. ** $P < 0.02$, * $P < 0.05$, paired t-test. (E) Plot of mean ± SEM of MC firing rate in 14 cell-attached recordings in NBQX+APV+GBZ. (F) Time course of MC depolarization following bath application of 5-HT (n=9 M Cs recorded in NBQX+APV+GBZ).
**Figure 2-2 Indirect excitation of granule cells by serotonin.**

(A) Diagram of recording configuration. (B) Example granule cell (GC) response to a 50 pA depolarizing current step. (C) Example intracellular GC recording before and during 5-HT application. Horizontal line at -74 mV in both traces. (D) Blockade of fast synaptic responses with NBQX+APV+GBZ occludes depolarizing effect of 5-HT on GC. Horizontal line at -68 mV in all three traces. Mean membrane potential change in synaptic blocker cocktail was 0.4 ± 0.7 mV; n =6; not significantly different from 0; P > 0.05. (E) Example cell-attached GC recording showing no spontaneous spikes before or after 5-HT application. Focal stimulation in the granule cell layer (GCL; asterisks) triggered a barrage of action currents in the same cell-attached recording (right trace). (F) Plot of change in mean intracellular membrane potential in 14 GCs treated with 200 µM 5-HT; ∆Vm from individual experiments indicated by symbols at left side. Serotonin triggered a similar membrane potential depolarization in subset of GCs with visualized and intact dendritic arbors within the external plexiform layer (Vis GCs; n=4). Granule cell 5-HT response was greatly attenuated when fast synaptic responses were blocked by NBQX+APV+GBZ (Syn Block; n=6). ** P < 0.01; * P < 0.05. (G) Plot of mean GC depolarization triggered by 5-HT in control conditions (n=14) relative to the membrane depolarization required to reach spike threshold from the same resting membrane potential (n=14). *** P < 0.001. (H) Plot of the proportion of GCs recorded in the cell-attached configuration which discharged spontaneously in control conditions (Ctrl; grey bar; 3/22), following bath application of 5-HT (3/22), and following focal GCL stimulation in 5-HT (12/13).
Figure 2-3 Serotonin triggers disynaptic inhibition onto mitral cells.

(A1) Example MC voltage-clamp recordings illustrating the increase in spontaneous IPSC frequency during 5-HT application (Cl\(^-\) reversal potential reversed because of CsCl internal solution). (A2) Gabazine (GBZ, 10 µM) blocked spontaneous and 5-HT triggered inward currents. (B) Plot of the time course of the spontaneous IPSC frequency during 5-HT application. Each symbol represents mean ± SEM from 61 MCs. (C) Plot of the increase in MC IPSC frequency with 5-HT after NMDARs were blocked with 25 µM d-APV. Example trace in APV and APV + 5-HT shown in inset. (D) Plot of the effect of 5-HT on MC IPSC frequency after AMPARs were blocked with 5 µM NBQX. Example traces shown in inset. (E) Summary of the increase in MC IPSC frequency with bath application of 5-HT in 61 MCs recorded in OB brain slices in control conditions (left bars) and in 1 µM TTX (right bars). *** \(P < 0.001\), paired t-test, n.s. \(P > 0.05\). (F) Plot of MC IPSC frequency increase with 5-HT in 5 experiments with dissected OB brain slices lacking glomerular layers. *** \(P < 0.001\), paired t-test. (G) Summary of the increase in MC IPSC frequency with 5-HT in the presence of glutamate receptor antagonists. ** \(P < 0.01\), paired t-test, n.s. \(P > 0.05\).
Figure 2-4 Serotonin promotes synchronous inhibitory synaptic responses in pairs of nearby mitral cells.

(A) Example intracellular responses in near pairs of MCs shown in left panel that include an IPSC recorded in both cells (white arrows; IPSC onset latency differed by 0.8 ms in the two MCs; 60 µm spacing between mitral cell bodies). Example recordings from a distant MC pair with no synchronous IPSCs (740 µm spacing between MCs). (B) Reconstructions of dual MC recordings following intracellular labeling with 150 µM Alexa594. Picture represents collage from multiple maximal Z-stack projections acquired using live 2-photon imaging. Neither MC had an intact apical dendrite; arrows indicate primary dendritic processes directed toward the glomerular layer that were truncated at the surface of the brain slice. Calibration bar, 50 µm. EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer. (C) Inset, example dual voltage-clamp recordings from two MCs that received synchronous IPSCs during 5-HT treatment. Arrow indicates example synchronous IPSC recorded in both MCs (IPSC onset latency difference = 0.4 ms in example shown). Plot of increase in clipped cross-intensity function (CIF; 2 ms analysis window) following 5-HT application. Grey bars represent results from bootstrap shuffles. 5-HT bar represents 140 sec following start of 5-HT application; Post bar represents 200 sec following 5-HT application. ** P < 0.01; paired Student's t-test; n = 4 episodes in each condition. Mean ± S.E.M. (D) Plot of MC IPSC synchrony estimated by clipped cross-intensity function (CIF) in near (< 200 µm separation between cell bodies), mid (200-1000 µm separation) and distant (>1000 µm separation) pairs of MCs. Grey columns represent average CIF computed from IPSC interval shuffles in each paired recording experiment. *** P < 0.005 paired t-test.
Number of paired recording experiments analyzed indicated at base of each bar. (E) Plot of mean IPSC frequency in control (Ctrl) and 5-HT in paired MC recordings from the same three groups of experiments presented in D. *** $P < 0.005$ paired t-test. Mean IPSC frequencies in 5-HT did not differ significantly between the three groups ($P > 0.05$, Kruskal-Wallis ANOVA; same MC paired recordings analyzed in D). (F) Plot of excess IPSC CIF (Actual – mean from interval shuffles) versus mitral cell body separation for 25 paired recording experiments. Dashed line represents no increase in frequency of coincident IPSCs over the frequency expected by chance (Excess IPSC CIF=0). (G) Summary of the increase in excess IPSC CIF following 5-HT in 13 MC pairs separated by < 200 µm; post bar indicates excess CIF at the same time window indicated in C. * $P < 0.05$, paired t-test.
Figure 2-5 Low Mg\textsuperscript{2+} external solution increase spontaneous MC inhibition without promoting synchronous IPSCs in paired MC recordings.

(A) Example traces in one MC under control conditions and at two time points following application of 0 Mg\textsuperscript{2+} ACSF. (B) Example cross-correlograms computed from spontaneous IPSC onset times illustrating frequent coincident inhibitory inputs promoted by 5-HT (black curve; peak at lag = +0.2 ms) with no correlated inhibitory input triggered by 0 Mg\textsuperscript{2+} ACSF (grey curve; separate experiment). (C) Plot of spontaneous IPSC frequency in the four experimental conditions shown in A for 8 MCs. ** P < 0.005. (D) Plot of MC IPSC CIF at two times following application of 0 Mg\textsuperscript{2+} ACSF in 4 MC paired recordings (< 200 µm cell body separation). Grey bars represent average CIF computed from 10,000 IPSC interval shuffles (Shuf). n.s. P > 0.05. (E) Plot of the relationship between duration of synchronous IPSC detection window and excess CIF (Actual – expected from IPSC interval shuffles) in near MC paired recordings (< 200 µm cell body separation). Serotonin (5-HT; black symbols) increased IPSC CIF significantly above expected values for all coincident detection windows while 0 Mg\textsuperscript{2+} (grey symbols) did not increase CIF beyond expected values at any coincidence window duration tested. Comparisons at all window durations were statistically significant (P < 0.01; paired t-test).
Figure 2-6 Synchronous MC inhibition triggered by afferent stimulation.

(A) Diagram of recording configuration. (B) Example recording of MC response to tetanic stimulation in GCL (3 bursts of 4 shocks at 100 Hz; 500 ms inter-burst interval).

(C) Enlargements of responses to GCL stimulation (left traces; arrows indicate IPSCs with onset latencies that differed by < 0.2 ms) in a paired MC recording with coincident inhibition. Bath application of 5-HT failed to evoke synchronous inhibition in the same pair of MCs (right traces). (D) Plot of the synchronous MC inhibition (IPSC CIF) present in control (Ctrl) conditions, following bath application of 5-HT, and following GCL stimulation (Stim). Estimates of IPSC synchrony expected by chance in each condition presented in grey columns (Shuf). * P < 0.05; *** P < 0.005, n.s. P > 0.05; paired t-test.

(E) Plot of excess CIF (Actual CIF – CIF expected by chance) in control conditions, 5-HT and following GCL stimulation. *** P < 0.005; paired t-test. (F) Plot of increase in IPSC frequency in 5-HT and following GCL stimulation. Both conditions increased IPSC rate from control conditions (P < 0.005, paired t-test). IPSC frequency did not differ significantly between 5-HT and GCL stimulation conditions (P > 0.05, n.s., paired t-test).

(G) Plot of relationship between excess MC IPSC CIF and the duration of the sliding analysis window in both 5-HT (open symbols; mean ± SEM; downward error bars) and following GCL stimulation (filled symbols; upward error bars). Solid line represents single divergent interneuron model with 2.0 ± 0.5 ms (mean ± SD) jitter associated with GABA release events. Dashed and dotted lines represent models of coincident IPSCs arising from excitatory synchronization of multiple interneurons. See Methods for details.

(H) Plot of the cumulative IPSC amplitude distributions for control conditions (solid line;
n = 20 cells), 5-HT (dash-dot line n = 20), 0 Mg²⁺ ACSF (dotted line; n = 8) and following GCL stimulation (dashed line; n = 16).
Chapter 3 – Discussion

Our findings on serotonin, GCL stimulation, and 0 Mg²⁺ can divided into three main sections: the effects on mitral cell (MC) synchrony, granule cell response to each condition, and the network level effects of each condition on the LFP. Investigations of MC synchrony was limited to inhibitory synchrony, however MC excitatory inputs and MC APs have also been the subject of some study. Granule cell responses to MC activation have remained sparse, however our dataset suggests that some of the proposed modes of GC activity may function within the same cell. The link between synchrony and oscillations was mentioned in Chapter 1, here theoretical predictions and our experimental findings will be examined and predictions will be made regarding future studies of serotonin’s network effects. The future studies which could branch off of the findings presented here will also be discussed.

§ 1 – Mitral cell synchrony

Synchronous activity in MCs can be divided into two classes, input and output. Mitral cell inputs can be further divided into synchronous excitation and synchronous inhibition. Of these divisions the present work has focused primarily upon synchronous inhibitory inputs.

Observed inhibitory synchrony of MCs was highly dependent upon distance between pairs, but not upon sharing a glomerulus (Fig. 2-4 B-E). This largely eliminates PG cells as a possible locus of shared synchrony of MCs. The decaying exponential function trend of the distance function of the synchrony measure corresponds well with the shape of an estimate of the number of connecting GCs that MC share as a function of
distance (Egger and Urban, 2006). It is unlikely that the distance dependence of inhibitory synchrony is due to the failure of MC APs from invading the full extent of the lateral dendrite as in vivo reports have shown that APs are detectable throughout the dendritic arborization of impaled MCs (Debarbieux et al., 2003). Some have theorized that glomeruli form functional units below them of MCs that project to the same glomerulus (Xu et al., 2000; Urban, 2002; Willhite et al., 2006). These functional units process odorant information with lateral inhibitory feedback between one another (Urban, 2002). This has been used to explain how gamma-band oscillations can vary spatially in both frequency and primarily amplitude across the bulb. Our data suggests that these proposed units experience significant lateral inhibition within 2-3 glomeruli diameters with pairs that are likely part of neighboring units having the highest levels of synchronous inhibition.

The finding that MC synchronous inhibition could be dependent upon AMPAR and not NMDAR activation (Fig. 2-3C-D, Fig. 2-4B-F) is relatively unsurprising given the fast dynamics of AMPAR mediated excitation versus that of the slower NMDAR which would be more conducive to fast synchrony in the former condition and less so in the latter (Schoppa, 2006a, 2006b). However, canonically the focus on NMDAR mediated inhibition of MCs through the recurrent synapse may in fact reflect the operation of the bulb when only a few glomeruli are activated, or during active periods of cortical feedback (Balu et al., 2007). During epochs when numerous glomeruli are active the inhibitory scheme of the bulb, given our data, likely switches to an AMPAR mediated one that generates fast synchronous inhibition of MCs. Raising the IPSC rate to similar levels as seen during serotonin bath application by removal of Mg$^{2+}$ from the external...
solution we were unable to elicit synchronous inhibition between MCs above chance, suggesting that activation of the GCs by NMDAR activation alone is not a sufficient component driving synchrony, and that synchronous inhibition was not the result of GC excitation alone. It is unlikely that MCs are projecting to the same glomerulus and receiving inhibition from periglomerular cells given the distances over which we find synchronous inhibition (Buonviso et al., 1991; Dhawale et al., 2010; Gerkin et al., 2013; Ke et al., 2013; Kikuta et al., 2013). To ensure that the increase in MC inhibitory tone was coming from GC activity and not from periglomerular cells we removed the glomerular layer via a micro-dissection. In this reduced preparation we were able to elicit a proportionally identical increase in the IPSC rate of MCs with bath serotonin. There was a 50% reduction in the baseline activity (Fig. 2-3F) which we attribute to physical disruption of the circuit as a consequence of the micro-dissection. In two experiments visualizing a pair of MCs, one in which both apical dendrites were truncated before the glomerulus, and another in which both MCs projected to different glomeruli, we observed significant increases in IPSC synchrony above chance levels. This provides another argument against the observed inhibitory synchrony arising from glomerular circuits.

While we do not detect increased somatic spiking in the GCs from which we have recorded in serotonin, the fact that NBQX, and not APV, is sufficient to block an increase in the IPSC rate of MCs in response to bath serotonin is indicative of calcium influx into GC apical dendrites through voltage gated calcium channels and not calcium influx through NMDA channels (Chen et al., 2000; Egger et al., 2003), presuming that the inhibition which we record arose in GCs. This lends itself to the idea that GCs may have dendritic spiking, unobservable in the somatic cell-attached recordings, that lead to
synchronous inhibition (Zelles et al., 2006), or that repetitive activation of GCs leads to synchronous lateral inhibition in local dendritic structures in a graded fashion as excitation spreads passively and leads to local calcium influx; though we cannot distinguish between these with our data set. We conclude therefore the reported inhibitory synchrony is the effect of lateral inhibition mediated by AMPAR activation of OB interneurons, and that calcium influx through NMDA channels is insufficient for lateral inhibition (Fig. 3-1A-B; shown as GC for illustrative purposes).

Lateral inhibition between MCs propagated using GCs as intermediaries, i.e. MCs laterally inhibiting their neighbors by exciting GC dendrites that synapse onto other MCs, within the OB has been of keen interest to many as it has been viewed as a potential mechanism of synchronizing bulbar activity giving rise to observed oscillations and to providing coincident excitation of upstream targets of the bulb. Lateral inhibition has been described by several groups (Phillips et al., 1963; Yokoi et al., 1995; Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Margrie et al., 2001; Urban and Sakmann, 2002; Arevian et al., 2008) Computational studies have argued that lateral inhibition is strongest at proximal sites nearest the MC soma (McTavish et al., 2012). Other computational work has examined the role lateral inhibition plays in OB models in improving contrast (Arevian et al., 2008) and sparseness of MC spiking (Yu et al., 2013), in addition to suggesting that spike timing of MCs is largely determined by lateral inhibition within local MC populations possibly giving rise to oscillations (Yu et al., 2013), though others have noted small effects of lateral inhibition on spike rate (Arevian et al., 2008).
Some have proposed that lateral inhibition can exist in a low and high state depending upon overall network activity (Egger and Urban, 2006). In the high state, overall network activity is low which allows for MC APs to spread throughout a greater distance of the lateral dendrite length. In such a condition, inhibition across glomeruli would be capable of forming much broader lateral inhibitory connections that have a much larger spatial extant than in the low lateral inhibition state in which high network level activity leads to lateral inhibition that stops AP propagation in lateral dendrites much closer to the soma. In this low lateral inhibitory state, MCs functional connections are greatly reduced spatially. Though lateral inhibition is an attractive explanation for the OB oscillatory synchrony, an argument has been made that the time constant of inhibition is too slow for lateral inhibition alone to give rise to fast oscillations (Galán et al., 2006).

Whether 5-HT recruited MC inhibitory synchrony promotes synchronous MC AP generation would be a natural next step in studying the effects of 5-HT on bulbar circuitry. The synchrony of these APs would be dependent upon inhibitory inputs, and occur in MCs to a maximum lateral extent as was observed with inhibitory synchrony. However, due to the relatively small amount of MC inhibition which is synchronous, and the large tonic excitatory conductance from 5-HTR activation it may be that bath application of serotonin will not produce synchronous MC APs. Instead, focal application of 5-HT through a puffing pipette could induce localized MC spiking and recruitment of synchronous MC inhibition that would synchronize MC spiking. Granule cell layer stimulation should also increase synchronous MC AP generation in whole-cell current-clamped MCs that are brought to spike with step potentials. This GCL stimulation would serve as a useful positive control for 5-HT puffing experiments.
Functionally, local synchrony within the OB may have diffuse effects on higher brain regions as activation of piriform cortex by MCs is sparse (Poo and Isaacson, 2009); however, neighboring MCs have been reported to have similar projection patterns (Buonviso et al., 1991) and coincident MC excitation of piriform pyramidal cells has been shown to be sufficient for pyramidal cell spike induction (Franks and Isaacson, 2006) suggesting that local synchrony may play some role in upstream processing as inputs converge providing precisely timed excitation. This is further reflected by the finding that piriform cortex oscillation patterns are tightly controlled by narrow windows in which pyramidal cell EPSCs are swiftly followed by IPSCs that provide precise spike timing at preferred phases of the oscillation (Poo and Isaacson, 2009).

Stimulation of the GCL elicited synchronous MC IPSCs in excess of levels expected by chance in MC pairs that did not experience significant increases ISPC synchrony in 5-HT (Fig. 2-6D-E). From the literature we can expect that GCL stimulation will activate predominantly centrifugal fibers (Nakashima et al., 1978; Balu et al., 2007; Gao and Strowbridge, 2009), but also axon collaterals of TCs (Schoenfeld et al., 1985) and interneuron processes in the stimulus area (Schneider and Macrides, 1978; Kosaka and Kosaka, 2011). The delay period following stimulation was imposed to account for this multimodal stimulation. Granule cells would often spike in response to GCL stimulation, in some recordings spiking for several hundred milliseconds after the cessation of stimulation. In the previous chapter it was argued that GCL stimulation and 5-HT activated different sets of interneurons based on the evidence that they recruited a different population of IPSCs as seen in the amplitude distribution. However, there could be an alternative explanation. Branch point failures within a GCs dendritic arbor (Egger
et al., 2005) are an attractive hypothesis, as such failures could account for the decreased lateral extent of coincident inhibition seen in 5-HT compared to GCL stimulation where a somatic spike would fully infiltrate the entire apical dendrite (Fig. 3-1 A-E, Egger et al., 2003; Egger and Urban, 2006). Due to the high basal activity in MCs during 5-HT, it is not inconceivable that Ca$^{2+}$ activated channels linked to NMDAR activation in GCs (Isaacson and Murphy, 2001) are providing modest negative feed-back that would promote such failures and depress the number of quanta released at the synapse (Robitaille and Charlton, 1992).

§ 2 – Granule cell response to mass mitral cell activation

Granule cell activity during oscillatory network activity has remained poorly understood. While thought to be an important mediator of cortical feedback projections believed to be necessary for β oscillations (Neville and Haberly, 2003), GC dynamics have remained largely unexamined in models of intrinsic bulbar oscillations. Models which predicted lateral inhibition being necessary for oscillations have minimized the requirement of sodium spikes in GCs as they appear to be uncoupled from field oscillations (Lagier et al., 2004), however some have suggested that Ca$^{2+}$ spikes are a potential mechanism for generating lateral inhibition during oscillations (Bathellier et al., 2006). Others have proposed that inhibition from GCs during oscillations is graded, requiring only local calcium influx to mediate reciprocal inhibition (Brea et al., 2009), with some suggestions that the predominant mode of GC inhibitory output is subthreshold (Rall et al., 1966; Egger and Urban, 2006). This low-threshold release is
triggered by sub-AP threshold depolarizations that depend solely upon low-threshold calcium channel activation. This mode of activation has been shown to reliably spread through GC dendritic branches (Egger et al., 2003, 2005; Pinato and Midtgaard, 2005).

Granule cell unit activity during awake odor sensing has been only recently reported (Cazakoff et al., 2014) and only to a unitary odorant stimulus. In contrast to studies of GC activity in anesthetized animals (Wellis and Scott, 1990; Cang and Isaacson, 2003; Tan et al., 2010), the recent study found that GC odor responses were not phase locked to the breathing rhythm, and represented more broadly tuned odor responses than was previously thought. In the awake state GCs did not display increases in spike rate that would significantly alter the perception that they rarely spike during normal activity.

In our in vitro slice preparation we found little spontaneous spiking activity in GCs recorded in the cell-attached configuration. Even with a presumptive large excitatory drive from induced MC spiking, GC somatic spiking was not significantly increased over baseline. Due to the electrotonic isolation of GC somas from the apical dendrites (Rall and Shepherd, 1968) it may be that spikes generated in the dendrites routinely fail to infiltrate the GC soma, but isolated spikelets (Pinato and Midtgaard, 2005; Zelles et al., 2006) were observed in some recorded GCs. We detected significant increases in GC EPSP frequencies, but these increases were minimal, and again likely reflected isolation of the GC soma from its dendritic arbor in the EPL, where it synapses with MC lateral dendrites. Unlike PV+ EPL interneurons (Van Gehuchten) GCs did not rapidly enter depolarization block in 5-HT (not shown). This is strongly supportive of the recent report by Cazakoff et al 2014. It also raises the question of the precise role of GCs in MC
activity given that their inputs to MCs appear in awake animals to not be specifically
timed to any particular phase of activity. One possibility is that GC somatic spiking does
not accurately reflect the output of the GC apical dendrite, which given our dataset seems
to be the case. Another possibility is that GC inhibition has a critical window of MC
activity in which it can effectively alter MC spiking behavior (Arevian et al., 2008) and
in the reported condition MC activity was not within this narrow band.

The absence of inhibitory synchrony during low Mg$^{2+}$ exposure was a surprising
experimental finding. However, there may be a reasonable explanation found in the
coupling of NMDAR activity to Ca$^{2+}$ activated potassium channels (Isaacson and
Murphy, 2001). In this paradigm large scale activation of NMDARs on GC apical
dendrites would produce localized calcium influx that would activate extra synaptic
potassium channels that would isolate dendritic spines from one another (Fig. 3-1F-G). In
this condition increasing GC dendritic activation produces ever increasing localization of
near simultaneous GABA release. This could in theory explain the surprising finding that
activation of large ensembles of MCs does not lead to spatially extended synchronous
inhibition as might be expected from MC morphology. Active potassium channels would
also explain the difference in IPSC amplitude distributions found between GCL
stimulation and 5-HT as they would depress the quantal number of vesicles released at
synapses (Robitaille and Charlton, 1992). This hypothesis could be easily examined by
blocking Ca$^{2+}$ activated potassium channels during 5-HT application.
§ 3 – Field potential generation

Rall and Shepherd (Rall and Shepherd, 1968) performed a series of lateral olfactory tract (LOT) stimulations coupled with a series of field recordings made at various depths within the OB. Using an early form of the ball and stick model they accurately modeled the activity of the OB’s primary circuit, and predicted the dendro-dendritic reciprocal inhibition between mitral and GCs before it was anatomically discovered. Their model consisted of successive stages wherein mitral cell somata would spike, the spike would back-propagate through the lateral dendrites where it would release excitatory neurotransmitters onto GCs which would then reciprocally inhibit the MC lateral dendrite. The interaction between the MCs and GCs would, as a result, oscillate through excitatory and inhibitory phases, which the authors hypothesized, underpinned the field potential oscillations observed by Adrian (Adrian, 1942). This idea was taken up by Freeman (Freeman, 1975), whereby through piecewise linear modeling of the same LOT stimulation he was able to determine that the population of MCs and GCs would be pushed into a stable limit cycle of activity of alternating excitation and inhibition. Freeman’s addition was to mathematically formalize the interactions between the two populations of cells as a single operating unit, and to definitively isolate their effects during the phases of the oscillation using current-source density techniques.

Stimulation of excitatory afferents onto MCs leads to oscillating network activity in the gamma band (Rall and Shepherd, 1968; Freeman, 1975; Friedman and Strowbridge, 2003; Lagier et al., 2004). Some have posited that gamma oscillations reflect the probability of MC spiking (Rojas-Libano and Kay, 2008; Kay et al., 2009). MCs appear to have a preferred beta/gamma phase for spiking (Cenier et al., 2009).
Given that synchronous inhibition in 5-HT is present in the OB slice, and several models predict that MC activation leads to OB oscillations (Li and Hopfield, 1989; Brea et al., 2009) investigating the predictions of these models using 5-HT or other 5-HTR agonists is likely to yield interesting results. Oscillations if present would be dependent upon both intact glutamatergic and GABAergic signaling. They would be present only during times in which significant ISPC synchrony was detected. If the Brea-Kay-Kopell model (Brea et al., 2009) is correct, oscillations would center around the average MC firing rate. These oscillations would not be observed with synchronous MC APs, though MC APs should exhibit phase preferences with respect to the oscillation. A positive control that oscillations were dependent upon synchronous inhibition, and not increased MC inhibitory tone could be found in zero Mg\(^{2+}\) experiments. Under these experimental conditions the LFP should resemble pink noise following a log-linear 1/f relationship.

If present such oscillations would conform to those first made by Rall and Shepherd (Rall and Shepherd, 1968) when they investigated the field potential from lateral olfactory tract (LOT) shocks, which were presumed to activate only MCs antidromically. In those experiments the field recorded in the EPL showed successive excitation of GCs by MCs and then inhibition of MCs, the combination of which generated an oscillation, as detailed exhaustively by Freeman (Freeman, 1972, 1975; Bressler and Freeman, 1980; Freeman and Schneider, 1982; di Prisco and Freeman, 1985; Eeckman and Freeman, 1990). Unlike in the LOT and glomerular stimulation experiments (Freeman, 1975; Friedman and Strowbridge, 2003; Lagier et al., 2004) where MC activation was synchronized to the initial shock and the circuit’s activity oscillated at a low γ-band frequency, possibly as an artifact of MC rebound spiking.
(Buonviso et al., 1996; Desmaisons et al., 1999; Schoppa, 2006a), serotonin mediated oscillations would be present due to tonic activation of MCs, through the activation of 5-HT2Rs. The finding would support strongly the theoretical model proposed by Brea, Kay, and Kopell (Brea et al., 2009) in which the activity of the MCs has the phase of its subthreshold oscillations (Desmaisons et al., 1999) reset by reciprocal inhibition such that the population activity oscillates around a given frequency of MC activity. In the model inhibition is stochastic, graded, and largely reciprocal in nature. Granule cell spiking if at all present, serves only to slow the oscillation’s frequency. A model wherein bulbar activity oscillated around a dominant MC spiking rate was proposed by (Li and Hopfield, 1989) and contained similar proposals to that of Brea-Kay-Kopell. Investigations of the effects of serotonin on the phase synchrony of MC subthreshold oscillations by holding two neighboring MCs in current-clamp and evoking subthreshold oscillations through current steps would provide the best evidence that the Brea-Kay-Kopell model was correct.

Most models of bulbar oscillations presume that the GCs are passive participants in shaping the oscillations of the bulb (Freeman, 1975; Li and Hopfield, 1989; Bathellier et al., 2006), that MC dynamics were the primary driver of the oscillation. If oscillations are observed in 5-HT, our data suggest that this may not faithfully represent OB oscillations, and that fast synchronous inhibition mediated by interneurons, presumably GCs, can generate oscillations in the OB slice.

§ 4 – Future Directions

Experiments that would build upon the findings of the present work could examine the possible synchrony between MCs and TCs, the cellular effects of serotonin
activation of MCs on GCs, the examination of other OB INs that could potentially mediate synchronous MC inhibition, the dependence of synchrony on synchronous release, and the effects of centrifugal input activation on OB function. Possible experiments examining these topics, potential results and their interpretation are presented.

**Mitral cell – tufted cell synchrony**

Given that MCs and TCs respond to odor input at different phases of oscillations (Fukunaga et al., 2012), tufted cells and MCs have different spatial extents of lateral inhibition evoked by glomerular stimulation (Christie et al., 2001), and that GCs appear to synapse onto either mitral, tufted, or both depending upon their position within the GCL (Mori et al., 1983; Orona et al., 1983; Greer, 1987; Mori, 1987; Imamura et al., 2006) it is thought that they may represent two distinct circuits (Shepherd et al., 2007). Identifying if middle tufted cells are synchronous with each-other as with MC’s in a spatially restricted manner could lead to an interesting finding where MCs are not synchronously inhibited to the same extent either in magnitude or spatially that like pairings are.

This difference in connectivity to inhibitory circuits in deeper layers of the bulb could be tested by performing paired recordings from simultaneously recorded tufted cells, and from a simultaneously recorded mitral and tufted cell pair utilizing the 5-HT driven approach detailed here. Our preliminary data on tufted cells did not indicate that they significantly increased their spiking frequency following the addition of serotonin, though they did depolarize. If true for a larger dataset, this would mean that we could identify if MCs activate interneuronal targets that inhibit tufted cells. If they did, we
could further examine the spatial extent and degree of synchronous inhibition shared by tufted cells with large ensembles of MCs being activated. As a potential control for this experiment stimulation of the olfactory nerve has been shown in external tufted cells to increase synchronous inhibition (Hayar et al., 2005). Any differences in synchronous inhibition between pairs of tufted cells in these conditions may be due to effects of inter-(Urban and Sakmann, 2002; McGann et al., 2005; Vucinic et al., 2006; Olsen and Wilson, 2008; Whitesell et al., 2013) or intra-glomerular inhibition (Murphy et al., 2005; Wachowiak and Shipley, 2006 p.200; Shao et al., 2009, 2012) By staining recorded cells with neurobiotin, or by performing the experiments utilizing 2-photon microscopy (2-P) illumination of the cells during recording we may rule out intra-glomerular inhibition by tracing the dendrites of the recorded cells. This has the added benefit of allowing us to examine the extent to which overlapping dendrites may correspond to the degree of inhibitory synchrony.

Schoppa (Schoppa, 2006a) utilized an olfactory nerve stimulus protocol which induced MC spike synchrony between neighboring MCs. He hypothesized that the spike synchrony was due to coincident inhibition received by the MCs. By utilizing a similar stimulus protocol the extent to which MC and TC pairs and TC-TC pairs spike synchronously can be assayed. It is likely that TC-TC pairs will spike synchronously with one another in a similar fashion as was reported for MCs. The results of MC and TC spike synchrony in the slice following electrical stimulation of the olfactory nerve is less certain, though presumably would not differ from the absence or presence of inhibitory synchrony.
Quantitating the degree of overlap of the dendrites would be assisted if each cell was filled with a different color fluorophore coupled with a dichroic mirror or filter setup so that the overlap could be easily calculated by separating the signal of the two cells and looking for overlap.

**Granule cell responses to mitral cell activation or low Mg^{2+}**

While it is possible that an uncharacterized interneuron may be contributing to the increase in MC inhibitory tone following serotonin bath application, given their well-characterized properties and large population, GC cellular activity is an attractive target to study following large MC ensemble activation with serotonin. Little is known about GC activity during odor sensing in complex odor environments when numerous glomeruli and consequently numerous MCs would be activated. Activity in the apical dendrite of the GC have been particularly challenging to study using somatic recordings due to the electrotonic distance of the dendrite from the soma. Utilizing 2-P microscopy dendritic patch recordings of the apical dendrite can be made prior to bath application of serotonin or TCB-2. This would allow for direct recording of the electrical activity taking place during MC activation. Experiments could be performed using a dichroic mirror apparatus that would allow for visualizing the calcium transients of the dendrite and its spines during such bath application by utilizing non-overlapping Alexa and calcium sensitive Oregon Green Bapta-1 (OGB-1) fluorescent dyes to simultaneously measure dendrite morphology and calcium spread. If GCs are the source of the large increase in MCs IPSC rate, large amplitude calcium or sodium spikes would be expected in the apical dendrite that would not be detected with the somatic recording pipette. It is known that NMDAR activation in GCs can lead to a calcium activated potassium current.
(Isaacson and Murphy, 2001). Care should be taken when utilizing the OGB-1 that its introduction into the cell does not alter the activity profile of the dendrite. Some experiments should be performed without the calcium sensitive dye present and compared to those performed with the dye.

Successful recording of calcium transients using calcium sensitive dyes would allow for a careful examination of the spatial extent to which calcium flows into the apical dendrite of GC in conditions of both serotoninergic drive of MCs, GCL stimulation, and in 0 Mg²⁺ coupled with a dendritic recording each EPSP could be matched to changes in calcium levels through a given plane of the dendrite. If GCs were the source synchronous and non-synchronous of inhibitory inputs onto MCs in both 5-HT and 0 Mg²⁺ conditions (Fig. 2-4C-E; Fig. 2-5) then it might be expected that in serotonin calcium transients would diffuse over a larger spatial extent than those of 0 Mg²⁺ which may be localized to individual spines or highly localized regions. Although, this localization may have been the result of the potassium current coupled to NMDAR activation (Isaacson and Murphy, 2001) and not easily observed with OGB-1 filling the dendrite, potentially affecting the potassium currents function.

Something that remained unexplored thus far and bears examination is the possibility that GCs may be receiving synchronous excitatory inputs. By recording from a pair of GCs in the voltage clamp configuration it may be possible to resolve the degree to which they are receiving synchronous excitation. Due to the electronic distance constraints previously mentioned such recordings may need to take place in the apical dendrite of the GC. The results of this experiment could prove invaluable to determining the nature of MC inhibitory synchrony in serotonin should it arise from GCs. If the GCs
do not receive synchronous excitation, and synchronous MC inhibition arises from GC activity, intrinsic dynamics in the GC give rise to synchronous release of GABA. Presumably some form of calcium spike. This would restrict synchronous inhibition between MCs to the lateral extent of a GC apical dendrite. If on the other hand synchronous excitation was present, it might be suggestive of diverging synchronous excitation from MCs that leads to synchronous inhibition of neighboring MCs mediated by GCs as was described by Schoppa (Schoppa, 2006a), but lacking the GC somatic spike. While our simulations (Fig2-6G) indicated that it was unlikely that synaptic integration and its associated jitter was giving rise to the coincident inhibition we observe, it is possible that these effects are lessened by the presence of the MC subthreshold oscillations (Desmaisons et al., 1999) which would provide an intrinsic timing mechanism to the circuit.

Isolating the synchronous inhibition of mitral cells

As previously stated it may be that GCs are not the sources of synchronous inhibition shared between MCs. Mitral cells share inhibitory connections with GCs exclusively in the EPL and other GCL interneurons may synapse onto MC somas or axon hillocks (Schneider and Macrides, 1978; Eyre et al., 2008; Kosaka and Kosaka, 2010). The two inputs can be distinguished by ensuring that mitral cell lateral dendrites do not overlap with a biocytin stain following a cut through the EPL that terminates just prior to the MCL. In pairs where a MC lacked a lateral dendrite that was proximal to the other MC, only inhibition that targeted the other dendrites and soma would be detected. From our present work, MCs with soma separations of ~ 80 μm will have significant synchronous inhibition without such a cut, and little to no synchronous inhibition with a
cut; provided that GCs and not somatically targeting INs are the source of synchronous MC inhibition.

Alternatively, in murine models where PV+, CRH+, or other unique OB interneurons have been identified, using Cre recombinant lines experimenters could express an inhibitory designer receptor exclusively activated by designer drug (DREADD) receptor hM3Di (Alexander et al., 2009; Ferguson et al., 2011) using adenovirus vectors. Repeating serotonin experiments in the presence of the DREADD receptor agonist clozapine-N-oxide (CNO) to silence specific populations of interneurons the precise identity of the interneuron could be determined. This would be observable as a lack of MC synchrony. Once identified, channel rhodopsin (Ch2R) experiments could be employed to selectively excite the identified interneurons to give a positive control of the identification. This would also allow for careful reconstruction of the MC lateral and apical dendrites to determine the relative location of coincident inhibition observed in serotonin.

Sufficiently distant pairs of MCs may be examined wherein one of the cells somas is bathed in the GABAaR blocker gabazine via puffing preventing proximal IPSCs from being observed with somatic recordings. In this condition, a MC pair will still be likely to have overlapping lateral dendrites that could be the site of shared inhibition. Control experiments for this puffing experiment would include photo uncaging of GABA to examine the extent of receptor inactivation with gabazine to ensure that any negative results of coincident inhibition were not due to blockade of receptors outside of the targeted area. Such an experiment may be technically difficult. Positive controls for this
experiment may be to puff NMDA in the GCL underneath a 2-P visualized MC lateral dendrite to ensure that synaptically evoked inhibition is also observable.

Directly recording from the interneuronal populations to determine if they are indirectly excited by 5-HT will also illuminate some of their connectivity, although this experiment could be performed using Ch2R expressed in MCs. Such an undertaking would necessitate characterizing the various populations of interneurons, making this approach unfeasible without some direction from earlier experimentation with DREADD receptors. However, regardless of the significant effort that would be required, few reports of GCL and EPL interneurons exist (Kosaka and Kosaka, 2011). Any positive results on circuit wiring from such experimentation would be highly valuable to the OB community and are thus highly desirable.

**Sensitivity of synchronous mitral cell inhibition to asynchronous release**

Within hippocampal and other brain circuits activity has been observed to switch from synchronous to asynchronous release following strong activation of a neuron (Barrett and Stevens, 1972; Goda and Stevens, 1994; Cummings et al., 1996; Atluri and Regehr, 1998; Lu and Trussell, 2000; Atwood and Karunanithi, 2002; Maximov and Südhof, 2005). Given that Schoppa (Schoppa, 2006a) was able to recruit synchronous MC inhibition through synchronous excitation of GCs in a timing dependent manner, presumably in which electrical stimulus of the ON produced less bulbar activation than is recruited by bath application of 5-HT, it may be that in 5-HT inhibition is recruited through a rate sensing mechanism that would be insensitive to changes in synchronous release.
If there is a difference in the sensitivity of MC inhibitory synchrony to varying concentrations of Sr\(^{2+}\) or Ba\(^{2+}\), both promoters of asynchronous release (McLachlan, 1977; Silinsky, 1978; Bekkers and Clements, 1999), it would be highly suggestive of differences in the mechanisms of inhibitory recruitment between the two experimental paradigms that led to synchronous inhibition (Fig 2-4BF, Schoppa, 2006a). If glomerular or ON stimulation but not serotonin was sensitive to asynchronous release it would argue that the tight coupling of MC spiking reported by Schoppa (Schoppa, 2006a) was the primary driver of synchrony. Given the differences in the level of MC activity, it could also reflect differences in bulbar processing of varying degrees of olfactory stimulation, something already seen in the lack of inhibitory synchrony following Mg\(^{2+}\) removal. This may belie a dual role for GCs in both temporal coding (Giridhar and Urban, 2012) and rate sensing. What might be puzzling about any potential differences is that both were reliant upon AMPAR activation, while coincidence detection in GCs has been associated with NMDAR activation (Chen et al., 2000).

**Centrifugal fiber activation and synchronous inhibition**

Beta-band oscillations in the bulb, present during fine discrimination tasks (Martin et al., 2004; Kay and Beshel, 2010) have been associated with centrifugal inputs to the OB (Neville and Haberly, 2003; Martin et al., 2006). Little work has been done to understand the mechanisms behind the generation of beta-band oscillations, but given what is known of cortical feedback projection circuitry to the bulb some have theorized that activity in the piriform acts in a predictive manner and sends this prediction back to the bulb to shape its output (Schoenbaum and Eichenbaum, 1995; Haberly, 2001). This feedback activity (Balu et al., 2007; Boyd et al., 2012) alters the dynamics of the bulb to
generate beta-band oscillations (Martin et al., 2006). One of the unique features of beta-band oscillations as compared to gamma-band oscillations in the bulb is the uniformity of the amplitude of beta band oscillations at differing sites in the bulb (Gervais et al., 2007; Lowry and Kay, 2007; Martin et al., 2007). This finding is suggestive of synchronous activation of GCs, or other INs, that impose a rhythm on the activity of the MCs. Utilization of a horizontal slice preparation (Balu et al., 2007) in which anterior piriform connections to the bulb remain partially intact would allow activation of centrifugal inputs and test for widespread inhibitory synchrony in MCs that could explain the seemingly global nature of beta oscillations.

DiI staining by Balu et al (Balu et al., 2007) showed that connections from the piriform projected broadly into the OB. By stimulating the piriform in the slice effects on MC synchrony and the field potential can be studied. If beta oscillations are projected into the bulb from piriform (Bressler and Freeman, 1980) then we might expect to see beta-band oscillations in the slice, and in the cross-spectra of the IPSC times of a pair of MCs, marking them as distinct from those induced by serotonin. The spatial extent of synchronous inhibition in this condition will also likely be much greater than that observed with serotonin bath application. Depending on the nature of serotonin induced coincident inhibition the window of inhibitory synchrony may be increased as a function of synaptic jitter associated with cortical excitation of GCs. Care should be taken when performing the stimulation to not anti-dromically stimulate MCs.

To put piriform stimulation experiments into context with ON and glomerular stimulation experiments (Friedman and Strowbridge, 2003; Schoppa, 2006a) it may be necessary to perform a series of experiments in which olfactory nerve stimulation,
piriform stimulation, and serotonin bath application are all performed on the same MC pair. This direct comparison would greatly enhance the rigor of any differences discovered between the varying protocols.

GCL stimulation showed an increase in MC inhibitory synchrony and a field potential that rapidly tapered off. It is unclear with the data presented if this was due to the direct activation of GC spiking or if it was due to activation of input fibers to the bulb. Their use in interpreting or predicting the effects of centrifugal input stimulation may be minimal. However, the general trend of increasing the lateral extent of MC inhibition would appear to be strongly supportive of predictions of increased lateral inhibitory extent with centrifugal input stimulation.
Figure 3-1 Schematic diagram of conclusions

(A) Schematic drawing of a mitral cell pair separated by short distance projecting apical dendrites to differing glomeruli. Note that they share a reciprocal dendrodendritic synapse with a granule cell (GC) on the same GC apical dendritic branch. (B) Glutamatergic excitation of AMPARs (orange) by one of the mitral cells leads to GABA release (red capped line) on both MC dendrites. (C) A pair of MCs who connect to a common GC though on different apical dendrite branches. (D) Granule cell layer stimulation leads to GABA release at both synapses. Serotonin would not have caused synchronous inhibition of this pair, potentially from a branch point failure. (E) Removal of Mg\(^{2+}\) from the ACSF in a similarly spaced pair as in A. (F) Removal of Mg\(^{2+}\) removes tonic blockade on NMDAR (light blue). Inhibition is localized within the GC apical dendrite and does not affect both mitral cells.
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