“Physiological analysis of oscillatory activity in excitatory neurons of the mouse accessory olfactory bulb”

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Chryssanthi Tsitoura
aus Thessaloniki

Berichter:    Universitätsprofessor Dr. rer. nat. Marc Spehr
         Universitätsprofessor Dr. rer. nat. Ivan Manzini

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Für meine Mutter
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1 Introduction

Sensory systems detect physical stimuli and transform this information within dedicated circuits to ultimately create a percept of the outside world. Sensory systems thus enable the interaction of individuals with their environment by eliciting appropriate behavioral responses to external cues. The chemical senses, comprised of the gustatory and olfactory system, detect a staggering number and variety of chemical compounds. This qualitative heterogeneity of stimuli distinguishes chemosensory systems from the other senses: the task of broad chemical recognition requires a massive repertoire of receptors to match the diversity in chemical structures (Mombaerts, 2004a). The gustatory system serves a primarily nutritional function. Here, the detection of non-volatile, water-soluble molecules elicits one of five distinct perceptual taste qualities: sweet, salty, sour, umami, and bitter (Barretto et al., 2015; Yarmolinsky et al., 2009; Zhao et al., 2003). The olfactory system, on the other hand, detects a myriad of chemically diverse volatile and non-volatile compounds, and serves a much broader behavioral context besides mediating the localization and evaluation of food. Specific olfactory cues can carry a chemical message among animals and are thus termed “semiochemicals”, from the Greek “σημείων” (sign) (Burger, 2005; Ferrero & Liberles, 2010). Semiochemicals are classified into pheromones and allelomones (Figure 1.1). The term “pheromone” was introduced by Karlson and Luescher in 1959 and is derived from the Greek “φέρειν” (to carry) and “ορμητής” (hormone, i.e. to excite, to stimulate) (Karlson & Lüscher, 1959). Pheromones are semiochemicals that mediate conspecific social communication by modulating the behavior and/or physiology of the recipient organism of the same species (Brennan & Kendrick, 2006; Liberles, 2014; Tirindelli, Dibattista, Pifferi, & Menini, 2009). Chemical signaling, however, can also occur on an interspecific level mediated by allelomones (greek “αλληλοοικονομικος”, of each other / reciprocal). Here, kairomones benefit the recipient, e.g. elicit predator avoidance in prey, whereas allomones benefit the emitter (e.g. skunk odor), and synomones benefit both emitter and receiver (Apfelbach et al., 2005; Liberles, 2014; Sbarbati & Osculati, 2006; Stowe et al., 1995).

To cope with the vast variety of environmental chemical cues that have to be identified, the olfactory system of rodents has evolved into several subsystems (Figure 1.2) (Ma, 2007; Munger, Leinders-Zufall, & Zufall, 2009; Zufall & Munger, 2001): the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the Gruneberg ganglion (Fleischer et al., 2006; Fuss et al., 2005; Gruneberg, 1973), and the septal organ of Masera (Rodolfo-Masera, 1943; Weiler & Farbman, 2003). These subsystems can be distinguished by the location of their sensory neurons in the nasal cavity, the receptors they use to detect chemosensory stimuli, the signaling mechanisms they employ to transduce those stimuli, and their axonal projections to specific regions of the olfactory forebrain (Munger et al., 2009). In higher structures of the central nervous system (CNS) sensory inputs by the olfactory subsystems likely converge to the same subnuclei where odor-driven activity is represented and integrated (Pérez-Gómez et al., 2015).
In recent years, the traditional separation between the MOE and VNO as detectors of general odors and semiochemicals, respectively (Scalia & Winans, 1975), has been challenged by a number of studies that show overlapping activation of both (Lin et al., 2005; Peter A. Brennan & Zufall, 2006; Spehr, Kelliher, et al., 2006; Spehr, Spehr, et al., 2006; Wang et al., 2006; Xu et al., 2005).

### 1.1 Main olfactory system

The main olfactory system comprises the MOE as the peripheral sensory structure and the main olfactory bulb (MOB) in the forebrain as the first processing station in the CNS. The MOE is located in the posterior nasal cavity where it lines the dorsal nasal septum and the dorsolateral surface of the endoturbinates. The MOE contains three principal cell types: olfactory sensory neurons (OSNs), sustentacular cells, and basal cells, the precursors of OSNs (Graziadei & Graziadei, 1979; Moulton & Beidler, 1967). OSNs have a bipolar anatomy. An apical dendrite terminating in a knob-like structure carries 20-30 cilia that extend into the olfactory mucus. Ciliary membranes are the site of olfactory signal transduction and are therefore characterized by highly enriched expression of odorant receptor (OR) and other transduction cascade proteins (Menco, 1997). At the basal pole, a thin unmyelinated axon emanates and projects to the MOB via the basal lamina and the cribiform plate (Levai et al., 2003; Mombaerts, 2006; Mombaerts et al., 1996) (Figure 1.3).

In 2004, Linda Buck and Richard Axel were jointly awarded the Nobel prize for Physiology or Medicine for their discovery of the OR gene family (Buck & Axel, 1991). ORs belong to the rhodopsin-receptor like family (family A) of G-protein coupled receptors (GPCRs) (Bockaert & Pin, 1999; Graul & Sadée, 2001; Joost & Methner, 2002) and constitute the largest gene superfamily in the vertebrate genome (Zhang & Firestein, 2002). Only one of ~1,000 functional receptor genes is thought to be expressed in each OSN in a mutually exclusive, monoallelic fashion (‘one neuron – one receptor’ rule) (Lewcock & Reed, 2004; S Serizawa et al., 2000). This popular notion has been
challenged, however, and a developmental phase of oligogenic expression was proposed (Mombaerts, 2004b). Recent studies using single-cell transcriptomics support this hypothesis (Hanchate et al., 2015; Tan et al., 2015). Nevertheless, expression of OR genes in mature OSNs is restricted to one per neuron. Here, this singular expression is stabilized by epigenetic mechanisms (Lyons et al., 2013). In recent years, a distinct class of GPCRs, trace amine-associated receptors (TAARs), have been shown to function as chemosensory receptors in olfactory sensory neurons (Liberles & Buck, 2006). Additionally, a small number of “necklace” OSNs utilize a noncanonical guanylyl cyclase-D (GC-D)-mediated pathway (Juilfs et al., 1997). Recently, however, necklace OSNs have been shown to express a previously unidentified non-GPCR class of chemoreceptor encoded by the Ms4a gene family (Greer et al., 2016) whose members are not expressed in the conventional one-receptor-one-neuron pattern.

In the MOB, OSN axons terminate in discrete spherical neuropil structures (50-100 µm diameter) termed glomeruli in a receptor-dependent pattern: the axons of all OSNs expressing a given receptor coalesce and terminate in 1-2 glomeruli per bulb (Figure 1.3) (Imai & Sakano, 2007; Shou Serizawa et al., 2006). Here, they form synaptic connections with mitral cell (MC) and tufted cell (TC) dendrites, the MOB’s principle cells. This way, a chemotopic map is formed on the MOB surface that provides the anatomical substrate for functional integration of OR-specific sensory information.

1.1.1 Architecture of the MOB

In the MOB, MCs and TCs are distinguished by their soma location and dendritic morphology, as well as their response profiles and axonal targeting (De Saint Jan et al., 2009; Haberly & Price, 1977; Igarashi et al., 2012; Mori et al., 1983; Orona et al., 1984). While the prevalent view postulates monosynaptic transmission from OSNs to MCs (Najac et al., 2011), increasing evidence points towards an indirect activation of MCs via TCs (Gire et al., 2012). Additionally, excitatory synaptic transmission from OSNs is not restricted to output neurons (Ennis et al., 1996; Keller et al., 1998). Extensive microcircuit formation between principal neurons and local interneurons further complicates network architecture within the MOB, making it the major site of integration for olfactory information. Most synaptic contacts between neurons in the MOB occur at reciprocal dendrodendritic synapses between different neuron types (Gire & Schoppa, 2009; Rall & Shepherd, 1968). At their apical tufts, mitral and tufted cells (M/TCs) form dendrodendritic synapses with juxtaglomerular neurons, such as periglomerular cells (PGCs) and short axon cells (Getchell & Shepherd, 1975; Kiyokage et al., 2010). These synapses mediate intraglomerular feedback inhibition and modulate M/TC activity within the glomerular layer (GL) (Dong et al., 2007). Here, additional reciprocal dendrodendritic connections between PGCs are implicated in temporal patterning of odor-evoked activity of M/TCs (Cang & Isaacson, 2003; Murphy et al., 2005). Contrary to their name, excitatory short axon cells extend their axons over distances of up to 1 mm and thus contact inhibitory PGCs in 2-4 glomeruli that may be located up to 20-30 glomeruli apart, thus providing center–surround inhibition among glomeruli (Aungst et al., 2003; Haberly & Price, 1977). Deeper in the MOB, a second inhibitory circuit is formed via dendrodendritic synapses between M/TC lateral dendrites and axonless inhibitory granule cells (GCs) that are located beneath the mitral cell layer (G M Shepherd, 1963; Gordon M. Shepherd et al., 2007; Haberly & Price, 1977; Rall & Shepherd, 1968). In addition to lateral inhibition providing contrast enhancement deep within the MOB (Aungst et al., 2003; Egger et al., 2003; Willhite et al., 2006), activation of GCs by MCs thus provides recurrent inhibition (Arevian et al., 2008; J S Isaacson & Strowbridge, 1998; Koukakov & Rinberg, 2011; Gordon M. Shepherd et al., 2007).

1.1.2 Downstream processing

Principal neurons in the MOB project to downstream centers in the ipsilateral hemisphere via the lateral olfactory tract (LOT). Regions receiving direct input from the MOB have been collectively termed primary olfactory cortex and include the piriform cortex (PIR), entorhinal cortex (EC), anterior
olfactory nucleus (AON), olfactory tubercle (OT), and lateral amygdala (LA) (Figure 1.4) (Ghosh et al., 2011; Miyamichi et al., 2011; Shipley & Ennis, 1996; Sosulski et al., 2011).

In the AON, M/TC axons topographically project to the pars externa with a matched dorsal-to-ventral pattern of projections (Lanuza & Halpern, 1998; Yan et al., 2008). Relative to M/TCs, however, AON neurons display a broader response profile, suggesting that individual AON cells integrate olfactory input from different M/TC classes (Lei et al., 2006). The AON provides feedforward modification of MOB-to-PIR information flow and is critical for the bilateral relay of olfactory signals (Hagiwara et al., 2012; Yan et al., 2008).

The piriform cortex is an archicortical region (Luskin & Price, 1983) with an extensive local associational network that constructs representations of odor identity. It is the largest and best-studied region of the olfactory cortex. Its trilaminar structure is heavily innervated by the olfactory bulb (Jeffry S. Isaacson, 2010; Wilson & Sullivan, 2011; Wilson et al., 2006). Odor responses are spatially distributed across the PIR, indicating that odor-evoked responses form overlapping ensembles (Illig & Haberly, 2003; Stettler & Axel, 2009).

The OT receives direct input from the OB, as well as extensive inputs from the other parts of olfactory cortex, including the PIR and cortical amygdala (CoA) (Scott et al., 1980). While MCs innervate large OT areas, external TCs preferentially target the anterolateral OT (Igarashi et al., 2012). Moreover, the OT is implicated in multimodal sensory processing (Wesson & Wilson, 2010). Both, PIR and the OT largely discard any topography present in the bulb.

MOB MCs also directly innervate the LA. Here, individual genetically identified glomeruli were found to project to focal and spatially stereotyped regions of the CoA (Sosulski et al., 2011) and infection of neurons within the CoA with a trans-synaptic retrograde virus revealed a higher overall density of innervation from the dorsal bulb (Miyamichi et al., 2011).

Finally, the EC represents the most caudal region of the olfactory cortex and receives direct bulbar input in addition to input from PIR. Despite strong feedback connections from the EC targeting both the MOB and PIR (Ferry et al., 2006; Moully et al., 2001), surprisingly little is known about olfactory sensory processing in this area that is largely known as a hub in a widespread network for memory and navigation (Sasaki et al., 2015).

1.2 Accessory olfactory system

In most vertebrates, an accessory olfactory system (AOS) plays a major role in detecting semiochemicals that convey information about the emitter’s identity, gender, social rank, and sexual
Introduction Accessory olfactory system

state (Ferrero et al., 2013; Luo et al., 2003; Peter A Brennan & Kendrick, 2006; Peter A. Brennan & Zufall, 2006; Tirindelli et al., 2009). It comprises the VNO as a peripheral sensory organ and the accessory olfactory bulb (AOB) as the first central processing station (Figure 1.5). The VNO was first described by Ludvig Jacobson in 1813 (Jacobson, 1813) and is therefore also referred to as the organ of Jacobson. He provided a detailed anatomi cal description of the organ in a variety of species but could only speculate about its function at the time. While a functional VNO is found in most amphibians, reptiles, and mammals (Heather L. Eisthen, 1992), old world monkeys, gorillas, chimpanzees and orangutans lack a functional VNO (Bhatnagar & Meisami, 1998). The VNO comprises a bilateral blind-ending tube that is enclosed in a cartilaginous capsule and is located at the base of the nasal septum (Jacobson et al., 1998; Keverne, 1999). A medial crescent-shaped sensory epithelium harbors the vomeronasal sensory neurons (VSNs), while a non-sensory epithelium lines the lateral wall. In between, a mucus-filled lumen is connected to the vomeronasal duct. Upon stimulation, a large blood vessel on the lateral side that is innervated by the autonomic nervous system begins to constrict and dilate. Upon constriction, negative pressure is generated in the VNO lumen, facilitating the transport of semiochemicals into the lumen via the narrow vomeronasal duct (DYGĐŶŶĞůů͕1979; Pankevich, Baum, & Cherry, 2003). This pumping mechanism allows for relatively large, non-volatile semiochemicals such as peptides or proteins to reach the vomeronasal sensory epithelium. Similar to the MOE, the vomeronasal sensory epithelium harbors two additional non-neuronal cell types: sustentacular cells and basal cells, having a supporting role and serving as VSN precursors, respectively (Ghiaroni et al., 2003; Keverne, 1999).

VSNs are bipolar neurons that extend an apical dendrite towards the lumen, where they terminate in a microvillous dendritic knob. Unmyelinated axons emanate from the basal poles and fasciculate to form the vomeronasal nerve (Figure 1.5). The vomeronasal nerve exits the cartilaginous capsule and ascends along the septum before it passes the cribriform plate. From here, VSN axons further course along the medial surface of the olfactory bulb in order to reach the AOB which is located dorsocaudally to the MOB (Belluscio, Koentges, Axel, & Dulac, 1999; Del Punta, Puche, Adams, Rodriguez, & Mombaerts, 2002; Rodriguez, Feinstein, & Mombaerts, 1999).

Vomeronasal receptors are GPCRs, which are distinct from the OR superfamily, and cluster in three classes: vomeronasal receptor type 1 (V1R), vomeronasal receptor type 2 (V2R), and formyl peptide receptors (FPR). V1Rs belong to GPCR family A, which contains >130 functional V1R genes, as well as ~150 pseudogenes. V1Rs are subdivided into 12 remarkably diverse subfamilies (V1ra-I) with 1-30 members (Rodriguez, Del Punta, Rothman, Ishii, & Mombaerts, 2002). Until today, V1R ligand identity remains largely unknown with only one receptor-ligand pair having been described so far (Boschat et al., 2002; Novotny, 2003). Structurally unrelated to V1Rs, the V2R genes encode family C GPCRs. The superfamily comprises ~120 functional genes and over 150 pseudogenes (Yang et al., 2005; Young & Hammock, 2007a, 2007b). V2Rs are characterized by a long hydrophobic extracellular N-terminal domain (Mombaerts, 2004a) that is thought to form the ligand binding site. In this family, functional genes are subdivided into four families (V2ra-d) with 95% of all V2Rs belonging to the families A, B, and D (Silvotti et al., 2007; Young & Hammock, 2007b). Members of the fourth family (C, also referred to as V2R2) are phylogenetically distinct from the other families and resemble mammalian Ca^{2+} sensors (Martini et al., 2001; Ryba & Tirindelli, 1997). While V2R ligand identity remains largely unknown, V2R-expressing neurons are implicated in the detection of non-volatile peptides or proteins. These ligands are derived for example from urine, skin, scent glands, or secretions from reproductive organs. Due to their non-volatile nature, direct physical contact between the nose and the stimulus source is necessary for VSN activation (Chamero et al., 2007; Haga et al., 2010; Kimoto et al., 2005; Leinders-Zufall et al., 2004, 2009). More recently, FPRs have been described as a third class of vomeronasal receptors (Liberles et al., 2009; Rivière et al., 2009). FPRs belong to the family A of GPCRs and were known from their expression in the immune system where they are activated by microbe- and / or
host-derived inflammation-associated metabolites such as formylated peptides (Migeotte et al., 2006; Schiffmann et al., 1975). In such, they serve as key mediators of leukocyte chemotaxis (H.-Q. He et al., 2013; Rivièrè et al., 2009). The FPR gene family comprises 7 members, 5 of which (FPR-rs1, rs3, rs4, rs6 and rs7) are predominantly or exclusively expressed in subsets of VSNs (Chamero et al., 2012; Liberles et al., 2009; Rivièrè et al., 2009). Similar to FPRs in the immune system, vomeronasal FPRs are suggested to detect inflammation-associated compounds, and to thus mediate identification of unhealthy conspecifics (Bufo et al., 2012; Liberles et al., 2009; Rivièrè et al., 2009).

VSNs are distinguished into two classes that form two distinct zones in the vomeronasal epithelium: apical and basal VSNs. The two classes are characterized by differential expression of receptor families and signaling components, as well as their discrete projections to the AOB. Apical VSNs are located to the more luminal side of the sensory epithelium and are characterized by the monoallelic expression of one member of the V1R family (Dulac & Axel, 1995; Rodriguez et al., 1999). In these neurons the vomeronasal receptor is coupled to the G protein Gα12 (Berghard & Buck, 1996; Mimi Halpern et al., 1995) and their axons project to the anterior part of the AOB (aAOB) (Belluscio et al., 1999; Del Punta et al., 2002). Basal VSNs, on the other hand, express V2Rs together with Gαo (Berghard & Buck, 1996; Mimi Halpern et al., 1995) and project to the posterior AOB (pAOB) (Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). Like apical VSNs, basal VSNs express a member of either subfamily A, B, or D of the V2R family in a monogenic, monoallelic fashion. Additionally, however, family-C receptors are coexpressed in all basal neurons, thus forming an exception to the one neuron – one receptor rule (Leonardo Belluscio, Gold, Nemes, & Axel, 1998; Bozza, Feinstein, Zheng, & Mombaerts, 2002; Rodriguez et al., 1999). Interestingly, Fpr-rs1 is expressed in basal sensory neurons, while the remaining vomeronasal Fpr-rs genes are all expressed in the apical layer of the VNO sensory epithelium (Liberles et al., 2009; Munger et al., 2009; Rivièrè et al., 2009).

1.2.1 Architecture of the AOB
Axons from both basal and apical VSNs project to the AOB in a topographic manner: while basal sensory neurons terminate in the pAOB, apical VSNs terminate in the aAOB. Similar to OSN axons in the MOB, they synapse onto the AOB’s principal neurons, AOB mitral cells (AMCs). There are, however, some fundamental differences between glomerular organization and architecture in the main and accessory olfactory bulbs. First, VSNs expressing the same receptor project to multiple glomeruli (Belluscio et al., 1999; Del Punta et al., 2002; Rodriguez et al., 1999; Wagner, Gresser, Torello, & Dulac, 2006). As a result, the axons from only a few hundred VSNs terminate in each glomerulus (C Dulac & Wagner, 2006; Hildebrand & Shepherd, 1997; Meisami & Bhatnagar, 1998). Second, glomeruli in the MOB are uniform in size (~50 μm in diameter) and lie side by side along the 1–2 glomeruli-deep glomerular layer, while periglomerular neurons and glial cells provide a clear anatomical and functional separation between individual glomerular units (Kosaka & Kosaka, 2005). In contrast, glomeruli in the AOB are smaller and variable in size (10-30 μm in diameter), are surrounded by only few PGCs, and are diffusely
organized (GL in Figure 1.6) (Meisami & Bhatnagar, 1998). The biology of PGCs in the AOB is almost unexplored, and any functional analogy with MOB juxtaglomerular neurons, which include periglomerular, short-axon, and external-tufted cells, is mostly speculative (C Dulac & Wagner, 2006). Nevertheless, some studies have shown the juxtaglomerular neuronal population in the AOB to include both GABAergic and glutamatergic neurons (Goldmakher & Moss, 2000; Quaglino et al., 1999; Shigeru Takami et al., 1992), whereas, in contrast to the MOB, dopaminergic neurons do not seem to be included (Meisami & Bhatnagar, 1998).

In the AOB, mitral cells do not form a distinct monolayer as their counterparts in the MOB do (MCL in Figure 1.6). Moreover, AMC morphology is strikingly different from their MOB counterparts. While MOB MCs extend one apical dendrite into the GL receiving receptor-specific sensory input from only one glomerulus (Schoppa & Urban, 2003), AMCs are smaller (Zibman et al., 2011) and extend several apical dendrites to contact 3 to 9 glomeruli in various locations of the GL (Larriva-Sahd, 2008; Takami & Graziaidei, 1991; Shigeru Takami & Graziaidei, 1990). Moreover, in contrast to MOB MCs, dendritic tufts of AMCs are highly variable in shape and size. In addition to apical dendrites, 1-2 lateral dendrites have been described to emanate from AMC somata. In the AOB, the LOT separates the MCL from the GCL. Similar, to the MOB, GCs in the AOB, spiny interneurons that lack an axon (Larriva-Sahd, 2008), form recurrent dendrodendritic synapses with AMCs (Castro et al., 2007; Jia et al., 1999; Taniguchi & Kaba, 2001). A controversy regarding the identity of sensory input to AMCs has yet to be resolved: whether AMCs receive homotopic input, similar to MCs in the MOB (Del Punta et al., 2002), or heterotopic input (L Belluscio et al., 1999; Wagner et al., 2006) is a matter of debate. A “mixed” model suggests that AMCs might receive input from multiple glomeruli, some of which receive input from VSNs with the same receptor identity, while others receive input from mixed VSN populations (Kevenere, 1999).

To date, central aspects of AOB physiology remain largely unexplored and functional analogies with neurons of the MOB are mostly speculative (C Dulac & Wagner, 2006). The morphological differences between the two processing centers, however, indicate that sensory computation in the AOB is fundamentally different compared to the MOB (Catherine Dulac & Torello, 2003). While important insights into the AOB’s connectivity, sensory input, and sensory integration have been gained in recent studies (Ben-Shaul et al., 2010; Castro et al., 2007; Tolokh et al., 2013; Wagner et al., 2006; Del Punta et al., 2002; Hammen et al., 2014; Hovis, Ramnath, Dahlen, Romanova, LaRocca, et al., 2012; J. Ma & Lowe, 2004; Kahan & Ben-Shaul, 2016; Leszkowicz et al., 2012; Shpak et al., 2012; Smith & Araneda, 2010), many basic physiological principles underlying information processing in the AOB remain poorly understood.
1.2.2 Downstream processing

A distinctive feature of the AOS is that it bypasses olfactory cortex with AMC axons directly targeting limbic circuits (Figure 1.7). These include the medial amygdala (MeA/MeP) and the postero medial cortical nucleus (PMCN), collectively termed the vomeronasal amygdala, as well as the bed nucleus of the stria terminalis (BNST) and the nucleus of the accessory olfactory tract (NAOT) (C Dulac & Wagner, 2006; Keshavarzi et al., 2014). Initially segregated sensory information from apical and basal VSNs is likely integrated in higher limbic structures such as the NAOT, MeA/MeP, PMCN, and BNST (Martinez-Marcos & Halpern, 1999; Mohedano-Moriano et al., 2008; Salazar & Brennan, 2001; von Campenhausen & Mori, 2000). Moreover, the MeA does not only receive direct sensory input from the AOS, but also from the MOS, largely via odor-processing regions such as the olfactory cortical amygdala (CoA) (Dhungel et al., 2011; Haberly & Price, 1977; Kang et al., 2011). Principal neurons in the MeA differentially process these converging inputs in distinct dendritic compartments (Keshavarzi et al., 2015).

From the vomeronasal amygdala, information is relayed to the ventromedial and medial preoptic area (MPOA), the ventromedial hypothalamus (VMH), and the premammillary and supraoptic nuclei in the hypothalamus (Keventer & Winans, 1981; Petrovich et al., 2001), areas that are involved in endocrine responses essential to evoke reproductive, parental and other social behaviors (Boehm et al., 2005; Moffatt, 2003; Yoon et al., 2005). Pheromonal effects on a recipient’s endocrine status are mediated by a group of ~800 hypothalamic neurons secreting gonadotropin-releasing hormone (GnRH) (Michael Meredith, 1998). These neurons form a tremendous number of synaptic connections with over 50,000 neurons in over 50 brain areas with some of these connections exhibiting sexual dimorphism (Boehm et al., 2005). GnRH neurons are master regulators of the hypothalamic-pituitary-gonadal (HPG) axis that controls the timing of puberty and estrous (Liberles, 2014; Sisk & Foster, 2004). Secreted GnRH is transported to the pituitary via the hypophyseal portal system, a system of blood vessels that connects the hypothalamus with the anterior pituitary. Here, it regulates the synthesis and secretion of gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH), into the blood stream. LH and FSH, in turn, stimulate gonadal release of sex steroids (Clayton & Catt, 1981; Sisk & Foster, 2004).

The small number of synaptic connections between the VNO and limbic system, in conjunction with the circumvention of cortical areas by the AOS, suggest a hardwired circuit diagram. Consequently, the AOS is thought to mediate innate, genetically pre-programmed responses. Nonetheless, experience and learning are clearly evident in the accessory olfactory system (Brennan & Keverne, 1997). The Bruce effect is the most extensively characterized example for an AOS-mediated effect that requires learning. It refers to a pheromone-mediated pregnancy termination (“pregnancy block”) that occurs when a newly mated female encounters the odor of a stranger male prior to embryo implantation, and it requires a functional VNO, but not a functional MOE (Kelliher et al., 2006; Peter A. Brennan, 2009). This selective pregnancy failure indicates that a female can effectively establish an olfactory memory...
of her mate that can last for up to 30 days (Kaba et al., 1988) and allows her to distinguish his odor from that of a stranger. Mate memory formation occurs at the level of the AOB (Kaba et al., 1989) where it requires noradrenaline signaling (Kaba & Keverne, 1988).

1.3 Oscillations

The first neuronal oscillation described was an 8 to 12 Hz rhythm, the alpha waves of Berger, in a human electroencephalogram (EEG) (Berger H, 1929). Berger called this large-amplitude rhythm, which was induced by eye closure in the awake, calm subject, the “alpha” rhythm because he observed this rhythm first. He named the faster, smaller amplitude waves, present when the eyes were open, “beta” waves. Today, slow oscillations with a strong 0.75–4-Hz component are associated with certain stages of sleep, whereas oscillations dominated by the 14–40-Hz band are typical of active, awake states (Sejnowski & Destexhe, 2000).

Traditionally, correlated neuronal activity is viewed as an additional coding dimension for building internal representations of the outside world. A more recent idea suggests that correlated activity might gate the flow of neural information and might thus serve to regulate the flow of information rather than its meaning (Salinas & Sejnowski, 2001). Either way, oscillations are phylogenetically preserved and can be generated in many ways, driven either by mechanisms within individual neurons or by interactions between neurons. Usually, however, they emerge from a dynamic interplay between intrinsic cellular characteristics and network properties (Bartos et al., 2007; Llinás, 1988). In individual neurons, oscillations can appear as oscillations in membrane potential and / or as rhythmic patterns of action potentials which in turn produce oscillatory activation of post-synaptic neurons. Moreover, oscillations can be induced by external stimuli, or arise spontaneously. Today, it is widely recognized that the brain’s ability to generate and sense temporal information is a prerequisite for both action and cognition. This temporal information is embedded in oscillations that exist at many different time scales (Figure 1.8) (Tonetti et al., 1976).

![Figure 1.8 Oscillatory classes in the rat cortex.](image)

In each oscillatory category in the cerebral cortex the frequency ranges (“bandwidth”) overlap with those of neighboring classes so that frequency coverage is more than four orders of magnitude. Note the linear progression of frequency classes on the log scale. (Adapted from Buzsáki & Draguhn, 2004.)

Burst firing is associated with oscillatory activity and is often thought to represent a distinct mode of neuronal signaling. A dominant point of view is that bursts are needed to increase the reliability of communication between neurons (Lisman, 1997; Pena & Konishi, 2002). More recent studies propose that bursting might additionally provide effective mechanisms for selective communication between neurons (Izhikevich et al., 2003).

1.3.1 Intrinsic oscillations

Several brain regions exhibit characteristic oscillations that emerge from the activity of a relatively small neuronal population dictating the firing rhythm. Such pacemaker neurons possess an intrinsic ability to generate rhythmic activity resulting from voltage- and time-dependent ion fluxes (Arshavsky,
A dynamic interplay among ion channels, second messenger pathways and intracellular Ca^{2+} concentrations may be involved in generating such periodic ion fluxes. Intrinsically oscillating neurons have been described to drive oscillatory circuits that play important roles in sensory perception, attention, memory formation, and decision making (Gutierrez et al., 2013). Moreover, a variety of rhythmic behaviors is reported to be controlled by such neurons (Bucher et al., 2006; Koizumi & Smith, 2008; Peña et al., 2004; Tazerart et al., 2008).

### 1.3.2 Network oscillations

Most neuronal oscillations emerge from changes in network activity that produce synaptic barrages of excitation and inhibition. Additionally, electrical coupling via gap junctions can aid to synchronize electrical activity (Bennett & Zukin, 2004). Oscillations with higher frequencies are usually confined to a small neuronal space, whereas slow oscillations can recruit very large networks (Csicsvari et al., 2003; M. Steriade, 2001). Network oscillations have been identified in a plethora of brain regions that include the thalamus, hippocampus, and cortex (Buzsáki & Draguhn, 2004) as well as the olfactory bulb (Bathellier et al., 2006; Fukunaga et al., 2012; Hayar et al., 2004; Lagier et al., 2007; Liu & Shipley, 2008; Schaefer et al., 2006). Moreover, spontaneous correlated neuronal activity represents a hallmark of the developing central nervous system (Hanganu-Opatz, 2010). The benefits of a particular oscillation depend on the function of the brain system in which it occurs. There are, however, some general principles regarding the function of oscillations, some of which are independent of the involved brain regions. Network oscillations may serve to transiently synchronize neurons by dynamic connections (Engel et al., 2001; Varela et al., 2001), or to refine input selection by phase biasing the open-time probability of a multitude of voltage-gated channels (Hutcheon & Yarom, 2000; Llinás, 1988). Moreover, oscillations play a role in memory formation, as well as in recalling stored information (Lisman, 1999) and may serve to represent information by phase for short-term storage of information (Buzsáki & Chrobak, 1995; Lisman & Idiart, 1995).

### 1.3.3 Olfactory oscillations

Various types of oscillations have been described in the MOB. These differ in frequency, and in the circuits and behavioral circumstances that produce them.

Gamma oscillations are the fastest oscillations described in the olfactory system and are the best studied. They are evoked by sensory stimulation and are initiated at the end of the inhalation cycle at ~40–100 Hz (Rojas-Libano & Kay, 2008). The dendrodendritic synapse between mitral and granule cells has been shown to support olfactory gamma oscillations (Halabisky & Strowbridge, 2003; Lagier et al., 2007; Neville & Haberly, 2003; Schoppa, 2006). During exploratory behavior correlated oscillations can be seen in the PIR, particularly when MOB gamma is large. Functionally, the power of odor-evoked gamma oscillations is associated with successful discrimination of closely related odorants (Beshel et al., 2007; Nusser et al., 2001).

Beta oscillation episodes (~15–30 Hz) can last on the scale of 2–4 inhalation cycles, and have been associated with some types of odor learning and odor sensitization (Gervais et al., 2007; Martin et al., 2007). Beta oscillations are seen in the MOB, PIR, EC and hippocampus of awake rats during exposure to volatile odorants (Lowry & Kay, 2007; Vanderwolf & Zibrowski, 2001) and are involved in entorhinal drive to the MOB in anticipation of odor stimuli (Kay & Stopfer, 2006). Beta oscillations are a network phenomenon that requires an intact MOB-PIR loop. Their cellular or synaptic origins, however, are not known.

Theta oscillations (~1-12 Hz) are driven by sensory input and are also called respiratory oscillations (Kay & Stopfer, 2006). Low-frequency burst firing of external tufted cells in the glomerular layer can support theta oscillations (Hayar et al., 2004) and has been attributed a role in setting sniff cycle-
Intrinsic rhythmogenesis in the accessory olfactory bulb

At the lower end of the time scale, oscillations extend into the slow (0.1–1 Hz) and infraslow (<0.1 Hz) range (Schroeder & Lakatos, 2009). Recently, we have shown that a group of AOB mitral cells is intrinsically rhythmic and exhibits infraslow stereotypical rhythmic discharge in absence of fast synaptic drive (Gorin, Tsitoura et al., 2016). Here, we sought to uncover the mechanistic basis behind intrinsic rhythmicity, and found that reciprocal interactions between depolarizing and hyperpolarizing conductances generate stable patterns of membrane potential (V_{mem}) oscillations within a broad spectral window.

The hyperpolarization-activated current (I_{h}), the low-voltage-activated T-type Ca^{2+} current (I_{T}), and the TTX-sensitive persistent Na^{+} current (I_{NaP}) are prototypical depolarizing pacemaker currents and, thus, major determinants of autorhythmicity (Chan et al., 2004; Crill, 1996; Perez-Reyes et al., 1998). I_{h}, in particular, is a predominant driving force of rhythmic oscillatory activity (Liu & Shipley, 2008; Maccaferri & McBain, 1996). Therefore, we tested whether any of these is involved in intrinsic rhythmogenesis in AOB projection neurons. We found that intrinsically oscillating AMCs (iAMCs) do not express I_{T}. I_{h}, on the other hand, was measured as a rebound depolarization (sag) at hyperpolarized potentials. However, pronounced voltage sags were only detected upon membrane hyperpolarization to values substantially more negative than the average downstate potential (V_{d}), indicating that I_{h} serves a minor, if any, role in iAMC rhythmogenesis. Turning towards I_{NaP}, we found that, in addition to disrupting action potential discharge, incubation with TTX also abolishes subthreshold V_{mem} oscillations (Figure 1.9 Ai) resulting in stable V_{mem} values that are statistically indistinguishable from V_{d} (Figure 1.9 Aii). In AMCs, TTX-sensitive I_{NaP} is first evident at ~-75 mV and steeply increases with voltage (Figure 1.9 Aiii). Moreover, we demonstrated that a hallmark of I_{NaP}, namely a shift in voltage dependence caused by changes in extracellular Ca^{2+} concentration ([Ca^{2+}]_{ex}) (Su et al., 2001) (Figure 1.9 Aiv, v), results in altered oscillation patterns (Figure 1.9 Bi, ii). In such, we identified I_{NaP} as a major determinant of autorhythmicity in the iAMC population.
**Figure 1.9** L_{Na_P} is a major determinant of iAMC autorhythmicity. A(i). Representative whole-cell current-clamp recording under isolation from fast synaptic input (gabazine + APS + NBQX) in absence and presence of TTX (300 nM; horizontal red bar). A(ii), Bar diagram quantifying average $V_{mem}$ values ($n = 13$) before (black; $V_d = -77.2 \pm 0.9$ mV; $V_h = -65.8 \pm 0.8$ mV) and after TTX incubation (red; $-73.5 \pm 0.8$ mV). Data are means $\pm$ SEM. Asterisks (*) denote statistical significance, $p < 0.0001$ (Kruskal-Wallis test); n.s., not significantly different. A(iii), Isolation of L_{Na_P}: representative i-V relationships of average whole-cell currents (3 consecutive recordings) evoked by a slow (10 mV/300 ms) ascending voltage ramp from -93 to -53 mV (inset) in absence (black trace) and presence (gray trace) of TTX (300 nM). When data recorded during drug treatment were digitally subtracted from control recordings and plotted as a function of the command voltage, the TTX-sensitive negative slope current becomes apparent (red trace). Note the TTX-insensitive linear leak current. A(iv), At different $[\text{Ca}^{2+}]_{o}$, TTX-sensitive currents (revealed by off-line subtraction) were converted to conductances and normalized quasi-steady-state activation curves were fit by the Boltzmann equation (0.1 mM $\text{Ca}^{2+}$) or extrapolated from monoexponential functions (solid lines on top of shaded traces). Dashed horizontal red line denotes a 5% conductance threshold. The gray rectangle indicates the “oscillatory” $V_{mem}$ range delimited by average $V_d$ and $V_h$ values. A(v), Bar chart depicting $V_{mem}$ values ($n = 7$; means $\pm$ SEM) that correspond to 5% activation of persistent Na$^+$ conductance. Data are plotted as a function of $[\text{Ca}^{2+}]_{o}$: -80.2 $\pm$ 0.4 mV (0.1 mM; orange); -73.4 $\pm$ 2.4 mV (0.5 mM; green); -70.3 $\pm$ 2.2 mV (1 mM; black); -65.2 $\pm$ 2.2 mV (5 mM; gray). Asterisks (*) denote statistical significance, $p < 0.05$ (one-way ANOVA with Tukey’s HSD post hoc test). B(i), B(ii), Original whole-cell current-clamp recordings from two representative iAMCs at different $[\text{Ca}^{2+}]_{o}$ levels (preincubation, $\sim$3 min). B(il), Note that oscillations recover after restoration of physiological $[\text{Ca}^{2+}]_{o}$. B(ii), Bar chart illustrating IBs as a function of $[\text{Ca}^{2+}]_{o}$. Data ($n = 5$; means $\pm$ SEM) are normalized to control conditions (1 mM $\text{Ca}^{2+}$): 0.44 $\pm$ 0.15 (0.1 mM; orange); 0.88 $\pm$ 0.14 (0.5 mM; green). Asterisk (*) denotes statistical significance, $p < 0.05$ (one-way ANOVA with Tukey’s HSD post hoc test). (Gorin, Tsitoura et al., 2016)

Next, we investigated the mechanistic basis of burst firing during the depolarizing envelope of iAMCs. While the predominant role of L_{Na} in AMC spike generation is well established (Castro et al., 2007; Shpak et al., 2012; Smith & Araneda, 2010), voltage-gated Ca$^{2+}$ (Cav) channels additionally shape various neuronal discharge parameters (Bean, 2007). While L-type, P-/Q-type, and N-type Ca$^{2+}$ currents were isolated using selective dihydropyridine or peptide toxin Cav-channel antagonists (Adams et al., 1993; Bossert & Vater, 1989; McCleskey et al., 1987), none of the tested channel blockers altered iAMC autorhythmicity. Only addition of SNX-482, a selective R-type Ca$^{2+}$ channel inhibitor (Bourinet et al., 2001), altered iAMC oscillations. Therefore, only R-type/Cav2.3 channels play a significant role in orchestrating autonomous iAMC oscillations. In search of the conductances responsible for burst termination in AMCs we asked whether Ca$^{2+}$-activated K$^+$ conductances might be involved. Underlying the afterhyperpolarization that follows single spikes or bursts, for example in the
hippocampus (Alger & Nicoll, 1980), small conductance $K_{Ca}$ (SK) channels are major determinants of firing rate. The selective SK channel antagonist apamin (Blatz & Magleby, 1986), however, does not affect rhythmic iAMC discharge. BK channels, which are cooperatively activated by depolarization and increased cytoplasmic $Ca^{2+}$ (Fakhler & Adelman, 2008), and are inhibited by both TEA and the selective organic blocker paxilline (Brenner et al., 2005) were identified to regulate burst duration via a negative feedback loop. In iAMCs, oscillatory discharge reversibly switched to irregular tonic firing by incubation with relatively low TEA concentrations (1 mM) (Figure 1.10 A). This suggested that coupling of $Ca^{2+}$ entry to activation and “build up” of $I_{BK}$ might cause burst termination and, consequently, the following transition to $V_d$. Changes in iAMC rhythmogenesis during incubation with low micromolar concentrations of paxilline (Figure 1.10 B) corroborated our hypothesis.

Figure 1.10 Functional interaction of $Cav$ and BK channels determines burst properties. A(i), Representative $V_{nem}$ recording illustrating oscillatory activity before and after, as well as irregular discharge during wash-in of relatively low TEA concentrations (1 mM; horizontal bar indicates drug incubation). A(ii), A(iii), Expanded view of periodic bursting before (A(ii)) and irregular activity during (A(iii)) TEA treatment, respectively. Rhythmicity (as well as the lack thereof) is also evident from the corresponding $V_{nem}$ all-points histograms. Red arrowheads indicate $V_{nem}$ states ($V_d$, $V_{U}$) under synaptic activation. B(i), Representative $V_{nem}$ recording of oscillatory discharge before (top) and during (bottom) paxilline treatment (5 μM; preincubation, >3 min). B(ii), Bar charts comparing iAMC discharge parameters ($n = 8$) under conditions of synaptic isolation (iso; black) versus additional inhibition of BK channels (paxilline; green). IBIs, burst durations, within-burst firing rates, and $V_{nem}$ states are plotted as means ± SEM. Paxilline treatment shortened IBIs ($13.9 ± 2.0$ vs $8.7 ± 0.8$ s) and increased burst durations ($5.5 ± 1.1$ vs $10.6 ± 1.9$ s). Firing frequencies ($3.4 ± 0.6$ vs $3.9 ± 0.5$ Hz) as well as $V_d$ and $V_{U}$ (-75.6 ± 0.8 vs -75.1 ± 1.4 mV; -65.2 ± 0.9 vs -62.0 ± 1.1 mV) were essentially unaffected. Asterisks (*) denote statistical significance, $p < 0.05$ (paired sample t-test). (Gorlin, Tsitoura et al., 2016)

In summary, rhythmogenesis in iAMCs appears to be driven by cyclic interplay of ≥3 voltage-dependent and / or $Ca^{2+}$-dependent conductances. Here, low threshold $I_{NaP}$ functions as the major excitatory element that drives iAMC transition from $V_d$ to $V_U$. R-type $Cav$ channels play a significant role in oscillation maintenance and shape, while the resulting increase in cytoplasmic $Ca^{2+}$ is coupled to progressive activation of BK channels which, in concert with slow voltage-dependent $I_{NaP}$ inactivation (Jasinski et al., 2013), ultimately causes burst termination. A recent study has reported phenotypically similar oscillations in AMCs of the adult rat (Vargas-Barroso et al., 2015), suggesting that they are functionally relevant.


**Aims**

For the majority of vertebrates, the sense of smell is a crucial sensory modality. The olfactory system plays a critical role in mediating diverse social behaviors. To be able to identify the vast variety of environmental chemical cues, the olfactory system of rodents has evolved into several subsystems. Most vertebrates possess an accessory system that plays a major role in the detection of semiochemicals that convey information about the emitter’s identity, gender, social rank, and sexual state. Bypassing the thalamocortical axis, the AOS is unique in that merely three synapses link sensory input to behavioral output. Thus, the AOS directly targets limbic circuits and controls an animal’s endocrine status. Receiving sensory information from peripheral vomeronasal neurons, the AOB is the first stage of AOS information processing. Therefore, the AOB is a major site of vomeronasal integration and information processing. Recently, we have shown that a group of AMCs, the AOB sole output neurons, is intrinsically rhythmogenic and exhibits spontaneous infraslow rhythmic discharge. To what extent intrinsic AMC rhythmogenesis shapes information processing and sensory coding in the AOB, however, remains unexplored.

The primary aim of my thesis is to gain a deeper understanding of the impact that iAMC oscillatory discharge exerts on the AOB network both in vitro and in vivo. Pursuing four lines of research, I aim to investigate how rhythmonic neurons differ from non-rhythmogenic neurons, and how intrinsic rhythmogenesis effects spontaneous activity in the remaining population. To target these issues, I will employ electrophysiological, pharmacological, computational, and imaging approaches.

First, in collaboration with Yoram Ben-Shaul (The University of Jerusalem), I will investigate spontaneous AOB neuron activity in vivo. Therefore, we will perform extracellular recordings of spontaneous activity from the AOB mitral cell layer over prolonged periods. Subsequently, I will analyze spike patterns in an attempt to classify rhythmic activity according to various physiological parameters.

Second, I will assess possible differences in the biophysical properties of rhythmogenic versus non-rhythmogenic AMCs in vitro. Upon characterization of basic passive and active properties, I will test whether or not \( I_{\text{NaP}} \), \( I_R \), and \( I_{\text{K}} \), the conductances driving intrinsic rhythmogenesis, are unique properties of iAMCs. Therefore, I aim to compare electrophysiological profiles of these currents in both AMC populations. Subsequently, taking a computational approach, I aim to investigate whether the interplay of \( I_{\text{NaP}} \), \( I_R \), and \( I_{\text{K}} \) is sufficient to drive autorhythmicity. This analysis will be done computationally in collaboration with Simon O’Connor (University of Hertfordshire).

Third, I will analyze differences in synaptic connectivity and synaptic drive in iAMCs and non-iAMCs. Rhythmic output of intrinsically oscillating AMCs, might periodically entrain neurons in the local network. Using pharmacology, I will dissect the nature of AMC synaptic drive and attempt to classify AOB projection neurons into subpopulations based on their functional wiring.

Fourth, I aim to take my investigation from a single-cell to the population level by establishing an experimental framework for paired patch-clamp recordings and by using a calcium imaging approach. Here, I will investigate whether and, if so, how heterogeneity of infraslow intrinsic AMC rhythmicity shapes spontaneous activity throughout the AMC layer and, thus, whether AMC oscillations in single neurons may synchronize, or interfere in a constructive or destructive manner.
2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Amplifier EPC-10 HEKA Elektronik
Amplifier EPC-10 USB double HEKA Elektronik
Binocular eyepiece S4E Leica Microsystems
CCD camera DFC350 FX Leica Microsystems
CCD camera DFC360 FX Leica Microsystems
Centrifuge mini spin Eppendorf AG
Cryostat CM 1950 Leica Microsystems
Hot plate magnetic stirrer Snijders
Microforge MF-830 Narishige
Micromanipulator Luigs-Neumann Device SM-5 Luigs & Neumann
Micropipette puller PC-10 vertical two-step puller Narishige
Microscopes Leica Microsystems
Leica DM6000 FS
Leica DM LFSA
Leica Multiphoton SP8
Noise eliminator 50/60 Hz (HumBug) Quest Scientific
Objectives Leica Microsystems
5x (N PLAN 5x/0.12)
10x (HC PL FL 10x/0.30 PH1)
10x (HCX APO L U-V-I 10x/0.30)
20x (HCX APO L U-V-I 20x/0.50)
25x (HCX IRAPO L25x/0.95 W)
25x (Fluotar VISIR/0.95 W)
63x (HCX APO L U-V-I 63x/0.90)
Oscilloscope TDS 1001B Tektronik
Osmometer osmomat 030 Gonotec
Perfusion system 8-in-1 AutoMate Scientific
pH electrode InLab routine Mettler Toledo
Materials and Methods

<table>
<thead>
<tr>
<th>Equipment/Chemical/Inhibitor</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Meter five easy</td>
<td>Mettler Toledo</td>
</tr>
<tr>
<td>Prior Lumen 200 Fluorescence Illumination System</td>
<td>Prior Scientific</td>
</tr>
<tr>
<td>ProScan™III control unit</td>
<td>Prior Scientific</td>
</tr>
<tr>
<td>Slice mini chamber</td>
<td>Luigs &amp; Neumann</td>
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<tr>
<td>Trigger interface TIB-14 S</td>
<td>HEKA Elektronik</td>
</tr>
<tr>
<td>Vibratome VT 1000 S</td>
<td>Leica Microsystems</td>
</tr>
<tr>
<td>Water bath</td>
<td>Memmert</td>
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2.1.2 Chemicals and inhibitors

<table>
<thead>
<tr>
<th>Chemical/Inhibitor</th>
<th>Supplier</th>
</tr>
</thead>
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<tr>
<td>Agarose</td>
<td>PeqLab</td>
</tr>
<tr>
<td>Agarose (low melt)</td>
<td>PeqLab</td>
</tr>
<tr>
<td>4-Aminopyridine (4-AP)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>ATP (Mg-ATP)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Biocytin</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Calcium chloride</td>
<td>Sigma Aldrich</td>
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<tr>
<td>D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5)</td>
<td>Abcam / Tocris</td>
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<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Ethanol</td>
<td>VWR/Merck</td>
</tr>
<tr>
<td>Ethylene glycol tetraacetic acid (EGTA)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Fluo-4, AM</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Fluo-4 pentapotassium salt</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Fura-2 pentapotassium salt</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Glucose</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>GTP (Na-GTP)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)</td>
<td>AppliChem</td>
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<tr>
<td>Hydrochloric acid</td>
<td>AppliChem</td>
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<td>Magnesium chloride</td>
<td>Sigma Aldrich</td>
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<td>2-(N-Morpholino)ethanesulfonic acid hydrate (MES)</td>
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<td>Mibefradil dihydrochloride</td>
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<tr>
<td>NBQX disodium salt</td>
<td>Abcam / Tocris</td>
</tr>
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</table>
2.1.3 Solutions and buffers

(1) HEPES-buffered extracellular solution (S1):
145 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, pH 7.3 (adjusted with NaOH); osmolarity = 300 mOsm (adjusted with glucose)

(2) Bicarbonate-buffered oxygenated (95% O$_2$/5% CO$_2$) artificial CSF (aCSF) (S2):
124 mM NaCl, 26 mM NaHCO$_3$, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 1.3 mM MgSO$_4$, 1.3 mM CaCl$_2$, 10 mM glucose; osmolarity = 300 mOsm (adjusted with glucose)

(3) Sucrose-based bicarbonate-buffered oxygenated aCSF (oxygenated 95% O$_2$, 5% CO$_2$) (S3):
220 mM Sucrose, 26 mM NaHCO$_3$, 3 mM KCl, 1.25 NaH$_2$PO$_4$, 2.6 mM MgSO$_4$, 10 mM glucose; osmolarity = 300 mOsm (adjusted with glucose)

(4) Bicarbonate-buffered oxygenated (95% O$_2$/5% CO$_2$) extracellular solution for VNO slices (S4):
120 mM NaCl, 25 mM NaHCO$_3$, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 5 mM BES, pH 7.3; osmolarity = 300 mOsm (adjusted with glucose)

(5) Elevated potassium extracellular solution for AOB slices (S5):
100 mM NaCl, 50 mM KCl, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 10 mM HEPES, pH 7.3; osmolarity = 300 mOsm (adjusted with glucose)

(6) Elevated potassium extracellular solution for VNO slices (S6):
100 mM NaCl, 50 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, pH 7.3; osmolarity = 300 mOsm (adjusted with glucose)
(7) TEA + 4-AP extracellular solution (S7) for isolation of Na\textsubscript{v} currents:
115 mM NaCl, 25 mM TEACl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, 10 mM 4-AP, pH 7.3; osmolarity = 300 mOsm (adjusted with glucose)

(8) Ba\textsuperscript{2+} extracellular solution (S8) for isolation of Ca\textsuperscript{2+} currents:
100 mM NaCl, 25 mM TEACl, 1 mM BaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, 10 mM 4-AP, pH 7.3; osmolarity = 300 mOsm (adjusted with glucose)

(9) 1 mM TEA extracellular solution (S9) for isolation of K\textsubscript{v} currents:
144 mM NaCl, 5 mM KCl, 1 mM TEACl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, pH 7.3; osmolarity = 300 mOsm (adjusted with glucose)

(10) Acidic extracellular solutions for VNO slices with different pH:
All acidic extracellular solutions were composed of the same ions as S4 with physiological pH. For acidic solutions with a pH ≤ 6, MES was used as buffer instead of HEPES. The pH of acidic solutions was adjusted with the same acids / bases as described for extracellular solutions with physiological pH.

(11) Potassium gluconate-based pipette solution (I1):
125 K\textsuperscript{+}-gluconate, 10 mM HEPES, 10 KCl, 2 mM MgCl\textsubscript{2}, 0.39 mM CaCl\textsubscript{2}, 1 mM EGTA (200 nM free Ca\textsuperscript{2+}), 2 mM ATP, 1 mM GTP, pH 7.1 (adjusted with KOH); osmolarity = 290 mOsm (adjusted with glucose)

(12) Symmetric Cl\textsuperscript{-} pipette solution (E\textsubscript{Cl} = 0 mV; I2):
143 KCl, 10 mM HEPES, 2 mM KOH, 0.3 mM CaCl\textsubscript{2}, 1 mM EGTA (110 nM free Ca\textsuperscript{2+}), 2 mM ATP, 1 mM GTP, pH 7.1 (adjusted with KOH); osmolarity = 290 mOsm (adjusted with glucose)

(13) EGTA- and Ca\textsuperscript{2+}-free potassium-gluconate-based pipette solution (I3):
125 K\textsuperscript{+}-gluconate, 10 mM HEPES, 10 KCl, 2 mM MgCl\textsubscript{2}, 2 mM ATP, 1 mM GTP, pH 7.1 (adjusted with KOH); osmolarity = 290 mOsm (adjusted with glucose)

(14) Low EGTA potassium-gluconate-based pipette solution (I4):
125 K\textsuperscript{+}-gluconate, 10 mM HEPES, 10 KCl, 2 mM MgCl\textsubscript{2}, 0.6 µM CaCl\textsubscript{2}, 1 µM EGTA (200 nM free Ca\textsuperscript{2+}), 2 mM ATP, 1 mM GTP, pH 7.1 (adjusted with KOH); osmolarity = 290 mOsm (adjusted with glucose)

(15) Phosphate-buffered saline (PBS):
150 Tris-HCl, 50 NaCl; pH = 7.4 (adjusted with NaOH); osmolarity = 300 mOsm (adjusted with glucose)

(16) Fixative solution:
4% (w/v) paraformaldehyde (PFA) in PBS\textsuperscript{-}\textsuperscript{−} (10 mM, pH 7.4)

(17) Blocking solution:
0.8% Triton-X-100, 5% normal bovine serum, 0.05% sodium azide in 10 mM PBS\textsuperscript{-}\textsuperscript{−}

(18) Staining solution:
3% BSA (IgG-free, protease-free), 0.05% sodium azide, streptavidin Alexa Fluor\textsuperscript{®} 488 (1:800)

(19) Agarose (4%):
4 g / 100 ml in S1

(20) Dye loading solution:
0.02% Pluronic, 9 µM Fluo-4 AM in S2
2.1.4 Antibodies and sera

Streptavidin Alexa Fluor® 488 conjugate  Molecular Probes
Albumin from bovine plasma  Sigma Aldrich
Normal bovine serum  Dianova

2.1.5 Consumables

Borosilicate glass capillaries with filament and fire-polished ends (1.50 mm OD / 0.86 mm ID)  Science Products
Cell culture dishes (35 x 10 mm)  Falcon
Cell culture dishes (145 x 20 mm)  Falcon
Eppendorf tubes, 0.5 ml  Eppendorf AG
Eppendorf tubes 1.5 ml  Eppendorf AG
Pasteur pipettes  VWR International
Filter IC Acrodisc 13 mm syringe filter with 0.2 μm Supor (PES) membrane  Life Sciences
Glass microscope slides ground edges frosted  VWR International
Gloves gentle skin sensitive  Meditrade
Syringe BD plastipak  Becton Dickinson
15/50 ml tubes  Sarstedt

2.1.6 Software

Corel Draw X7  Corel Corporation
Office 2013  Microsoft
Igor 6.37  WaveMetrics Inc.
JPCalcW  Barry, 1994
Leica LAS X  Leica Microsystems
Leica MM AF 1.8  Leica Microsystems
Matlab  Mathworks
Patchmaster 2.7 / 2.8  HEKA Elektronik
Photoshop CS5  Adobe Corporation
PPT (Patcher’s Power Tools) 2.19  Dr. F. Mendez
Materials and Methods

2.1.7 Mouse strains

C57BL/6                              Charles River
Tbet::Cre                            kindly provided by Catherine Dulac
Ai95(RCL-GCaMP6f)-D (Ai95D)          The Jackson Laboratory

2.1.8 Primers

Tbx21-cre_fwd  AGA GAA AGC CCA GGA GCA G
Tbx21-cre_rev  CAT GTC CAT CAG GTT CTT GC
GCaMP6f_fwd   ACG AGT CGG ATC TCC CTT TG
GCaMP6f_rev   CCG AAA ATC TGT GGG AAG TC

2.2 Methods

2.2.1 Breeding and Genotyping

In the Tbet::Cre driver line Cre cDNA was inserted at the start ATG of the Tbx21 gene in a bacterial artificial chromosome (BAC; RP23-237M14). The recombined BAC was linearized and injected into C57BL/6 x CBA oocytes. Tbet::Cre mice express Cre recombinase in projection neurons of the MOB and AOB (Haddad et al., 2013). Hemizygous transgenic mice (kindly provided by Dr. Catherine Dulac, Harvard University) were maintained by breeding with wild type (WT) C57BL/6J mice. Ai95D reporter mice (The Jackson Laboratory, Bar Harbor, Maine) are a Cre-dependent, fluorescent, calcium-indicator tool strain. They harbor the Rosa-CAG-LSL-GCaMP6f::deltaNeo conditional allele, designed with a floxed-STOP cassette upstream of the GCaMP6 fast variant calcium indicator (GCaMP6f) coding sequence. Although under control of the endogenous Gt(ROSA)26Sor promoter/enhancer regions and the CAG hybrid promoter, widespread expression of GCaMP6f is prevented by the floxed-STOP cassette. Heterozygous transgenic Ai95D mice were maintained by breeding with WT C57BL/6J mice.
Figure 2.1 Breeding scheme for mice expressing GCaMP6f in projection neurons of the AOB and MOB. Tbet::Cre mice that express Cre recombinase under control of the Tbx21 promoter specifically in mitral cells of the MOB and AOB were mated to heterozygous Ai95D reporter mice. The Ai95D strain harbors a floxed-STOP cassette upstream of the GCaMP6f coding sequence under control of the endogenous ROSA 26 promoter/enhancer regions and the CAG hybrid promoter. Expression of GCaMP6f is prevented by the floxed-STOP cassette. Approximately 25% of the offspring is hemizygous for the Cre allele and heterozygous for the floxed-STOP-GCaMP6f allele. Here, Cre recombinase will recombine DNA at the loxP sites resulting in an excision of the STOP-cassette. Thus, the housekeeping locus ROSA 26 and the CAG hybrid promoter can drive expression of GCaMP6f resulting in a cell-type-specific expression of the transgene (Rogan & Roth, 2011).

In order to express GCaMP6f in AMCs we made use of the Cre-Lox recombination system (Rogan & Roth, 2011). Ai95D reporter mice were mated to Tbet::Cre mice to produce offspring in which GCaMP6f is expressed in AMCs (Figure 2.1). To determine the offspring’s genotype we used the DirectPCR® Lysis Reagent Ear kit from Peqlab (Peqlab, Erlangen, Germany). Briefly, a 2 mm ear biopsy was submerged in 250 µl lysis buffer with 5 µl Proteinase K solution (AppliChem, Gatersleben, Germany). Lysis was performed in a 1.5 ml tube on a rotating thermoblock (Eppendorf, Germany) at 55°C for 12 - 16 h to complete lysis. The lysate was incubated at 85°C for 45 min to heat-inactivate Proteinase K. For DNA amplification, a standard polymerase chain reaction (PCR) protocol was performed resulting in two bands at approximately 300 bp (Cre) and 450 bp (GCaMP6f), respectively.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
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</thead>
<tbody>
<tr>
<td>1. Initialization</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95°C</td>
<td>30 s</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>61°C</td>
<td>30 s</td>
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<tr>
<td>4. Elongation</td>
<td>72°C</td>
<td>45 s</td>
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<tr>
<td>5. Final elongation</td>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>6. Cooling</td>
<td>4°C</td>
<td>end</td>
</tr>
</tbody>
</table>

35 cycles (steps 2-4)


Materials and Methods

2.2.2 Animal preparation

All animal procedures were in compliance with local and European Union legislation on the protection of animals used for experimental purposes (Directive 86/609/EEC) and with recommendations put forward by the Federation of European Laboratory Animal Science Associations (FELASA). Mice were housed in groups of both sexes at room temperature on a 12 h light/dark cycle with food and water available ad libitum. Experimental procedures used adults of either sex. No obvious gender-dependent differences were observed in electrophysiological recordings.

Preparation of sagittal AOB acute vibratome slices

Mice were killed by brief exposure to a CO₂ atmosphere and decapitation. Upon removal of the lower jaw and fur all further steps were carried out with the cranium / tissue submerged in ice-cold S3. A bilateral craniotomy gave access to the brain. The left and right olfactory bulbs were rapidly dissected and embedded in 4% low-gelling agarose. Parasagittal slices (250 µm) were cut in ice-cold S3 with a Leica VT1000S vibratome (speed: 3.5 a.u. = 0.15 mm/s; frequency: 5.5 a.u. = 55 Hz; amplitude = 0.6 mm; Leica Biosystems, Nussloch, Germany). Acute slices containing the AOB were transferred to a storage chamber and left to recover for ≥1 h in oxygenated S2 at RT.

Preparation of coronal VNO acute vibratome slices

Mice were killed by brief exposure to a CO₂ atmosphere and decapitation. Removing the lower jaw and soft palate gave access to the vomeronasal capsule. The VNO was dissected by removing the cartilage and was embedded in 4% low-gelling agarose (Ackels et al., 2016). Coronal slices (150 µm) were cut in ice-cold S4 with a Leica VT1000S vibratome (speed: 3.5 a.u. = 0.15 mm/s; frequency: 7.5 a.u. = 75 Hz; amplitude: 0.6 mm). Acute slices were transferred to a storage chamber and submerged in oxygenated and chilled S4 until use.

2.2.3 Electrophysiology

The patch clamp technique

In 1949, Kenneth Cole developed the voltage clamp to stabilize the membrane potential of neurons for experimental purposes (Kandel et al., 2013). This technique was used by Alan Hodgkin and Andrew Huxley in the early 1950s in a series of experiments that revealed the ionic mechanisms underlying the action potential (Hodgkin & Huxley, 1952). The patch clamp technique is a refinement of the voltage clamp that allows for the measurement of currents across biological membranes and has become widely used in physiological laboratories. It was developed in the late 1970s and early 1980s by Bert Sakmann and Erwin Neher (Hamill et al., 1981; Neher & Sakmann, 1976) who were jointly awarded the Nobel Prize in Physiology or Medicine in 1991 for their pioneering work (Nobelprize.org, 2014). The underlying principle is the electrical isolation of a small spot of cell membrane (“patch”) beneath the tip of a fire-polished glass micropipette filled with a salt solution. A recording electrode in contact with the electrolyte inside the pipette and a reference electrode in the bath solution surrounding the cell allow for the measurement of current through ion channels in the cell membrane. In order to prevent clogging of the tip of the pipette (1-2 µm in diameter) a slight positive pressure is applied via a tube connected to a syringe. The pipette is then positioned in close proximity to a cell’s membrane, resulting in a slight increase in pipette resistance as well as formation of a small dent in the cell’s plasma membrane. Once the positive pressure is released, the plasma membrane gets sucked toward the tip of the pipette, leading to a steep increase in pipette resistance. Gentle suction to the patch pipette
increases the tightness of the seal between the pipette and membrane further, resulting in a seal with an extremely high resistance of several gigahms between the inside and outside of the pipette ("gigaseal"). This lowers electronic noise considerably allowing for tight-seal recordings in the so-called “cell-attached” mode (Figure 2.2). In this configuration, recordings from single or few channels situated in the patch beneath the pipette tip can be performed with any intracellular mechanisms that could possibly influence a channel’s function remaining unperturbed.

![Figure 2.2 Schematic representation of the procedures leading to a variety of electrophysiological recording configurations. The cell-attached configuration is the starting point for all other possible configurations: the whole-cell configuration is used to record currents across the plasma membrane of the entire cell, while the cell-free outside-out and inside-out configurations allow for single-channel recordings (adapted from Hamill et al., 1981).](image)

The whole-cell configuration allows for the recording of ionic currents over the entire cell membrane. To attain this configuration, the patch beneath the pipette tip is ruptured without damaging the seal between the pipette rim and cell membrane by a gentle suction pulse (Figure 2.2). Upon “break-in” the cytosol is dialyzed by the pipette solution. Hence, experiments with defined ionic concentrations in the outer and inner cellular milieu can be performed. Dialysis, however, can result in the loss of important cytosolic constituents such as small molecular weight proteins and other soluble substrates, and can thus alter some of the cell’s physiological properties.

The loose-seal cell-attached or “loose-patch” configuration does not perturb the cell’s intracellular environment. Moreover, no gigaseal between patch pipette and plasma membrane is required. Here, seal resistance is kept in the MΩ range (Marrero & Lemos, 2007). This configuration is adequate for recording capacitive currents during action potential discharge (Perkins, 2006) and has the advantage that the pipette can be repositioned to sample currents from a number of patches either from the same cell or from multiple cells (Sherman-Gold, 1993). Moreover, as the intracellular milieu is kept intact, input resistance and resting membrane potential remain unperturbed. It thus presents the least invasive method for monitoring the endogenous electrical activity of single cells (Nunemaker et al., 2003).

**Electrophysiological recordings from AOB mitral cells in acute tissue slices**

For electrophysiological recordings from AMCs, acute parasagittal tissue slices containing the AOB were transferred to a Slice Mini Chamber (Luigs & Neumann, Ratingen, Germany). Slices were anchored to the bottom of the chamber using a stainless steel anchor stringed with approximately 0.1 mm thick human hair (Figure 2.3 A). For single-cell recordings, an upright fixed-stage video-microscope
with infrared-optimized differential interference contrast (IR-DIC) optics (Leica DM LSFA) equipped with a 10x / 0.30 NA, a 20x / 0.50 NA water immersion, and a 63x / 0.90 NA water immersion objective, as well as a cooled CCD-camera (Leica DFC350FX) was used to visualize neurons. Paired recordings were conducted using an upright fixed-stage video-microscope (DM6000FS, Leica Microsystems) equipped for IR-DIC (Figure 2.3 B). Here, neurons were visualized using a 5x / 0.12 NA and a 25x / 0.95NA water immersion objective, a three-position magnification changer (0.35x, 1.25x, and 4.0x) and a cooled CCD camera (DFC360FX, Leica Microsystems). Slices were continuously superfused with oxygenated S2 (~3 ml/min; gravity flow; RT). Patch pipettes (5–8 MΩ) were pulled from borosilicate glass capillaries (with filament, outer diameter 1.50 mm / inner diameter 0.86 mm; Science Products) on a PC-10 micropipette puller (Narishige Instruments), fire-polished (MF-830 Microforge, Narishige Instruments), and filled with pipette solution (II-I3, depending on experimental design). Alexa Fluor 488 hydrazide (20 μM) was routinely added to the pipette solution to enable on-line evaluation of cell morphology (Figure 2.3 D). In some recordings, biocytin [0.3% (w/v)] was added to the pipette solution to enable post hoc morphological reconstruction. Neither Alexa Fluor 488 nor biocytin showed an evident effect on mitral cell electrophysiology. Slight positive pressure was applied to the pipette before entering the bath solution to prevent clogging of the tip with dirt or debris. Single neurons were targeted under optical control using Luigs & Neumann micromanipulators. An agar bridge (150 mM KCl) connected the reference electrode and bath solution. For single-cell AMC recordings, an EPC-10 amplifier controlled by Patchmaster 2.7 / 2.8 software (HEKA Elektronik) was used for data acquisition. Paired AMC recordings were performed using an EPC-10 USB double amplifier (HEKA Elektronik) controlled by Patchmaster 2.8 software (Figure 2.3 C). To minimize electrical network noise, a 50/60 Hz noise eliminator (HumBug, Quest Scientific) was connected to the amplifier. Pipette and membrane capacitance as well as series resistance were monitored and compensated. Only neurons exhibiting relatively low (<30 MΩ) and stable access resistances were used for analysis. Liquid junction potentials were calculated using JPCalcW software (Barry, 1984) and corrected on-line. Signals were low-pass filtered [analog three-pole and four-pole Bessel filters (-3 dB, adjusted to one-quarter to one-fifth of the sampling rate (6.67 kHz or 10 kHz, depending on protocol)]. In some experiments action potential-driven capacitive currents were recorded in the “loose-seal” cell-attached configuration (seal resistance 150–200 MΩ). Mitral cells were identified according to their location [residing in the external cellular layer between the AOB glomerular layer and the LOT (Larriva-Sahd, 2008)], relatively large soma size, and dendritic morphology (Figure 2.3 D). Solutions and pharmacological agents were applied either by bath or from air-pressure-driven reservoirs via an eight-in-one multibarrel “perfusion pencil” (AutoMate Scientific; Figure 2.3 B). Changes in focal superfusion (Veitinger et al., 2011) were software-controlled and synchronized with data acquisition by transistor–transistor logic input to 12 V DC solenoid valves using a TIB 145 digital output trigger interface (HEKA Elektronik; Figure 2.3 C). Final solvent concentrations for pharmacological agents were ≤0.1%.

**Figure 2.3** Electrophysiology setup for patch clamp recordings in acute slices. (A) Infrared DIC image showing the layered structure of the AOB in an acute parasagittal section of the olfactory bulb. (B) An upright fixed-stage video-microscope (DM6000FS, Leica Microsystems) with a CCD-camera (CCD, DFC360FX, Leica Microsystems) used for visualization of tissue slices. The microscope is equipped with peripheral experimental devices: two patch-clamp amplifier headstages (probe 1 & 2).
and a perfusion system for precise stimulus application. Both probes, as well as an eight-in-one multibarrel “perfusion pencil” (AutoMate Scientific) are mounted on a Luigs-Neumann device (LN) for micromanipulator-controlled movement. (C) External devices for electrophysiological recordings include a patch-clamp amplifier (amp, HEKA EPC-10 USB double), trigger interface board (TIB, HEKA TIB 14S), an oscilloscope (osci), and a Luigs-Neumann (LN) control box. (D) Addition of Alexa Fluor 488 hydrazide to the pipette solution allows for on-line evaluation of cell morphology. (GL) glomerular layer, (MCL) mitral cell layer, (LOT) lateral olfactory tract, (GCL) granule cell layer.

**Loose patch extracellular recordings from VSNs in acute tissue slices**

For loose patch extracellular recordings from VSNs, acute coronal tissue slices containing the VNO’s sensory epithelium were transferred to a Slice Mini Chamber. Slices were anchored to the bottom of the chamber as described in section 0. An upright fixed-stage light microscope with IR-DIC optics (Leica DM LSFA) equipped with a 10x / 0.30 NA, a 20x / 0.50 NA water immersion, and a 63x / 0.90 NA water immersion objective as well as a cooled CCD-camera (Leica DFC360FX) was used to visualize neurons. Slices were continuously superfused with oxygenated S4 (~3 ml/min; gravity flow; RT). Patch pipettes (5–8 MΩ) were pulled as described in section 0 and filled with S1. Action potential-driven capacitive currents were recorded in a loose-seal cell-attached configuration (seal resistance, 30-150 MΩ). Application of stimuli and pharmacological agents was software-controlled and synchronized with data acquisition as previously described. Interstimulus intervals were 55–60 s.

**Pulse protocols for data acquisition**

Measurement of membrane time constant (τ_{mem}) and input resistance (R_{input}) / gap junction protocol for paired recordings:
Injection of a -70 pA hyperpolarizing current step to measure the steady-state voltage response.

Current-clamp IV step protocol: Stepwise depolarization (5 pA intervals; 500 ms duration) to induce voltage sags and action potential firing.

Slow ramp recording protocol in voltage-clamp mode for I_{NaP} recordings: The cell is gradually depolarized from -90 mV up to -50 mV over a time course of 1.2 s.

Fast ramp recording protocol in voltage-clamp mode for Ca^{2+} current recordings: The cell is gradually depolarized from -100 mV to +80 mV over a time course of 500 ms.

Pulse protocol in voltage-clamp mode for recordings of I_{BK} activation: The cell is depolarized from -100 mV to +90 mV in a stepwise manner (10 mV intervals; 150 ms duration).

Burst stimulus for paired recordings: Consecutive rectangular pulses (1 nA amplitude; 2 ms duration) to induce a burst of action potentials (4.22 Hz; 5.45 s duration). Sweep intervals were adjusted to obtain interburst intervals of 17.8 s.
2.2.4 Ca$^{2+}$ imaging

Changes in cytosolic Ca$^{2+}$ concentration of AMCs were monitored using either chemical indicators, such as fura-2 or Fluo-4, or the genetically encoded calcium indicator (GECl) GCaMP6f.

Single cell Ca$^{2+}$ imaging

For combined electrophysiological and fluorometric recordings, AMCs were targeted and patched as described in section 2.2.3. Pipettes were filled with either 13 or 14 and fura-2 pentapotassium salt (10, 30, or 100 µM) or Fluo-4 pentapotassium salt (3, 5, or 10 µM), respectively. The setup was equipped with a Prior Lumen 200 Fluorescence Illumination System and a ProScan™III control unit (Prior Scientific, Rockland, MA, U.S.A) including external filter wheels, as well as a fura-2 HC Monochromator filter cube (AHF Analysentechnik, Tübingen, Germany) and a GFP filter cube (Leica Microsystems). A 63x / 0.90 NA water immersion objective and a cooled CCD camera (Leica DFC360FX) was used to visualize dye-loaded neurons.

Widefield Ca$^{2+}$ imaging in AM-loaded acute AOB tissue slices

For AM dye loading, slices were transferred to a loading chamber consisting of an inner and an outer compartment (Figure 2.4). The outer compartment was filled with approximately 3 ml oxygenated S2. The bottom of the inner compartment consisted of a gas-permeable membrane to ensure oxygenation of the loading solution inside the inner chamber. This allowed for a substantial reduction in the dye solution’s volume. Acute slices were placed on the porous membrane of the inner loading chamber submerged in 0.5 ml loading solution. 0.5 µl Fluo-4 AM dissolved in DMSO (10 µg/µl) was pipetted directly onto the AOB for initial exposure to high concentrations of Fluo-4 AM that is gradually diluted through passive diffusion (Ikegaya et al., 2005). Slices were incubated for 30 minutes at RT in the dark and were washed for another 30 minutes in oxygenated S2 prior to imaging. Cells were visualized using a 10x (HCX APO I U-V-I 10x/0.30) water immersion objective (Leica Microsystems). A 2 s–long control stimulation with elevated potassium solution (S6) that depolarizes the plasma membrane by reversing the driving force for K$^+$ was applied at the end of each experiment. Changes in cytosolic Ca$^{2+}$ were monitored over time at 1 Hz frame rate. Pharmacological agents and S6 were applied as described in section 2.2.3.

![Figure 2.4 Schematic drawing of AM dye loading chamber. Slices were submerged in a small volume of loading solution inside the inner chamber. A larger volume of S2 in the outer chamber was constantly oxygenated (95% O$_2$/5% CO$_2$). A porous, gas-permeable membrane at the bottom of the inner chamber allowed for oxygenation of the loading solution (adapted from Ikegaya et al., 2005).](image)

GCaMP6f

Widefield imaging experiments in acute AOB slices from animals expressing GCaMP6f in AMCs were performed on an upright fixed-stage setup equipped with an EL6000 Fluorescence Illumination System. The AOB was visualized using a 25x / 0.95NA water immersion objective, a three-position magnification changer (0.35x, 1.25x, and 4.0x) and a cooled CCD camera (DFC360FX, Leica Microsystems). Confocal imaging experiments were performed using an upright Leica SP8 microscope equipped for fluorescence imaging, IR-DIC and brightfield, as well as confocal and multiphoton imaging. GCaMP6f was excited using the 488 nm line of a diode laser. Changes in cytosolic Ca$^{2+}$ were monitored over time.
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using PMT detectors, a 25x / 0.95NA water immersion objective, a 3-position magnification changer (0.35x, 1.25x, and 4.0x) and a DFC365 FX CCD camera (Leica). In one experiment GCaMP6f was excited with a multiphoton laser (Mai Tai DeepSee) at 900 nm. Here, emitted light was directed to a HyD detector (Leica). Pharmacological agents and S6 were applied as described in section 2.2.3.

2.2.5 Data analysis

All data were obtained from independent experiments performed on ≥ 3 days using at least three different animals. Pharmacological agents were allowed to preincubate for at least 60 s for data to be analyzed.

Electrophysiology

Offline analysis was performed using different software (Patchmaster, IGOR Pro, and Excel). If not stated otherwise, results are presented as mean ± SEM. Statistical analysis was performed using paired or unpaired student’s t-tests or one-way ANOVA tests (as dictated by data distribution and experimental design). For figurative presentation, stimulus artifacts in continuous recordings are corrected. Treated as a “biological constant” with a value of 1 µF/cm² (Gentet et al., 2000), C_{mem} was determined using a square-pulse (5 mV, 10 ms) routine. Input resistance (R_{input}) at the mitral cell soma was determined by measuring the steady-state voltage response to a hyperpolarizing current step of -70 pA. Linear passive voltage responses were also used to estimate \( \tau_{mem} \) from monoexponential fits to the voltage responses (from onset to steady state). Synaptic currents in continuous recordings were analyzed using Igor Pro functions (SpAcAn, written by Guillaume Dugué and Charly Rousseau) for detection and analysis of spontaneous events by a threshold/waveform detection algorithm. TTX-, SNX-, and TEA-sensitive currents were isolated by digital subtraction of averaged traces from 3 consecutive recordings under the respective pharmacological condition from averaged control traces. Activation curves were fitted by the Boltzmann equation to calculate the membrane potential \( (V_{mem}) \) of half-maximal activation \( (V_{1/2}) \). Conductances were calculated using Ohm’s Law, where \( G = I / U \). Spikes in VSN loose patch recordings were analyzed with SpAcAn. Neuronal responses (or the lack thereof) were classified according to the following criteria: (1) discharge was time locked to stimulus presentation; (2) spike patterns clearly deviated from prior baseline activity (Frequency histograms (1 s bin width) were calculated, and responses were evaluated according to \( \Delta f > 3 \times SD f_{(baseline)} \) criterion). For paired recordings spontaneous and evoked activity were analyzed in customized experiments using the Experimentor Analysis plugin in SciWorks (DataWave Technologies, Loveland CO, USA). Here, systematic exploration of the postsynaptic signal was performed by averaging both pre- and postsynaptic signals after aligning both with reference to the presynaptic spike. Experiments that tested for electrical coupling between AMC pairs were analyzed in Igor Pro. Here, consecutive sweeps were averaged in both pre- and postsynaptic recordings in search of an attenuated voltage signal in the postsynaptic neuron.

Calcium Imaging

The average pixel intensity within a user-selected region of interest (ROI) encompassing the soma and/or proximal dendrites of identified cells was digitized and stored using Leica MM AF or LAS X imaging software for single cell and AM bulk loading experiments, respectively. For recordings using fura-2 Ca^{2+}-dependent fluorescence, signals at 505 nm were calculated as the f340/f380 intensity ratio in Leica MM AF. For population imaging experiments using bulk loading with Fluo-4 AM, relative fluorescence intensity was calculated in LAS AF. Here, exported data were baseline corrected and normalized in Igor Pro (Wavemetrics). For periodicity detection in noisy recordings, the linear autocorrelation function of Igor Pro was used to calculate normalized autocorrelograms. The linear
crosscorrelation function was used to identify correlated cellular activity. Multi-peak fit analysis of either the baseline-corrected and normalized relative fluorescence, or of the autocorrelogram in case of noisy recordings, was used to analyze the frequency of oscillating AMCs. In experiments utilizing slices from GCaMP6f-expressing mice there was considerable fluorescence throughout the neuropil of the MCL. Here, image stacks were analyzed in Fiji (Schindelin et al., 2012). Two ROIs were specified for each cell: one encompassing the soma, and a second that encompasses the adjacent neuropil background. The average pixel intensity of the second ROI was subtracted from that of the somatic ROI to obtain a higher signal-to-noise ratio in Excel (Microsoft). Auto- and crosscorrelograms were calculated in Igor Pro. For analysis of oscillation frequencies data were smoothed in Igor Pro using a binomial smoothing algorithm with 3 passes. To calculate the oscillation frequency of a given AMC interpeak intervals (IPIs) were inverted and averaged. If not otherwise specified changes in cytosolic Ca\(^{2+}\) were monitored over time at 1 Hz frame rate. Pairwise analysis of cross-covariance, calculation of power spectral densities, and pairwise correlation analyses of signal correlation (zero-lag cross-covariance) and physical distance was performed using custom routines written in Matlab (MathWorks) by Yoram Ben-Shaul with the help of Sebastian Malinowski.
3 Results

The accessory olfactory system (AOS) is a key component in rodent conspecific chemical communication (reviewed in Brennan & Zufall, 2006; Liberles, 2014). Despite its fundamental function, however, sensory coding in the accessory olfactory bulb (AOB), the first stage of information processing in the AOS, is poorly understood. Here, mitral cells (AMCs) receive sensory input from peripheral vomeronasal neurons and relay this information to the vomeronasal amygdala and the hypothalamus, bypassing olfactory cortex (C Dulac & Wagner, 2006). Recently, we demonstrated that a subpopulation of mouse AMCs is intrinsically rhythmogenic and exhibits slow stereotypical oscillatory discharge triggered by cyclic activation of three interdependent ionic conductances: subthreshold persistent Na\(^+\) current, R-type Ca\(^{2+}\) current, and Ca\(^{2+}\)-activated big conductance K\(^+\) current (Gorin, Tsitoura et al., 2016). The majority of data presented in sections 3.1 and 3.2 has been published in this study.

3.1 Slow oscillatory bursting AOB neurons in vivo

In our recently published study, we performed in vivo recordings from the AOB mitral cell layer of anesthetized mice in collaboration with Yoram Ben-Shaul and Anat Kahan (Department of Medical Neurobiology, The Hebrew University of Jerusalem). Here, we continuously monitored spontaneous multi-unit activity using a 32-channel multisite electrode positioned in the AOB (Figure 3.1 A-C). AOB neurons display large heterogeneity in their spontaneous firing patterns that ranges from apparently random (Figure 3.1 Ci) to regular firing (Figure 3.1 Cii). Based on these findings we classified the observed patterns into three groups (Figure 3.1 D-H) by quantifying multiple measures. These included deviation from Poissonian spiking, burst firing parameters, autocorrelation analysis, as well as each unit’s TI (see section 2.2.5). 339 of 466 (72.8%) units were classified as irregular nonbursting (Figure 3.1 D), whereas 127 of 466 (27.2%) were classified as bursting (Figure 3.1 E-H). 49 neurons (10.5%) exhibited non-Poissonian ISI distributions (p < 0.05; two-sample Kolmogorov–Smirnov test) and lacked “side” peaks in their autocorrelation histogram (ACH), and were thus classified as irregularly bursting (Figure 3.1 E). By contrast, 78 (16.7%) bursting neurons were classified as oscillating (Figure 3.1 F-H). These were characterized by highly rhythmic spontaneous discharge with prominent ISI and ACH peaks, corresponding to large TI values. Interburst intervals (IBIs), burst durations, and firing rates were highly variable and non-normally distributed within the oscillating population (Figure 3.1 I). IBIs ranged from 1.4 to 112.2 s and bursts lasted for ≤15.0 s indicating the lack of one prominent characteristic rhythm within the oscillating AOB neuron population. The average activity of oscillating neurons was markedly increased compared with previously observed low baseline rates (1–2 Hz) of irregularly firing neurons (Ben-Shaul et al., 2010; Luo et al., 2003). Next, we plotted histograms of burstiness (Figure 3.1 Ji), deviation from Poissonian spiking (Figure 3.1 Jii), and regularity (Figure 3.1 Jiii) for the entire sample population (n = 466). These analyses indicate that oscillating AOB neurons form a distinct group and do not represent the extreme of a continuous, normally distributed neuronal population in vivo.
Figure 3.1 Spike patterns of AMCs in vivo. A, Schematic drawing illustrating a 32-channel multi-site electrode positioned in the AOB. B, Photomicrograph of a 30 μm sagittal section of the olfactory bulb (nuclear staining; blue; DAPI). Location of a Dil-labeled 32-site recording electrode from a single fluorescent tract (red). Prior to each experiment recording electrodes were dipped in fluorescent dye (Dil) allowing confirmation of correct targeting to the AOB external cellular layer from electrode tracts post mortem. Dashed white line delimits the AOB. C, Ciii, Example 10 minute long extracellular recordings (0.3 – 5 kHz bandpass filtered) of spontaneous activity in the anesthetized mouse AOB. Red boxes illustrate 20 s time windows shown at an enlarged scale below. Cii, Civi, Single unit activity from two presumptive AMCs with irregular and bursting discharge, respectively, obtained by off-line spike waveform sorting of the multiunit activity recordings displayed in C and Ciii. Red traces are average spike shapes from overlaid spike waveforms (black). D-H, Raster plots of spike times recorded from 5 representative AOB neurons in vivo. The corresponding ISI distribution (Dii, Eii, Fii, Gi, Hi) and discrete autocorrelograms (Diii, Eiii, Fiii, Gi, Hi) enable classification of AOB neurons as either irregular nonbursting (D; corresponding to Cii, Ciii), irregular bursting (E), or oscillating (F-H; F corresponds to Ciii, Civi). Di, Ei, Fi, Gi, Hi, ISI histograms are superimposed with the probability (red curve) and cumulative probability (gray curve) functions, corresponding to random Poisson spiking with the same mean rates. The light red vertical line indicates the median ISI interval (ISImed). Regularity (or the lack thereof, D, E) is mirrored by the shape of the autocorrelogram (F-H; 200 ms bin width). Insets, Autocorrelograms (3 s duration; 50 ms bin width) show narrow initial peaks close to zero (E-G), indicative of bursting units. Red asterisks (*) and boxes mark the time windows of either spike times (Di, Ei, Fi, Gi, Hi) or autocorrelograms (Diii, Eiii, Fiii, Gi, Hi) displayed at an expanded time scale; red horizontal bars in Ei, Fi, Gi and Hi denote epochs defined as bursts. I, Scatter dot plots depicting the distributions of
3.2 Spontaneous oscillations in a subpopulation of AOB mitral cells in vitro

Similar to our findings in vivo, AMCs display two characteristic firing patterns in vitro. We recorded spontaneous activity from individual AOB mitral cells in acute parasagittal sections of the mouse olfactory bulb and found neurons to either fire action potentials irregularly with no apparent periodicity (Figure 3.2 Ai, ii), or rhythmically with alternating periods of activity and silence (Figure 3.2 Bi, ii). In contrast to the steady resting membrane potential ($V_{\text{rest}}$) of irregular AMCs (Figure 3.2 Aiii), $V_{\text{mem}}$ in rhythmic AMCs is bistable and oscillates between recurring up and down states ($V_u$ and $V_d$; Figure 3.2 Bi). Essentially identical patterns of spontaneous activity were observed when mitral cell firing was recorded in “loose-seal” cell-attached configuration (Figure 3.2 C). Here, input resistance and resting membrane potential remained unperturbed since the intracellular milieu was kept intact. Loose-patch recordings from neurons in the mitral cell layer (MCL) of a juvenile mouse (P8) revealed rhythmic activity to already be present at this stage in 3 of 3 neurons (Figure 3.2 D). In vitro, about half of the AMC population (56.3%; 196 of 348) was classified as oscillating (Figure 3.2 E).

Figure 3.2 Spontaneous activity of AOB mitral cells in vitro. Ai, Bi. Representative whole-cell current clamp recordings of two distinct types of spontaneous discharge found in AMCs: mitral cells either spike irregularly (Ai), or exhibit periodic discharge patterns (Bi). Rhythmicity of action potential discharge (or the lack thereof) is evident in the corresponding autocorrelation
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Histograms (Aii, Bii; 1 s bin width). Whereas irregularly firing AMCs have a steady baseline membrane potential (V_{mem}), reflected in a single peak in the all-points histogram (Aii; 82 µV bin width), periodically discharging AMCs alternate between two membrane potentials. Membrane bistability of rhythmic AMCs is reflected in the two distinct peaks of the all-points histogram (Biii; 122 µV bin width) that correspond to a relatively hyperpolarized downstate membrane potential (V_d) and a more depolarized upstate membrane potential (V_u). Periodic bursting is also observed in extracellular loose-seal recordings (Ci, ii), and is found in juvenile mice as young as P8 (Di, ii). E, Irregular spontaneous activity was found in 43.7% (152/348) of cells. 56.3% (196/348) of AMCs displayed an oscillatory discharge pattern with alternating periods of activity and silence.

Previously, we have shown that periodic activity patterns in a group of AMCs are generated in absence of fast synaptic drive (Gorin, Tsitoura et al., 2016). Experiments revealed that the physiological mechanism underlying mitral cell autorhythmicity emerges from coordinated reciprocal interaction of I_{NaP}, I_R, and I_{BK} (section 1.4). In this study, we also tested whether or not I_{NaP}, I_R, and I_{BK} are unique properties of intrinsically rhythmogenic AOB mitral cells (iAMCs). We therefore compared electrophysiological profiles of these currents in iAMCs to the remaining AMC population. All three currents were found across the entire AMC population (Figure 3.3 A-G). With the exception of SNX-sensitive I_R (Figure 3.3 D-E), currents were found to differ substantially between iAMCs and irregularly spiking AMCs. The I_{NaP} activation threshold was ~5 mV lower in iAMCs (Figure 3.3 A, Bi). Moreover, maximum amplitudes calculated from sigmoidal fits to each cell’s individual I-V curve were significantly increased in iAMCs (Figure 3.3 Bii). TEA-sensitive K^+ currents also differed between oscillating and irregularly firing AMCs (Figure 3.3 F-G). Intrinsically oscillating AMCs exhibited larger I_{BK} amplitudes. Steady-state activation curves in these neurons are considerably shifted toward more hyperpolarized V_{mem} values (Figure 3.3 G). These results show that, although not exclusively expressed in the intrinsically oscillating mitral cell population, I_{NaP}, I_R, and I_{BK} confer a distinct electrophysiological phenotype on iAMCs. In collaboration with Simon O’Connor (Biocomputation Group, University of Hertfordshire) we next asked whether reciprocal interaction of these conductances is sufficient to drive autorhythmicity in a model AOB mitral cell. V_{mem} simulations were run using the Neuron simulation environment (Hines & Carnevale, 1997) based on the volume-rendered 3D reconstruction of a representative iAMC (Figure 3.3 Hi). The model neuron expressed I_{Kdr} and I_{KA} (based on voltage-clamp experiments performed in our lab by Monika Gorin), as well as I_{NaT} (Migliore et al., 2005) in addition to I_{NaP}, I_R, and I_{BK}. Simulations qualitatively reproduced the experimental data, with relatively small changes in Kdr channel density accounting for much of the experimentally observed heterogeneity in both IBIs and burst duration (Figure 3.3 Hii-Hiii). These data demonstrate that I_{NaP}, I_R, and I_{BK} are sufficient to generate autorhythmicity in AMCs.
Figure 3.3 A distinct combination of currents is sufficient to generate autorhythmicity in iAMCs. A, I-V relationship of TTX-sensitive whole-cell currents (300 nM; mean of 3 consecutive recordings; I_{NaP} isolated by digital subtraction) evoked by slow (10 mV/300 ms) ascending voltage ramps from -90 to -50 mV (inset) in a representative iAMC under synaptic isolation (gabazine (10 μM) + AP5 (100 μM) + NBQX (10 μM)). B, Bil, Bar diagrams quantifying either (B) the average activation
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threshold of $I_{NaP}$ in iAMCs (oscill.; red; $V_{threshold} = -80.1 \pm 1.2 \text{ mV}$) versus nonrhythmogenic neurons (irreg.; black; $V_{threshold} = -74.6 \pm 0.7 \text{ mV}$) or (Bii) the maximum $I_{NaP}$ amplitude in oscillating (red; -164.0 ± 36.8 pA) versus irregular neurons (black; -87.9 ± 9.7 pA). Data are means ± SEM. Asterisks (*) denote statistical significance, $p < 0.0001$ (Bi) or $p < 0.05$ (Bii; unpaired sample $t$ test). Numbers of experiments are denoted above bars. C, Average I–V relationships of TTX-sensitive $I_{NaP}$ recorded from oscillating (red; n = 6–17) versus irregular mitral cells (black; n = 16–23). Data points depict means ± SEM. Curves are sigmoidal Boltzmann-type equation fits assuming threshold $V_{mem}$ values shown in Bi. $V_{mem}$ values corresponding to half-maximal activation are -60.4 ± 0.8 mV versus -60.6 ± 14.5 mV in oscillating and irregular neurons, respectively. D, SNX-482 (100 nM) sensitive $I_{R}$ recorded from an irregularly firing mitral cell under synaptic isolation in response to a fast ascending voltage ramp (-100 to +80 mV; 100 ms duration) and plotted as a function of command voltage. E, Bar charts comparing $I_{R}$ characteristics in oscillating (violet) versus irregular (black) mitral cells. Ei–Eiv, SNX-sensitive peak current (Ei; 10.3 ± 3.1 vs 9.9 ± 1.6 pA/pF), activation threshold (Eii; -38.8 ± 3.1 vs -35.1 ± 4.2 mV), $V_{mem}$ inducing maximum currents (Eiii; -20.6 ± 6.9 vs -17.1 ± 4.3 mV), and R-type channel conductance (Eiv; 0.73 ± 0.2 vs 0.64 ± 0.5 nS) are not significantly different between both mitral cell populations (unpaired sample $t$-test). Data are means ± SEM. Numbers of experiments are denoted above bars. F, $K^+$ current sensitive to TEA (1 mM) recorded from a representative iAMC (whole-cell voltage clamp). Inset depicts pulse protocol. G, Normalized $I_{BK}$ activation curve recorded from oscillating (green, n = 3) versus irregular (black, n = 3) mitral cells. Steady-state current amplitudes are measured upon stepping back to 0 mV (+ in F) and plotted as a function of prepulse $V_{mem}$. Solid lines represent sigmoidal fits to data points (mean ± SD; $V_{1/2} = -2.0 \pm 0.8 \text{ mV}$). $V_{1/2}$ shift as indicated. Inset depicts average steady-state current densities of TEA-sensitive $I_{BK}$ (* in F) as a function of command voltage (n = 3; means ± SD). Hi, Volume-rendered 3D reconstruction of a representative iAMC used for modeling spontaneous discharge. Hii, Hiii, Model-based $V_{mem}$ simulations (top) qualitatively reproduce experimental recordings (bottom) with respect to both IBIs and burst durations. Note that the different $V_{mem}$ simulation outcomes result mainly from model variation in $K_{dr}$ channel density and deactivation time constant. (Gorin, Tsitoura et al., 2016)

3.3 An excitatory circuit entrains oscillations in a subpopulation of AOB mitral cells

In Gorin, Tsitoura et al. we have demonstrated that oscillation frequency in iAMCs changes as a function of depolarizing or hyperpolarizing current injection as illustrated in Figure 3.4 A: while hyperpolarization increased IBIs (Figure 3.4 Ai), depolarization reduced IBIs (Figure 3.4 Aiii, Aiv). This positive causal correlation between oscillation frequency and “baseline” $V_{mem}$ (Crunelli & Hughes, 2010) is similar to intrinsic oscillators described in other circuits (Blethyn et al., 2006; Hayar et al., 2004; Tazerart et al., 2008). Moreover, neurons in this population exhibited a characteristic $V_{mem}$ threshold below which $V_{mem}$ bistability is abolished (Figure 3.4 Av). These results allowed us to conclude that the neuronal population examined in Gorin, Tsitoura et al. is intrinsically rhythmogenic.

In a second group of oscillating AMCs, however, we found that oscillation frequencies were not affected by negative current injection (Figure 3.4 Bi). Moreover, subthreshold oscillations persisted under hyperpolarized conditions (Figure 3.4 Bii). This finding suggests oscillatory activity to be entrained through a network-dependent mechanism in these cells. While autorhythmicity was found in 39% of oscillating neurons (iAMCs), rhythmic discharge was found to be entrained in 61% of oscillating AMCs (eAMCs; Figure 3.4 C).
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Figure 3.4 Continuous current injection reveals two populations of oscillating AMCs. A, B, Original whole-cell current-clamp recordings from two representative oscillating AOB mitral cells during continuous current injection of variable amplitude (-10 to -30 pA). While depolarization decreases iBIs, hyperpolarization increases intervals (AI and AIII-IV, respectively) in iAMCs. In this population, the pattern of periodically recurring up and down states switches to a stable resting state below a characteristic V_{rest} threshold (A; see also Gorin, Tsitoura et al., 2016). In another group of oscillating AMCs (Bi), oscillation frequency does not change as a function of depolarizing or hyperpolarizing current injection. Moreover, as illustrated in Bi, subthreshold oscillations persist and are qualitatively unaffected under hyperpolarized conditions, thus, indicating network-dependent mechanisms as the driving force in this entrained AMC population. C, Oscillations were generated intrinsically in 35% of oscillating AMCs (47 of 122 cells). The majority of AMC oscillations (61%, 75 of 122 cells) were entrained.

Next, we asked which mechanism underlies oscillatory entrainment. We therefore combined pharmacology with negative DC current injections. As previously demonstrated (Gorin, Tsitoura et al., 2016), iAMC oscillations persisted when both GABAergic and glutamatergic fast synaptic transmission were blocked (Figure 3.5 A). Moreover, oscillations were abolished upon hyperpolarization below a characteristic threshold (Figure 3.5 Aii). Performing these experiments in entrained AMCs, however, unveiled the existence of two distinct eAMC subpopulations. In one group, oscillations were sensitive to block of glutamatergic fast synaptic transmission. Here, rhythmic bursting and membrane bistability were abolished upon pharmacological inhibition of AMPA/Kainate and NMDA receptors (Figure 3.5 B). In another group, however, oscillations were not affected by isolation from fast synaptic transmission (similar to iAMCs; Figure 3.5 Ci). Here, additional hyperpolarization revealed the entrained nature of membrane potential oscillations (Figure 3.5 Ci, Ciii). Our combinatorial experimental strategy showed that oscillatory activity in 44% of eAMCs is sensitive to block of fast glutamatergic transmission (glut.-sensitive eAMCs). In 56% of eAMCs, however, entrained rhythmic activity was not abolished upon isolation from fast synaptic transmission (glut.-insensitive eAMCs; Figure 3.5 D).
3.4 Passive and active membrane properties of AMC subpopulations

Passive and active membrane properties determine a neuron’s basic biophysical parameters and thus critically influence how information is integrated and transformed (Fortune & Rose, 1997; Kowalski et al., 2016). Therefore, we asked whether AMCs of different subpopulations (Figure 3.5 D) differ in their passive electrical properties. We compared membrane capacitance ($C_{\text{mem}}$), membrane time constant ($\tau_{\text{mem}}$), and input resistance ($R_{\text{input}}$) in whole-cell patch-clamp recordings from AMCs. $C_{\text{mem}}$ was determined using a square pulse routine (see section 2.2.5). To calculate $\tau_{\text{mem}}$ and $R_{\text{input}}$ a hyperpolarizing current step was injected (Figure 3.6 B). When comparing irregular to oscillating AMCs neither $C_{\text{mem}}$ nor $\tau_{\text{mem}}$ differed significantly (Figure 3.6 Ai, Ci). $R_{\text{input}}$, however, was increased in oscillating AMCs (Figure 3.6 Di). No differences were found in either parameter between iAMCs and eAMCs (Figure 3.6 Ai, Ci, Di). Differentiating between glut.-sensitive and glut.-insensitive eAMCs, again, did not reveal any significant differences. However, we observed a tendency toward increased $\tau_{\text{mem}}$ and $R_{\text{input}}$ in glut.-insensitive eAMCs (Figure 3.6 Ci, Di).
Figure 3.6 Passive membrane properties of AMC subpopulations. A, C, D, Box-and-whisker plots comparing \( C_{\text{mem}} \), \( \tau_{\text{mem}} \), and \( R_{\text{input}} \) respectively. Bottoms and tops of boxes indicate the first and third quartiles, bottoms and tops of whiskers represent the 10th and 90th percentiles, respectively. Outliers are plotted as individual points. The central band represents the population median. Numbers of experiments are denoted above boxes. Analysis of \( C_{\text{mem}} \) reveals no differences between irregular and oscillating AMCs [Ai; 12.01 ± 11.99 pF (median); 12.01 ± 0.15 vs 11.77 ± 0.16 pF (means ± SEM)], IAMCs and eAMCs [Aii; 11.99 ± 11.99 pF (median); 11.99 ± 0.4 vs 11.96 ± 0.23 pF (means ± SEM)], or between glut.-sensitive and glut.-insensitive eAMCs [Aiii; 11.75 ± 12 pF (median); 11.62 ± 0.47 vs 12.32 ± 0.29 pF (means ± SEM)]. B, Mean of 3 consecutive current clamp recordings illustrating a representative response to a hyperpolarizing current step (inset; -70 pA, 500 ms duration). A monoeponential fit (red curve) was used to calculate \( \tau_{\text{mem}} \). \( R_{\text{input}} \) was calculated by measuring the steady-state voltage response. Comparison of \( \tau_{\text{mem}} \) between irregular and oscillating AMCs [C, 36.29 vs 43.44 ms (median); 37.94 ± 2.58 vs 44.75 ± 3.25 ms (means ± SEM)], between IAMCs and eAMCs [D, 49.72 vs 19.96 ms (median); 45.27 ± 9.67 vs 31.42 ± 10.1 ms (means ± SEM)], or between glut.-sensitive and glut.-insensitive eAMCs [Dii, 17.43 vs 47.35 ms (median); 17.86 ± 4.05 vs 44.99 ± 18.36 ms (means ± SEM)] reveals no differences. \( R_{\text{input}} \) was found to differ significantly between irregular and oscillating AMCs [Di, 342.89 vs 514.43 MO (median); 435.66 ± 39.07 vs 574.47 ± 47.66 MO (means ± SEM), \( p < 0.05 \) (unpaired t-test)]. No differences were found when comparing \( R_{\text{input}} \) in IAMCs vs eAMCs [Dii, 667.85 vs 314.46 MO (median); 618.97 ± 113.22 vs 403.88 ± 107.67 MO (means ± SEM)], or in glut.-sensitive vs glut.-insensitive eAMCs [Diii, 278.09 vs 539.23 MO (median); 266.95 ± 46.26 vs 540.82 ± 198.64 MO (means ± SEM)].

Next, addressing active neuronal properties, we compared mean instantaneous spike frequencies as a function of stationary current input in irregular AMCs, IAMCs, and eAMCs (\( f-I \) curve; Figure 3.7). For irregular AMCs and eAMCs, curves were essentially indistinguishable with maximum average firing frequencies of \( \sim 20 \) Hz (Figure 3.7 C; black and green curves, respectively). IAMCs, however, displayed evoked action potentials at significantly higher frequencies across the entire stimulation range (Figure 3.7 B vs A; blue curve in C). For this population, maximum average firing frequencies increased to \( \sim 35 \) Hz.
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**Figure 3.7** Spiking characteristics of AMC populations. A, B, Representative responses evoked by depolarizing (A1 II, B1 II) or hyperpolarizing (AII, BII) stationary current step injections (inset; -20 to 85 pA, 5 pA intervals, 500 ms duration) in an irregular (A) and an intrinsically oscillating AMC (B) recorded in current clamp. C, F-I curve depicting average instantaneous discharge frequencies in irregular AMCs (black; n = 17-39), iAMCs (blue; n = 4-9), and eAMCs (green; n = 6-7). Maximum frequencies are 21.14 ± 1.01 Hz (irregular), 34.5 ± 8.77 Hz (iAMCs), and 20.33 ± 6.86 Hz (eAMCs), respectively. Individual data points are means ± SEM. Curves are monoexponential fits. Asterisks (*) denote statistical significance between iAMCs and irregular AMCs (p < 0.02, unpaired t-test).

**3.5 Pattern variability among oscillating AOB mitral cells**

In Gorin, Tsitoura et al. we demonstrated a wide spectrum of rhythmic discharge patterns in iAMCs that was also observed in extracellular in vivo recordings from AOB neurons (see Figure 3.1. F-I). Therefore, we next sought to investigate whether similar heterogeneity in oscillatory discharge is observed in eAMCs. We measured burst duration, interburst intervals (IBIs), as well as downstate (V_d) and upstate (V_u) membrane potentials in prolonged whole-cell current-clamp recordings, and calculated burst frequencies (f_burst) and within-burst firing rates (f_AP) across all oscillating AMC populations (Figure 3.8). Parameters describing the temporal patterns of activity and silence, namely burst durations, IBIs, and f_burst, as well as f_AP were similarly diverse in eAMCs (Figure 3.8 A - C, F). Here, no differences were found, neither between iAMCs and eAMCs, nor between glut.-insensitive and glut.-insensitive eAMCs. While V_d did no differ in iAMCs and eAMCs, glut.-sensitive eAMCs were depolarized (~6 mV) as compared to glut.-insensitive eAMCs [Figure 3.8 D; -67.18 ± 4.45 vs -74.81 ± 5.26 mV (means ± SD); p < 0.05, unpaired t-test]. The entrained AMC subpopulations (glut.-sensitive and –insensitive) did not differ in their upstate membrane potential. V_u, however, was more hyperpolarized in eAMCs compared to their autorhythmic counterparts [Figure 3.8 E; -63.65 ± 5.55 vs -59.49 ± 5.55 mV (means ± SD); p = 0.02, unpaired t-test].

Taken together, these data demonstrate a wide distribution of oscillatory discharge parameters in all rhythmic AMC populations. Although the observed rhythms (f_burst; Figure 3.8 C) clustered at the lower end of the bandwidth scale of neuronal oscillators (Tonetti et al., 1976), the wide spectrum within this infraslow range argues against a distinct prevalent AOB rhythm. The similarities in distribution of burst durations, IBIs, and f_burst, however, led us to hypothesize that intrinsically generated oscillations in iAMCs might entrain rhythmic activity in eAMCs via a hitherto undescribed excitatory connection between the two classes of AOB projection neurons.
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Figure 3.8 Oscillatory discharge parameters are highly variable in all oscillating AMC subpopulations. A–F, Box-and-whisker plots comparing burst duration, IBI, burst frequencies, downstate and upstate membrane potentials, and within-burst spike frequencies, respectively. Bottoms and tops of boxes indicate the first and third quartiles, bottoms and tops of whiskers represent the 10th and 90th percentiles. The central band represents the population median. Numbers of experiments are denoted above boxes. A, No differences in burst duration were found between IAMCs and eAMCs [6.13 vs 5.88 s (median); 6.77 ± 4.88 vs 7.32 ± 5.48 s (means ± SD)], or glut.-sensitive and glut.-insensitive eAMCs (7.97 vs 8.93 s (median); 7.19 ± 3.71 vs 7.58 ± 1.7 s (means ± SD)]. B, Analysis of IBI revealed no significant differences between IAMCs and eAMCs (10.14 vs 11.65 s (median); 11.75 ± 7.01 vs 12.79 ± 5.76 s (means ± SD)]. C, Burst frequencies do not differ between either IAMCs and eAMCs (0.056 vs 0.064 Hz (median); 0.066 ± 0.031 vs 0.064 ± 0.040 Hz (means ± SD)) or glut.-sensitive and glut.-insensitive eAMCs (0.045 vs 0.044 Hz (median); 0.059 ± 0.035 vs 0.063 ± 0.041 Hz (means ± SD)]. D, Quantification of $V_{m}$ is not significantly different in IAMCs and eAMCs [-71.59 vs -73.14 mV (median); -71.64 ± 6.2 vs -73.14 ± 5.67 mV (means ± SD)]. Glut.-sensitive eAMCs, however, have a more depolarized $V_{m}$ compared to glut.-insensitive eAMCs [-67.27 vs -76.32 mV (median); -67.18 ± 4.45 vs -74.81 ± 5.26 mV (means ± SD); p < 0.05, unpaired t-test]. E, Comparison of $V_{m}$ reveals a significantly more depolarized upstate in IAMCs compared to eAMCs [-59.55 vs -63.54 mV (median); -59.49 ± 5.55 vs -63.65 ± 5.55 mV (means ± SD); p = 0.02, unpaired t-test]. F, Analysis of within-burst spike frequencies revealed no differences between either IAMCs and eAMCs [3.41 vs 3.61 Hz (median); 4.49 ± 2.3 vs 4.37 ± 2.24 Hz (means ± SD)] or between glut.-sensitive and glut.-insensitive AMCs [3.20 vs 3.88 Hz (median); 3.23 ± 1.24 vs 4.99 ± 2.15 Hz (means ± SD)].
3.6 Synaptic drive in intrinsically oscillating and entrained AMCs

Analysis of passive electrical properties revealed that iAMCs and eAMCs do not display readily distinguishable biophysical phenotypes. We next asked whether these neurons differ in synaptic connectivity. Therefore, we analyzed spontaneous synaptic input to eAMCs and iAMCs by continuous whole-cell voltage-clamp recordings of spontaneous postsynaptic currents (PSCs) in neurons of both populations. In these experiments, both excitatory and inhibitory PSCs (EPSCs and IPSCs) were recorded as downward deflections from the current baseline due to the combination of extra- and intracellular solutions (S1/I1; see section 2.1.3; $E_{Na^+} = 144 \text{ mV}$, $E_{K^+} = -84 \text{ mV}$, $E_{Ca^{2+}} = 0 \text{ mV}$, $E_{Cl^-} = -59 \text{ mV}$). PSCs were detected using a threshold/waveform detection algorithm in Igor Pro (SpAcAn, written by Guillaume Dugué and Charly Rousseau). Following event detection, frequency histograms and discrete autocorrelograms were calculated (Figure 3.9 Aii, Bii) and PSC amplitudes were plotted over time for each cell (Figure 3.9 Aiii, Biii). To analyze decay kinetics, individual PSCs were aligned at half-rise and averaged for each cell (Figure 3.9 C, F). Integrals of averaged events were calculated to estimate the charge transfer across the plasma membrane during an average PSC. Moreover, distribution of rise times (Figure 3.9 Di, Gi) and amplitudes (Figure 3.9 Ei, Hi) were plotted together with the corresponding cumulative probabilities (Figure 3.9 Dii, Gii and Eii, Hii, respectively). In these recordings, excitatory postsynaptic currents are likely to be strongly overrepresented within the mixed population owing to the increased driving force for cations through AMPA/Kainate and NMDA receptors compared to chloride ions through GABA\textsubscript{A} receptors (driving force $V_{DF} = -80 \text{ vs } -20 \text{ mV}$).
Figure 3.9 Synaptic input to iAMCs and eAMCs. A(i), B(i), Representative whole-cell continuous voltage-clamp recordings (V_{hold} = -80 mV) of spontaneous postsynaptic currents (PSCs) in an iAMC and eAMC, respectively. Note the oscillation reflected in the current baseline of the eAMC (B(i)) and lack thereof in the iAMC (A(i)). Traces exemplify PSCs as downward deflections of varying amplitudes under control conditions. Arrowheads indicate relatively large events. A(ii), B(ii), PSC frequency histograms (1 s bin width) illustrating how synaptic input to A(i) and B(i) changes over time (mean, 0.68 and 5.79 Hz, respectively). Note (lack of) rhythmicity in the corresponding autocorrelation histograms (insets). A(iii), B(iii), Amplitudes of individual PSCs as recorded
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over time (means, 6.23 and 6.08 pA, respectively). Red horizontal lines mark detection thresholds at 3.5 (Al) and 3.7 pA (Bii). C, F. Average of detected events in Al and Bi aligned at half-rise, respectively. Note noisy average in C compared to F due to small number of PSCs (n = 82 vs 1042; see also Al, III vs Bii, III). Indicated decay constants of fast component (τdec 15.22 vs 3.48 ms) were calculated by fitting a double exponential function to averaged events. D, E and G, H. Analysis of spontaneous PSCs detected in Al and Bi, respectively. Di, Gl. Rise time histograms (0.2 ms bin width). Note that skewed distributions are not well fitted by a Gaussian function, indicative of multiple PSC populations. Di, Gl, Cumulative rise time histograms (0.2 ms bin width) of PSCs in Al and Bi, respectively. Vertical lines indicate median (2.1 ± 1.75 ms). Amplitude histograms (Ei, Hii) and cumulative amplitude histograms (Eii, Hi; vertical lines indicate medians, 4.05 and 5.79 pA, respectively) reveal no distinct populations in detected PSCs with respect to this parameter.

In the majority of eAMCs an oscillation of the current baseline was evident during the recording (Figure 3.9 Bi). Here, deflections to more negative values correspond to periodic membrane potential depolarization and bursting epochs. In contrast, the majority of recordings from iAMCs lacked any obvious baseline oscillations (Figure 3.9 Ai, Al). PSC analysis in intrinsically oscillating and entrained AMC populations (Figure 3.10) revealed that the frequency of PSCs (fPSC) was markedly increased in eAMCs compared to iAMCs [Figure 3.10 A; 10.26 ± 1.39 vs 1.07 ± 0.19 (means ± SEM); p = 0.0003, unpaired t-test], as is evident in the two representative recordings in Figure 3.9 Aii, Bii. With detection thresholds ranging from 2.2 to 7 pA [3.9 ± 1.3 pA (mean ± SD)], average PSC amplitudes were relatively small (2.86 – 10.1 pA) with the vast majority of individual PSC amplitudes in each cell clustering close to the respective detection threshold (see Figure 3.9 Aii, Bii). No differences were found between PSC amplitudes or integrals (fPSC) in iAMCs and eAMCs (Figure 3.10 B, C; see examples in Figure 3.9 E, H). Moreover, quantification of PSC kinetics did not reveal significant differences in either rise time or decay (Figure 3.10 D, E; examples in Figure 3.9 C, D, and F, G, respectively).

![Figure 3.10 Quantification of synaptic input to iAMCs and eAMCs. A-E. Box-and-whisker plots comparing spontaneous PSC frequencies, amplitudes, charge transfer, rise times and decay constants, respectively. Bottoms and tops of boxes indicate the first and third quartiles, bottoms and tops of whiskers represent the 10th and 90th percentiles. The central band represents the population median. Numbers of experiments are denoted above boxes. A. The frequency of spontaneous synaptic input is significantly increased in eAMCs compared to iAMCs [9.96 vs 0.9 Hz (median); 10.26 ± 1.39 vs 1.07 ± 0.19 (means ± SEM); p = 0.0003, unpaired t-test]. No differences are found in amplitude [B; 5.42 vs 6.50 pA (median); 6.36 ± 0.44 vs 5.78 ± 0.99 pA (means ± SEM)], charge transfer [C; -67.01 vs -54.45 fC (median); -63.21 ± 9.86 vs -67.49 ± 9.50 fC (means ± SEM)], rise time [D; 2.52 vs 2.39 ms (median); 2.56 ± 0.11 vs 2.32 ± 0.18 ms (means ± SEM)], or τfast [E; 5.13 vs 8.81 ms (median); 8.54 ± 1.95 vs 6.96 ± 2.31 ms (means ± SEM)] of spontaneous PSCs in iAMCs compared to eAMCs.

The frequency of spontaneous postsynaptic currents in eAMCs was not merely increased on average compared to iAMCs. In the above exemplary experiments (Figure 3.9), the variation of PSC frequency over time was increased in the entrained AMC (Figure 3.9 Bii). Here, epochs of increased synaptic activity were interrupted by periods in which synaptic drive was decreased. This was not observed in the intrinsically rhythmonic neuron (Figure 3.9 Aii). The periodicity in synaptic drive (or lack thereof) is highlighted in the corresponding autocorrelation histograms calculated for detected events in each neuron. Comparison of discrete autocorrelograms for each iAMC and eAMC shows that these findings
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mostly hold true across the intrinsic and entrained populations. In eAMCs, spontaneous PSCs tended to occur more periodically (Figure 3.11 Bi-viii vs Bix-x; eight of 12 neurons). In contrast, synaptic input to iAMCs was mostly irregular (Figure 3.11 Ai-ii-vi). Here, synaptic drive in only two of six neurons displayed some degree of regularity (Figure 3.11 Ai, ii).

\[ \text{Figure 3.11 Rhythmicity is more strongly reflected in spontaneous synaptic input to eAMCs, but not to iAMCs. A, B, Autocorrelation histograms (1 s bin width) of spontaneous PSCs detected in 6 iAMCs (Ai-ii-vi) and 12 eAMCs (Bii-xii). In 2 out of 6 iAMCs PSCs exhibited some degree of regularity, resulting in weak formation of regular side-peaks in the corresponding discrete autocorrelationgrams (Ai, ii). Periodicity is more pronounced in synaptic input to entrained AMCs. Here, regular ACH side peaks are evident in 8 of 12 cells (Bi-viii) and, though relatively weak, in two eAMCs (Bix, x). Regularity of synaptic input was not observed in two of 12 eAMCs (Bxi, xii), compared to 4 of 6 iAMCs (Ai-ii-vi).} \]

To further investigate the mechanisms underlying oscillatory entrainment we sought to assess whether the nature of synaptic drive differs between burst epochs and IBIs. We recorded spontaneous PSCs in continuous voltage-clamp recordings from eAMCs and compared PSCs during bursting epochs and IBIs (purple vs grey sections in Figure 3.12A). We next calculated frequency histograms (purple vs grey bins in Figure 3.12 B) and analyzed amplitudes of PSCs during bursts versus IBIs (purple vs grey dots in Figure 3.12 C; Dii, iii). Moreover, we compared both charge transfer and decay kinetics of average events (Figure 3.12 Di). Averaged PSCs during bursts (purple) compared to average of PSCs detected in IBIs (gray) might indicate a slower decay of events occurring in IBIs for this cell. This should be interpreted with caution, however, since averaging those relatively few PSCs between bursts (n = 67 vs 374; see also Figure 3.12 B) results in larger ‘noise’.
Figure 3.12 Synaptic input to an eAMC during vs between bursts. A, Representative continuous whole-cell voltage-clamp recording (V_{hold} = -80 mV) of spontaneous PSCs in an eAMC. Note oscillation reflected in the current baseline. Intervals defined as bursting epochs are highlighted in purple in the background. B, PSC frequency histogram (1s bin width) illustrating how synaptic input to A changes over time (mean, 2.53 Hz). Note how PSC frequency is increased during bursts (purple bars; within-burst vs interburst frequency means, 3.65 vs 0.96 Hz). C, Amplitudes of individual PSCs recorded over time (mean, 3.99 pA; median, 3.50 pA). Red horizontal line indicates detection threshold at 2.8 pA. No changes in PSC amplitudes are evident during burst epochs (purple dots; within-burst vs interburst amplitude medians, 3.44 vs 3.42 pA). D(iii), Quantification of PSCs detected in A. Averaged PSCs (D(i), aligned at half-rise) detected during bursts (purple) compared to average of PSCs detected in IBIs (gray) might indicate a slower decay of events occurring in IBIs for this cell. No differences in PSC amplitude distributions (medians, 3.44 vs 3.42 pA) are obvious from either amplitude histogram (D(ii), 0.25 pA bin width), or cumulative amplitude histogram (D(iii), vertical line indicates median).

In eAMCs, PSC frequency was reduced by ~50% during IBIs compared to burst epochs [Figure 3.13. A; 7.11 vs 13.71 Hz (medians); p = 6x10^{-4}]. Moreover, PSC amplitudes were slightly reduced by 6% [Figure 3.13. A; 7.11 vs 13.71 Hz (medians); p = 0.01] during IBIs. This moderate, yet significant reduction during IBIs did not suffice to exert a significant effect on the charge transferred across the plasma membrane during an averaged event (Figure 3.13. C). Finally, PSCs detected during IBIs were not significantly altered in their decay kinetics (Figure 3.13. D).

Figure 3.13 Quantification of synaptic input to eAMCs within-burst vs interburst epochs. Data are normalized for visualization. Statistical analysis was performed on raw data using paired t-tests. Asterisks (*) denote statistical significance, p < 0.01. Data points for cell depicted in Figure 3.12 are highlighted in green. A, Within-burst PSC frequency (f_{PC}) to eAMCs...
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is significantly increased compared to interburst fPSC. [1 vs 0.48; ± 0.08; 13.71 vs 7.11 Hz (medians); p = 6*10^{-4}; n = 10]. B, Median amplitudes in IBIs are moderately decreased to 0.94 ± 0.02 [5.64 vs 5.28 pA (medians); p = 0.01; n = 10]. Across the population of eAMCs no differences were found in either charge transfer [C; 1 vs 0.93 ± 0.05; 61.11 vs 67.29 fC (medians); n = 10] or τ_{fast} [D, 1.93 ± 0.62; 4.7 vs 6.06 ms (medians); n = 8] of averaged PSCs. Two cells were excluded from analysis in D because the small number of events in IBIs did not allow for an accurate measurement of τ_{fast} (see Figure 3.12 DI).

The data presented in this section demonstrate that synaptic input to entrained AMCs is periodic and markedly increased relatively to the sparse, uncorrelated postsynaptic activity detected in intrinsically oscillating AMCs. While basic biophysical properties (section 3.4) do not distinguish iAMCs from eAMCs, the above findings strongly suggest the aforementioned populations to be differentially wired within the local AOB network. Moreover, increased postsynaptic activity in eAMCs during burst epochs clearly demonstrates the synaptic origin of rhythmic bursting in eAMCs, as hypothesized in section 3.3.

3.7 Inhibitory synaptic drive in a mixed AMC population

In pharmacological experiments we have shown that inhibition of GABAergic fast synaptic transmission does not qualitatively affect rhythmic discharge in iAMCs. It does, however, alter some oscillation parameters (Gorin, Tsitoura et al., 2016). Similarly, selective block of inhibitory input did not qualitatively alter oscillatory activity in eAMCs (data not shown). Inhibition, however, plays a key role in rhythmic neural activity in other parts of the brain (Bartos et al., 2007; Buzsáki & Chrobak, 1995; Traub et al., 1996; Whittington et al., 2000). In the AOB, granule cells mediate lateral and recurrent inhibition in AMCs (Jia et al., 1999; Larriva-Sahd, 2008). In the MOB, inhibition at dendrodendritic synapses has been found to tune γ-oscillations (Lagier et al., 2007) and mediate mitral cell synchrony (Schoppa, 2006). What role does GABAergic synaptic transmission play in AOB oscillatory activity? Does periodic excitation of granule cells by oscillating AMCs manifest in rhythmic lateral and/or recurrent inhibition? To address these questions we recorded spontaneous PSCs in whole-cell continuous voltage-clamp recordings from mitral cells using a combination of intra- and extracellular solutions (S1/I2; section 2.1.3) that allowed us to mainly record inhibitory postsynaptic currents (IPSCs) by increasing the driving force for chloride (Figure 3.14).
Results

Figure 3.14 Inhibitory synaptic input to AMCs. A, Representative whole-cell continuous voltage-clamp recording \((V_{hold} = -60 \text{ mV})\) of spontaneous inhibitory PSCs (iPSCs; downward deflections of varying amplitudes) in an AMC. AMCs receive extensive inhibitory synaptic input under control conditions (Ai, no blockers). Red dashed rectangle corresponds to segment displayed in enlarged scale above trace. Inset illustrates shift of \(E_{Cl}^*\) for these experiments (S1/12; \(E_{Cl}^* \approx 0 \text{ mV}\)). Inhibition of fast inhibitory synaptic transmission (Aii; 10 \(\mu\text{M}\) gabazine) abolishes the vast majority of PSCs \((n = 7289 \text{ vs } 98)\). Arrowheads indicate baseline deflection in regular intervals (less evident in Aii), suggesting that we recorded from an oscillating AMC. B, PSC frequency histogram (1 s bin width). Under control conditions (Bi) the cell receives extensive (mean, 40.49 Hz), tonic synaptic input as indicated by the autocorrelation histogram of detected events (inset, 1 s bin width). Upon application of 10 \(\mu\text{M}\) gabazine (Bii) the few detected PSCs (mean, 0.54 Hz) occur periodically (inset; autocorrelation histogram, 1 s bin width). In C the amplitudes of individual PSCs are depicted over time. Red horizontal line indicates detection threshold at 3 pA. Amplitudes are markedly diminished upon gabazine application (Cii) compared to control (CI) [20.84 vs 8.03 pA (mean); 13.0 vs 7.68 pA (median)]. This finding is not unexpected since \(E_{Cl}^* \approx 0 \text{ mV}\) increases the amplitudes of events that should be sensitive to gabazine, namely iPSCs. D, F, Analysis of events detected in Aii \((n = 7289)\). D, Average of all PSCs aligned at half-rise. Red curve indicates monoexponential fit used to calculate decay kinetics \((\tau_{decay} = 3.54 \text{ ms})\). Distribution of rise times is skewed (Ei; 0.2 ms bin width) and thus not well fitted by a Gaussian function. Fii, Cumulative rise time histogram. Median (1.20 ms) indicated by vertical line. The majority of PSC amplitudes clusters below 50 pA (Fi; amplitude histogram, 1 pA bin width). Fii, Cumulative amplitude histogram with median (13.0 pA) indicated by vertical line.

Shifting the chloride reversal potential \((E_{Cl}^* \approx 0 \text{ mV})\) resulted in an apparent increase in spontaneous synaptic activity (Figure 3.14 Aii). Incubation with gabazine (10 \(\mu\text{M}\)) abolished the vast majority of PSCs (Figure 3.14 Aii) confirming that the experimental design (S1/12) is suitable for analysis of inhibitory synaptic drive in AMCs. Similar to experiments in section 3.6, we analyzed PSC frequency (Figure 3.14 B) and amplitudes (Figure 3.14 C, F), as well as rise and decay kinetics (Figure 3.14 D, E).
AMCs were found to receive extensive inhibitory drive (f_{PSC}, 29.36 ± 3.81 Hz; mean ± SEM; p < 0.0001; Figure 3.15 A) that is sensitive to pharmacological block of GABA_A receptors (10 μM gabazine; 0.30 ± 0.11 Hz). The increased driving force for chloride resulted in increased average amplitudes (24.06 ± 4.88 vs 6.68 ± 1.19 pA; mean ± SEM; p = 0.01; Figure 3.15 B) and charge transfer per average event (287.5 ± 53.4 vs 58.3 ± 15.8 fC; mean ± SEM; p < 0.01; Figure 3.15 C) compared to PSCs that were not sensitive to gabazine. IPSC rise times were faster than events not mediated by GABA_A receptors (1.30 ± 0.09 vs 1.82 ± 0.09 ms; mean ± SEM; p < 0.002; Figure 3.15 D).

*Figure 3.15 Quantification of inhibitory synaptic input to a mixed population of AMCs. A-D, Diagrams comparing PSC parameters under control conditions to conditions under inhibition of inhibitory synaptic transmission. Individual experiments are depicted in black. Data points for cell depicted in Figure 3.14 are highlighted in violet. Experiment numbers for each condition are indicated. Asterisks (*) denote statistical significance (unpaired t-tests). Average values are shown ± SEM (red). Frequency (A), amplitude (B), charge transfer (C), and rise time values (D) are shown before and during gabazine (10 μM) incubation. All parameters are significantly affected by gabazine: frequencies, 29.36 ± 3.81 vs 0.30 ± 0.11 Hz (p < 0.0001); amplitudes, 24.06 ± 4.88 vs 6.68 ± 1.19 pA (p = 0.01); charge transfer, 287.5 ± 53.4 vs 58.3 ± 15.8 fC (p < 0.01); rise times, 1.30 ± 0.09 vs 1.82 ± 0.09 ms (p < 0.002).

Most importantly, inhibitory synaptic drive in AMCs was non-rhythmic. Figure 3.16 depicts the autocorrelation histograms of spontaneous PSCs recorded in ten neurons under control conditions (black) and during gabazine incubation (violet). In nine of ten neurons, no periodicity was detected in their inhibitory synaptic drive (Figure 3.16 A-I). Regular side peaks in the ACH of one neuron (Figure 3.16 J) indicate that inhibitory input to this cell was rhythmic. Upon pharmacological block of GABA_A receptor-mediated transmission, however, remaining PSC rhythmicity became apparent in two of five neurons (Figure 3.16 Gi, Hi). Non-rhythmic inhibitory input to AMCs supports the notion that the majority of PSCs recorded experiments that are presented in section 3.6 (solutions S1/I1) were not mediated by chloride, and thus were most likely excitatory in nature.
3.8 Excitatory synaptic drive in eAMCs

The above results (section 3.3) show that in a subpopulation of eAMCs oscillatory activity is sensitive to block of fast excitatory synaptic transmission (glut.-sensitive eAMCs). In a second entrained population, however, rhythmic activity persists under conditions of synaptic isolation from both fast excitatory and inhibitory transmission (glut.-insensitive eAMCs). To further unravel the mechanistic basis of oscillatory entrainment we recorded spontaneous excitatory postsynaptic activity in both glut.-sensitive and glut.-insensitive AMCs in presence of 10 µM gabazine.

Figure 3.17 and Figure 3.18 show a representative recording from a glut.-sensitive and a glut.-insensitive eAMC respectively. In the glut.-sensitive eAMC, excitatory synaptic drive was found to occur in periodic surges during burst epochs with periods of relative silence in between (12.02 vs 0.54 Hz; mean; Figure 3.17 A, B). Block of NMDA receptor-mediated transmission decreased average \( f_{PSC} \) from 5.70 to 2.82 Hz and was not sufficient to abolish the oscillation of the current baseline or rhythmic input to the glut.-sensitive eAMC (7.30 vs 0.08 Hz; mean; burst vs IBI). Additional block of AMPA / Kainate receptors, however, abolished baseline oscillation. Parallel baseline drift to more positive values indicates a concomitant hyperpolarization of the neuron’s membrane potential (Figure 3.17 A). Here, no periodicity was apparent in the remaining PSCs (3.13 Hz; mean; Figure 3.17 B). Interestingly, EPSC amplitudes were bimodally distributed (Figure 3.17 C), forming two distinct populations that have amplitudes either >10 pA or <10 pA (Figure 3.17 F, ii). Under conditions of isolation from fast synaptic transmission, only events with amplitudes <10 pA remained. While decay kinetics did not differ substantially in any of the tested blocker combinations (Figure 3.17 D), distribution of rise times shifted to faster kinetics upon application of AP5, thus becoming less skewed (Figure 3.17 E).
Results

Figure 3.17 Excitatory input to glutamate-sensitive eAMCs is rhythmic and drives oscillations. A, Representative whole-cell continuous voltage-clamp recording (V_m = -75 mV) of spontaneous excitatory PSCs (EPSCs; downward deflections of varying amplitudes) in a glutamate-sensitive eAMC recorded during incubation with gabazine (10 μM). Note EPSCs situated upon the oscilating current baseline only during burst epochs. Inhibition of NMDA receptor-mediated transmission (100 μM AP5) is not sufficient to abolish the oscillation. However, the oscillation is sensitive to addition of 10 μM NBQX to block AMPA/Kainate receptors. Here, the baseline deflection to more positive values indicates a hyperpolarization of the cell’s membrane potential.

B, EPSC frequency (f_EPSC) histogram (1 s bin width) illustrating how, under incubation with gabazine (violet), excitatory synaptic input to the cell (mean, 5.70 Hz) occurs in barrages during burst epochs (mean, 12.02 Hz), and is nearly absent during IBIs (mean, 0.54 Hz). Incubation with AP5 (blue) reduces mean f_EPSC to 2.82 Hz. Average f_EPSC remains elevated during burst epochs, compared to IBIs [7.30 vs 0.08 Hz (means)]. Rhythmicity of excitatory input is evident in the highly periodic autocorrelation histograms (inset; 1 s bin width) for both pharmacological conditions. Isolation from fast excitatory transmission by addition of NBQX (red) abolishes rhythmicity of f_EPSC (mean, 3.13 Hz). Note “side” peaks in autocorrelation histogram (inset) resulting from last burst of EPSCs in the very beginning of synaptic blocker cocktail application (red arrowhead). C, Diagram depicting amplitudes of individual EPSCs over time with detection threshold indicated at 3.5 pA (red horizontal line). Two distinct populations of EPSCs with respect to amplitude are evident. Upon synaptic isolation only events with small amplitudes remain.

D-F, Analysis of EPSCs detected in A. D, Averages of EPSCs detected under incubation with gabazine (violet, n = 1367), gabazine...
Results

+ AP5 (blue, n=338), and addition of NBQX to the blocker cocktail (red, n=188), aligned at half-rise. Fast component of decay kinetics is somewhat slower under gabazine + AP5 (3.54 ms) compared to 2.64 ms under gabazine alone, or 2.73 ms under conditions of synaptic isolation. Distribution of rise times (Ei; 0.2 ms bin width, color-coded according to pharmacological condition) and cumulative rise times (Eii) does not reveal multiple populations with respect to this parameter. The existence of 2 distinct populations of EPSCs with respect to amplitude, however, is evident in the (cumulative) amplitude histograms (Fi, ii) under control conditions (10 µM gabazine, violet) and upon additional inhibition of AP5 receptors (+ 100 µM AP5; blue). Under inhibition of all fast synaptic transmission EPSCs with amplitudes over 10 pA are blocked.

Similar to glut.-sensitive eAMCs, excitatory synaptic drive occurred in periodic barrages in glut.-insensitive eAMCs (burst vs IBI $f_{EPSC}$: 4.20 vs 0.18 Hz; mean; Figure 3.18 A, B). In this representative recording, inhibition of AMPA / Kainate receptor-mediated transmission decreased average EPSC rates from 1.96 Hz (10 µM gabazine) to 1.26 Hz (10 µM gabazine + 10 µM NBQX). Under both conditions, synaptic drive was rhythmic (Figure 3.18 A, B; ACH insets). Subsequent isolation from fast synaptic transmission, however, did not abolish the oscillation of the current baseline. Moreover, average $f_{EPSC}$ was not further decreased by NMDA receptor block, remaining periodic with increased activity during burst epochs (Figure 3.18 B; ACH inset). In this neuron, the fast component of average event decay became slower upon incubation with NBQX [4.45 ms (gabazine) vs 6.34 ms (gabazine + NBQX); Figure 3.18 D]. EPSC rise times were skewed under control conditions (10 µM gabazine; Figure 3.18 E, violet) and lasted up to 8 ms. Upon incubation with NBQX (10 µM; green) rise time distribution became less skewed with maximal rise times of up to 4 ms. NBQX also slightly decreased EPSC amplitudes as evident in Figure 3.18 C, F.
Results

Figure 3.18 Excitatory input to glut.-insensitive eAMCs is rhythmic and not sensitive to inhibitors of fast synaptic transmission. A, Representative continuous voltage-clamp recording (V_hold = -80 mV) of spontaneous excitatory PSCs (EPSCs; downward deflections of varying amplitudes) in a glut.-insensitive eAMC recorded during incubation with gabazine (10 μM). EPSCs are situated upon the oscillating current baseline predominantly during bursts. Neither inhibition of AMPA / Kainate receptor-mediated transmission (10 μM NBQX), nor additional block of NMDA receptors (100 μM AP5) abolishes the oscillation. B, EPSC frequency histogram (1 s bin width) illustrating how f EPSC (mean ± 1.96 Hz) is markedly increased during burst epochs compared to iIBs (violet; 4.20 ± 0.18 [means]). Incubation with NBQX reduces mean f EPSC to 1.26 Hz (green). Here, average f EPSC during bursts remains elevated, as it does under subsequent addition of 100 μM AP5 (red). Rhythmicity of excitatory input is evident in the autocorrelation histograms (insets; 1 s bin width). Amplitudes of individual EPSCs over time are depicted in C. Detection threshold indicated at 3.5 pA (red line). D-F, Analysis of EPSCs detected in A. For each condition, EPSCs were aligned at half-rise and averaged (D, color-coded according to pharmacological condition as in B). The fast component of decay kinetics increased from 4.45 ms (gabazine) to 6.34 and 6.41 ms under additional incubation with NBQX and NBQX + AP5, respectively. While distribution of EPSC rise times (Ei, 0.2 ms bin width) becomes narrower upon incubation with NBQX, addition of AP5 has no evident effect on this parameter. Cumulative rise time probabilities (Eii) indicate a shift to faster rise times due to AMPA / Kainate block. Amplitude histogram (Fi, 1 pA bin width) and cumulative amplitude probabilities (Fii) indicate a slight decrease in EPSC amplitudes due to incubation with NBQX. Subsequent addition of AP5 does not further alter either distribution, or cumulative probabilities.

Across a mixed population of glut.-sensitive and glut.-insensitive eAMCs, EPSC frequencies were markedly increased during bursts and were almost absent in iIBs (Figure 3.19 A; violet). Accordingly, block of fast glutamatergic transmission only affected within-burst f EPSC. Here, block of NMDA receptors (100 μM AP5; Figure 3.19 A; blue) reduced average f EPSC by 22%. Additional inhibition of AMPA / Kainate receptors further reduced average within-burst f EPSC to 50% of control values (100 μM
AP5 + 10 μM NBQX; Figure 3.19 A; red). Incubation with NBQX in absence of AP5 similarly reduced average f_{EPSC} (Figure 3.19 A; green). Analysis of excitatory fast synaptic drive in eAMCs revealed a tendency towards increased charge transfer per average event during bursts, compared to IBIs (10 μM gabazine; 1.0 vs 0.7 ± 0.13, within-burst vs IBI; Figure 3.19 B; violet). This trend was abolished upon pharmacological block of either AMPA / Kainate (10 μM NBQX; green), or NMDA receptors (100 μM AP5; blue), as well as upon a complete block of fast glutamatergic transmission (10 μM NBQX + 100 μM AP5; red). Figure 3.19 C indicates how excitatory charge transfer in eAMCs changed over time. During IBIs, charge transfer in eAMCs was reduced to 14% relative to within-burst epochs. During bursts, we found a reduction of 40% upon inhibition of NMDA receptors (100 μM AP5; blue). Incubation with NBQX (10 μM; green) alone or NBQX + AP5 (red) reduced charge transfer during bursts more strongly by 60% and 62%, respectively.

So far, these experiments show that entrained oscillatory activity in eAMCs is driven by periodic surges of excitatory synaptic transmission that is mediated, at least in part, by AMPA / Kainate and NMDA receptors. The increase in excitatory input to eAMCs manifests mainly as an increase in spontaneous EPSC frequency, and to a lesser degree as an increase in charge carried by individual events. Moreover, in contrast to inhibitory synaptic drive (section 3.7), excitatory drive is practically absent during IBIs.

Figure 3.19 Quantification of excitatory synaptic input to eAMCs during burst epochs compared to IBIs for all pharmacological conditions tested. Normalized data are plotted as means ± SEM. A, Under control conditions (10 μM gabazine, violet, n = 10) f_{EPSC} during IBIs is reduced to 0.17 ± 0.05 compared to burst epochs. Block of NMDA receptor-mediated transmission (100 μM AP5, blue, n = 3) markedly reduced within-burst f_{EPSC} to 0.78 ± 0.08, while frequency of interburst EPSPs is not affected (0.15 ± 0.08). Inhibition of AMPA / Kainate receptors (10 μM NBQX, green, n = 5) affected within-burst f_{EPSC} more strongly, reducing it to 0.50 ± 0.15. Again, f_{EPSC} during IBIs is not strongly altered (0.12 ± 0.05). Similarly, isolation from fast synaptic transmission (10 μM gabazine + 10 μM NBQX + 100 μM AP5, red, n = 4) reduces within-burst f_{EPSC} by half (0.5 ± 0.14). Interburst f_{EPSC} remains unaffected at 0.19 ± 0.06. B, Analysis of charge transferred per average EPSC (f_{EPSC}). Under control conditions charge transfer during IBIs is decreased to 0.70 ± 0.13. Incubation with AP5 reduced f_{EPSC} in both within-burst and interburst epochs to 0.78 ± 0.01 and 0.38 ± 0.11, respectively. Application of NBQX reduced f_{EPSC} similarly to 0.66 ± 0.10 (within-burst) and 0.53 ± 0.07 (interburst). Complete block of fast excitatory synaptic transmission decreased f_{EPSC} to similar levels during burst epochs and IBIs (0.72 ± 0.11 vs 0.73 ± 0.12). C, Analysis of charge transferred over time (f_{EPSC} - f_{EPSC}) during bursts and IBIs indicates how synaptic input to eAMCs is strongly increased during bursting epochs, and is mediated, at least in part, via fast excitatory synaptic transmission. Control: 1 vs 0.14 ± 0.05, AP5: 0.60 ± 0.06 vs 0.08 ± 0.05, NBQX: 0.40 ± 0.16 vs 0.08 ± 0.04, AP5 + NBQX: 0.38 ± 0.11 vs 0.15 ± 0.06 (within-burst vs interburst intervals).

We next asked how excitatory fast synaptic transmission differentially shapes spontaneous synaptic drive in glut.-sensitive and glut.-insensitive eAMCs and compared several parameters under control conditions (10 μM gabazine) and under synaptically isolated conditions (10 μM gabazine + 10 μM NBQX + 100 μM AP5). Block of glutamatergic transmission reduced frequency of spontaneous EPSCs more strongly in glut.-sensitive eAMCs compared to glut.-insensitive eAMCs (0.53 ± 0.10 vs 0.77 ± 0.12; Figure 3.20 Ai, Bi). No clear effects were observed for any other parameter (amplitude, charge transfer, rise time, τ_{fast}; Figure 3.20 Aii-v, Bii-v), likely due to the relatively small sample size. Nevertheless,
some tendencies were observed and need to be addressed in future experiments: EPSC amplitudes, rise times, as well as charge transfer per average event seem to decrease in both glut.-sensitive and glut.-insensitive eAMCs subpopulations (Figure 3.20 Ai-iv, Bii-iv), while $\tau_{\text{fast}}$ seems to increase in glut.-insensitive but not in glut.-sensitive eAMCs (Figure 3.20 Av, Bv).

In summary, these data demonstrate that excitatory fast synaptic drive is more strongly pronounced in glut.-sensitive eAMCs. Pharmacological block of AMPA / Kainate and NMDA receptors in this population abolished baseline oscillation. In glut.-insensitive eAMCs, however, such oscillations were insensitive to NBOX and APS incubation (see also section 3.3). Here, rhythmicity in synaptic drive was maintained upon isolation from fast synaptic transmission indicating a different mechanistic basis for oscillatory entrainment in this entrained AMC subpopulation.

![Figure 3.20](image)

Figure 3.20 Comparison of how fast excitatory synaptic transmission affects EPSC parameters in glut.-sensitive vs glut.-insensitive eAMCs. Data are normalized to control conditions (10 µM gabazine). Number of experiments is denoted in parentheses. Data are presented as means ± SEM. A, Effects of AMPA / Kainate and NMDA receptor inhibition on glut.-sensitive eAMCs. Cell depicted in Figure 3.17 is highlighted in green. While incubation with APS and NBOX reduces $f_{\text{EPSC}}$ to 0.53 ± 0.10, no clear effects are observed in either EPSC amplitude (0.85 ± 0.14), $f_{\text{EPSC}}$ (0.70 ± 0.19), EPSC rise times (0.87 ± 0.11), or $\tau_{\text{fast}}$ (0.87 ± 0.39). B, Isolation from fast synaptic transmission has no strong effects on EPSCs in glut.-insensitive eAMCs. Cell depicted in Figure 3.18 is highlighted in light red. $f_{\text{EPSC}}$: 0.77 ± 0.12, amplitude: 0.92 ± 0.04, $f_{\text{EPSC}}$: 0.80 ± 0.07, rise times: 0.87 ± 0.03, $\tau_{\text{fast}}$: 1.44 ± 0.16.

### 3.9 “Dual” rhythmic synaptic drive in entrained AOB mitral cells

Recordings of spontaneous synaptic activity in entrained oscillatory AMCs revealed that amplitudes of spontaneous (E)PSCs were multimodally distributed in three of 23 eAMCs. Hence, we analyzed and compared distinct EPSC populations within these cells. The neuron depicted in Figure 3.17 showed bimodally distributed EPSCs, forming two distinct populations with amplitudes either <10 pA (“small”) or > 10 pA (“large”); Figure 3.21 Ai, ii). Under control conditions (10 µM gabazine) these occurred with a mean frequency of 0.83 or 2.02 Hz, respectively. Large amplitude events had a faster decay ($\tau_{\text{fast}}$;
3.20 vs 8.01 ms; Figure 3.21 Aiii) and narrower rise time distribution with the majority of EPSPs exhibiting rise times between 1 and 2.5 ms, compared to small events. Here, rise times ranged from 0.5 to 4 ms (Figure 3.21 Av). Occurrence of both large and small events was highly periodic (Figure 3.21 Av). Block of NMDA receptors (100 μM AP5) did not affect bimodal distribution of amplitudes (Figure 3.21 Bi, ii). The fast component in decay (τfast) of EPSPs <10 pA was reduced to 4.84 ms, whereas that of events >10 pA became somewhat slower (4.53 ms; Figure 3.21 Bii). However, EPSPs >10 pA still occurred more frequently than EPSPs <10 pA (2.82 vs 1.31 Hz), and exhibited faster kinetics (Figure 3.21 Biii; iv). Rhythmicity in synaptic drive was evident in both populations (Figure 3.21 Bv). Additional block of AMPA / Kainate receptors with 10 μM NBQX abolished all events with amplitudes >10 pA (Figure 3.21 Cii, ii). Remaining EPSPs (<10 pA) decayed even faster (τfast = 2.46 ms; Figure 3.21 Ciii) but did not show any evident changes in rise time distribution (Figure 3.21 C-iiv). Most notable, periodicity in synaptic drive was abolished upon isolation from fast synaptic transmission even though mean occurrence throughout the recording was increased (fEPSC; 3.31 Hz).

Figure 3.21 Analysis of two distinct populations of EPSCs with respect to amplitude in a glut.-sensitive eAMC (see Figure 3.17). Events were assigned to one of two populations, either above, or below 10 pA (vertical dashed lines in Ai ii, Bi ii, Ci ii). A, Control conditions [gabazine (10 μM)]. B, Additional block of NMDA receptor-mediated transmission [gabazine (10 μM) + AP5 (100 μM)]. C, Analysis of PSCs detected in isolation from fast synaptic transmission [gabazine (10 μM) + AP5 (100 μM) + NBQX (10 μM)]. Under control conditions, as well as upon application of AP5, two distinct populations of EPSCs are evident in both amplitude histograms (Ai, Bi, 1 pA bin width), and corresponding cumulative probabilities (Aii, Bii). Under synaptic isolation only one population with events <10pA remains (Cii). A-Ciii. Within each population EPSCs were aligned at half-rise and averaged for comparison of their decay kinetics (τfast in average EPSCs >10pA or <10 pA). Traces are color-coded as in A-Cii. ii. Red curves are biexponential fits used to calculate decay kinetics. Aiii, 3.20 vs 8.01 ms. Biii, 4.53 vs 4.84 ms. Ciii, 2.46 ms for average of remaining population <10 pA. A-Cvi. Rise time histograms of EPSCs within each population (color-coded) reveal a relatively narrow distribution of events >10 pA. Here, the rise times of the majority of EPSCs lies between 1 and 2.5 ms (Aiv, Biv). For small events (<10 pA) the distribution is much broader, ranging from 0.5 to 4 ms. Autocorrelation histograms (A-Bv, Cv).
Results

colors as in A-C. ii) illustrating periodicity of recorded EPSCs in both large and small amplitude populations. Rhythmicity is abolished under synaptic isolation in the remaining population of EPSCs with amplitudes <10 pA (Cv). See arrowheads in Figure 3.17 A-B regarding origin of side-peak (* in Cv).

Bimodal distribution of PSCs was also found in an eAMC where no pharmacological agents were applied (Figure 3.22). As outlined in sections 3.6 and 3.7, while both excitatory and inhibitory events are recorded in this configuration, EPSCs are strongly overrepresented. This bias towards excitatory events stems from the increased driving force (V_{DF}) for cations, whereas V_{DF} for chloride is small (solutions S1/S11). Figure 3.22 A depicts a continuous voltage clamp recording where spontaneous PSCs are evident as downward deflections from baseline. Analysis of f_{PSC} over time (Figure 3.22 B) illustrates how f_{PSC} gradually increased, driving the neuron into bursting mode. During each cycle, a steep drop in f_{PSC} resulted in burst termination, causing the current baseline to drop back to zero (Figure 3.22 A). Bimodal distribution of PSC amplitudes is evident with two distinct populations that have amplitudes either above or below 5 pA (Figure 3.22 C, Di-ii). In this recording large events (>5 pA) occurred more frequently than small events (<5 pA) at 4.59 and 1.24 Hz, respectively. In this neuron average EPSC decay was particularly slow in both populations (t_{fast}: 18.63 vs 19.71 ms; PSCs <5 pA vs >5 pA; Figure 3.22 Diii). Rise times, however, were slower and more widely distributed in small events compared to PSCs >5 pA (Figure 3.22 Div). Both large and small PSCs occurred periodically throughout the recording.

Figure 3.22 Example for distinct PSC populations with regard to amplitude in an eAMC with no pharmacological perturbation of network activity. A. Representative whole-cell continuous voltage-clamp recording (V_{hold} = -80 mV) of spontaneous PSCs. B. Frequency histogram (1 s bin width) illustrating how f_{PSC} (mean, 4.59 Hz) is markedly increased during burst epochs compared to IIBs. Note how a steep drop in f_{PSC} causes burst termination, while a subsequent gradual increase drives the current baseline back to more negative values. C. Diagram depicting individual PSC amplitudes over time. Detection threshold at 2.5 pA indicated by red horizontal line. Two distinct populations are evident. D. Bimodal distribution is evident in
both the amplitude histogram (Di, 1 pA bin width), and corresponding cumulative probability (Dii). For further analysis events were assigned to one of two populations, either above (dark green), or below (light green) 5 pA (vertical dashed lines in Di-ii). Within each population EPSCs were aligned at half-rise and averaged for comparison of their decay kinetics ($\tau_{fast}$): 19.71 ms for events >5 pA, 18.63 ms for events <5 pA. Red curves are biexponential fits (Diii). Div. Distribution for the majority of rise times of PSCs >5 pA ranges from 1.5 to 3.3 ms (peak around 2.2 ms). Rise times of small PSCs (<5 pA) are more widely distributed and range from =0.4 to over 6 ms. Autocorrelation histograms (Dv) illustrate rhythmicity in both PSC populations.

The third neuron with multimodal EPSC amplitude distribution, was a glut.-insensitive eAMC in which isolation from fast synaptic transmission did not abolish baseline oscillation (Figure 3.23 A). Nevertheless, block of fast glutamatergic transmission strongly attenuated the oscillation. Successive incubation with AP5 (100 µM) and NBQX (10 µM) progressively reduced EPSC frequencies and blurred boundaries between silent IBIs and burst epochs with increased activity (Figure 3.23 B). This decorrelation of synaptic drive is also evident in the diagram depicting the amplitudes of individual EPSCs over time (Figure 3.23 C). Here, distinct populations of EPSCs with respect to their amplitude are less evident compared to the aforementioned examples (Figure 3.21 and Figure 3.22). Amplitude distribution, as well as cumulative amplitude probabilities, however, illustrate a trimodal distribution. Detected events cluster into three populations that have amplitudes either <6 pA (“small”), between 6 pA and 11 pA (“intermediate”), or >11 pA (“large”) (Figure 3.23 D-Fi, ii). Under control conditions (10 µM gabazine), large events occurred frequently (5.52 Hz) compared to 0.73 and 0.97 Hz for intermediate and small events, respectively. Large events remained the most frequent to occur upon inhibition of NMDA receptor-mediated synaptic transmission (100 µM AP5; 4.12 vs 0.68 vs 1.07 Hz), as well as upon complete isolation from fast synaptic transmission (10 µM gabazine + 100 µM AP5 + 10 µM NBQX; 3.99 vs 0.77 vs 0.85 Hz). Similar to the above example (Figure 3.21 and Figure 3.22), decay kinetics of averaged events for each population indicate a progressive increase of $\tau_{fast}$ with decreasing amplitude for all experimental conditions: 4.21 vs 7.42 vs 7.60 ms (10 µM gabazine; Figure 3.23 Diii), 3.81 vs 6.25 vs 5.77 ms (gabazine + AP5; Figure 3.23 Eiii), and 7.15 vs 7.15 vs 7.49 ms (gabazine + AP5 + NBQX; Figure 3.23 Fiii). Likewise, rise times tended to increase and become more widely distributed with decreasing amplitudes (Figure 3.23 D-Fiv). With regard to rhythmicity in synaptic drive, large and intermediate events occurred highly periodic, while regularity in EPSCs <6 pA was reduced under control conditions (Figure 3.23 Dv). This reduced correlation in small events is also evident in Figure 3.23 C where EPSCs with amplitudes close to the detection threshold were recorded during IBIs when larger events were absent. Incubation with AP5 did not affect regularity in large and intermediate events. Small EPSCs, however, became more regular (Figure 3.23 Ev) despite small EPSCs still being recorded during IBIs (Figure 3.23 C). Subsequent block of AMPA / Kainate receptors and concomitant isolation from fast synaptic drive led to general decorrelation in EPSC occurrence, regardless of amplitude (Figure 3.23 Fv), although some degree of regularity is still evident in weakly pronounced side-peaks in the autocorrelation histograms for all three EPSC populations. The decrease in regularity was accompanied by the aforementioned strong attenuation of the baseline oscillation.
Results

A

10 μM gbz

-40 pA

10 μM gbz + 100 μM AP5

10 μM gbz + 100 μM AP5 + 10 μM NBQX

20 s

20 pA

B

f_{EPC} (Hz)

0 50 100 150

0 50 100 150

0 50 100

C

amplitude (pA)

0 50 100 150

0 50 100 150

0 50 100

D (i)

count

0 10 20 30

0 10 20 30

0 10 20 30

(ii)

P (amplitude (pA))

0 10 20 30

0 10 20 30

0 10 20 30

(iii)

20 ms

5 pA

(iv)

count

0 50 100

0 50 100

0 50 100

(v)

Z-score

E (i)

count

0 10 20 30

0 10 20 30

0 10 20 30

(ii)

P (amplitude (pA))

0 10 20 30

0 10 20 30

0 10 20 30

(iii)

20 ms

12 pA

(iv)

count

0 50 100

0 50 100

0 50 100

(v)

Z-score

F (i)

count

0 10 20 30

0 10 20 30

0 10 20 30

(ii)

P (amplitude (pA))

0 10 20 30

0 10 20 30

0 10 20 30

(iii)

20 ms

12 pA

(iv)

count

0 50 100

0 50 100

0 50 100

(v)

Z-score

Figure 3.23 Multimodal distribution of EPSC amplitudes in a glut.-insensitive eAMC. A, Representative whole-cell continuous voltage-clamp recording (V_{hold} = -80 mV) of spontaneous EPSCs during incubation with gabazine (10 μM, violet), gabazine + AP5 (100 μM, blue), and gabazine + AP5 + NBQX (10 μM, red). B, Frequency histogram (1 s bin width). Under gabazine f_{EPC} (mean, 5.52 Hz) is strongly increased during bursts compared to IBIs (means, 10.24 vs 3.33 Hz). Block of NMDA-mediated transmission decreases f_{EPC} to 4.12 Hz (mean). An increase in f_{EPC} during burst epochs is maintained nonetheless (6.42 vs 1.46 Hz). Isolation from fast synaptic transmission further decreases mean f_{EPC} to 3.99 Hz. Here, oscillation of the current baseline is strongly diminished, and thus prohibits further breakdown of the recording into burst and interburst intervals. Individual amplitudes of EPSCs are depicted in C. D-F, Event analysis of EPSCs detected under all tested pharmacological conditions. Amplitude histograms (1 pA bin width) reveal a multimodal distribution with 3 peaks for all three pharmacological conditions (D-Fii). This is also reflected in the shape of the cumulative probability curves (D-Fii). For further analysis events were assigned to one of three populations (see vertical dashed lines in D-Fii), either above 11 pA (large; dark colors), below 6 pA (small; light colors), or between 6 and 11 pA (medium; medium colors). Within each population EPSCs were aligned at
Results

half-rise and averaged for comparison of their decay kinetics (D-Fiii). Red curves are biexponential fits. $t_{fast}$ is given for EPSCs with large, medium, and small amplitudes. Diii, Gabazine: 4.21 vs 7.42 vs 7.60 ms. Eiii, Gabazine + AP5: 3.81 vs 6.25 vs 5.77 ms. Fiii, Gabazine + AP5 + NBQX: 7.15 vs 7.15 vs 7.49 ms. D-Fiv, Comparison of rise time distributions of the three populations in all pharmacological conditions. Under gabazine (Div) the majority of rise times of large and medium EPSCs clusters between 0.8 and 2.8 ms, peaking at $t=1.7$ ms. Small amplitude events are distributed between 0.4 and up to $>$5 ms, lacking a prominent peak. Upon additional inhibition of NMDA receptors (Eiv) large events exhibit a narrow distribution (1-2 ms; peak at $t=1.35$ ms) in their rise time. Medium-sized events are more widely distributed (0.9-2.6 ms), peaking at 1.5 ms. Again, rise times of small EPSCs are widely distributed (0.5-$>$6 ms), with no peak evident. Isolation from fast synaptic transmission (Fiv) does not strongly alter rise distributions in either of the three populations. Rise times of big EPSCs range from 1-1.8 ms (range: $t=0.75-2.1$ ms). Small event rise times range from 0.4-$>$6 ms, lacking a peak in their distribution. D-Fv, Autocorrelation histograms of EPSCs. Colors correspond to pharmacological conditions, shades correspond to amplitude populations. Under control conditions (Dv, 10 µM gabazine), both big and medium-sized events exhibit a high degree of periodicity. Small events ($<6$ pA) are less well correlated. Additional block of NMDA receptors (Ev) does not affect rhythmicity in big and medium-sized events. Small EPSCs, however, display a higher degree of correlation (see regularity in more prominent side peaks, compared to Dv). Upon isolation from fast synaptic transmission rhythmicity is less evident in all three EPSC populations (Fv).

In summary, multimodal EPSC amplitude distribution was detected in 13% of eAMCs. In all cases, EPSC kinetics and amplitudes were inversely correlated, pointing towards dendritic low-pass filtering (Rall, 1967) as a probable cause for the observed phenomenon. EPSCs from distal synapses must traverse more dendritic length than the latter and are expected to decrease in amplitude because axial resistance of the membrane causes the voltage to drop between a synaptic input site and the soma. Additionally, charging of the membrane capacitance between synaptic input site and soma slows the kinetics of postsynaptic currents. A potential change produced at a distal synapse will thus be slower at the soma than at the synaptic input site itself. Therefore, EPSCs with smaller amplitudes could stem from synapses located at more distal locations, while synapses that trigger EPSCs with larger amplitudes might be located closer to the soma.

3.10 Paired whole-cell patch-clamp recordings from AOB mitral cells

Intrinsically rhythmogenic AOB mitral cells are highly heterogeneous with respect to their oscillatory discharge parameters. A similarly wide spectrum of rhythmic discharge patterns was also found to describe oscillatory activity in entrained mitral cells (Figure 3.8). Therefore, we hypothesized that iAMCs might entrain rhythmic activity in eAMCs via a hitherto undescribed excitatory connection between the two classes of projection neurons (section 3.5). Our findings that iAMCs and eAMCs are differentially wired (section 3.6) and that excitatory but not inhibitory synaptic drive in eAMCs is periodic (sections 3.7 and 3.8) support this hypothesis. To further investigate connectivity between AOB mitral cells we established an experimental framework utilizing paired whole-cell patch clamp recordings. The use of paired recordings to study neuronal communication was developed in the 1960s in the ganglion of Aplysia in vitro (Hughes & Tauc, 1968) and thrived with the spread of acute mammalian brain slice preparations (reviewed in Miles & Poncer, 1996). The main advantage of paired-recordings in the analysis of synaptic transmission resides in the fact that this is the only method that guarantees triggering of a presynaptic action potential (AP) (Debanne et al., 2008).

To begin studying connectivity between AMCs we utilized three recording protocols, each focusing on a specific aspect of neuronal communication. Biocytin was routinely added to the intracellular solution, to allow for connectivity to not just be functionally, but also anatomically explored. To assess chemical synaptic transmission between AMC pairs a burst of action potentials was induced in one neuron while synaptic activity was monitored in a second (see section 2.2.3 and 2.2.5).

Figure 3.24 shows an example paired recording from an intrinsically oscillating and an irregular AMC. Here, a burst of 23 APs was elicited by consecutive injection of 23 rectangular current pulses (Figure
3.24 Bi; see section 2.2.5) while synaptic activity was monitored in the second neuron (Figure 3.24 Bii). This protocol was repeated 25 times. Systematic exploration of the postsynaptic signal was performed by averaging both pre- and postsynaptic signals after both signals were aligned with reference to the presynaptic spike (see section 2.2.5). No PSC was detected after either first, second, third..., or 23rd AP (Figure 3.24 C-E). Here, 25-fold averaging (25 sweeps, each containing a burst of 23 APs) already markedly reduced noise in the recording from the postsynaptic neuron (compare Figure 3.24 Bii to Figure 3.24 Cii–Eii). To further minimize noise, all elicited APs and corresponding postsynaptic signals were averaged (Figure 3.24 F). Again, no postsynaptic response was detected, indicating that the two neurons (Figure 3.24 A) were not synapticially connected.

Figure 3.24 Paired recording from two AMCs illustrating experiment for study of chemical synaptic interactions. A, Epifluorescence photomicrograph for Alexa 488 depicting the recording pipettes attached to two AMCs classified as intrinsically oscillating (1) and irregularly spiking (2). B, Representative sweep of a whole-cell continuous current clamp (Bi) and voltage-clamp recording (Bii, Vhold = -80 mV) from cell 1 and 2, respectively. In each sweep a burst of 23 action potentials was induced in cell 1 (black trace) by consecutive rectangular current pulse injection. Synaptic activity was continuously monitored in cell 2 (red trace). C-Eii, Averages of peak-aligned 1st, 12th, and 23rd action potentials from all consecutive sweeps. C-Eii, Corresponding averages from voltage clamp recordings in cell 2. Average from all evoked action potentials in cell 1 (Fi) and corresponding voltage clamp traces in cell 2 (Fii). No evoked PSCs are evident.

In total, chemical synaptic connectivity was assessed in thirteen pairs of AMCs (Table 3.1; four pairs were tested reciprocally). No evoked PSCs were recorded in any postsynaptic neuron.

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Table 3.1 Numbers of AMC pairs tested for chemical synaptic connectivity. Numbers in brackets denote numbers of experiments where connection was tested reciprocally.

In addition to chemical synaptic interaction, electrical connectivity via gap junctions might serve a role in oscillatory entrainment of eAMCs. We tested for electrical coupling between AMC pairs by injecting a hyperpolarizing current step into one cell, and monitored $V_{mem}$ simultaneously in the second cell (Figure 3.25). A potential coupling of AMCs via gap junctions was assessed in 5 pairs (Table 3.2). In four
pairs the experiment was performed in both directions to assess for rectifying gap junctions (Edwards et al., 1998).

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Table 3.2 Numbers of AMC pairs tested for electrical coupling via gap junctions. Numbers in brackets indicate numbers of experiments where connection was tested reciprocally.

In all but one pair no evidence for electrical coupling was detected. Figure 3.25 A, B illustrates an example where no response was measured in one neuron (iAMC; Figure 3.25 Bi, iii) upon hyperpolarizing current injection to the other neuron (eAMC; Figure 3.25 Bii). In one pair of irregularly spiking AMCs, however, a current-induced hyperpolarization triggered in the first neuron (Figure 3.25 Di) produced an attenuated voltage signal in the second neuron (Figure 3.25 Dii, iii) that corresponds to a coupling coefficient of 0.018 between these cells.

![Figure 3.25 Paired recording from two AMCs illustrating experiment for study of electrical synaptic interactions. A, C, Epifluorescence photomicrographs for Alexa 488 depicting the recording pipettes attached to two AMCs. AMCs in A were classified as entrained (1) and intrinsically oscillating (2). In C both AMCs were non-oscillating. Bi, Di, Injection of a -70 pA hyperpolarizing current step to cell 1 in current clamp. Average of 50 (Bi) and 25 (Di) consecutive sweeps. Note rebound action potentials elicited in Di (*). Bi, Di, Traces of corresponding averages (whole-cell voltage clamp) recorded in cell 2. While no activity is recorded in the iAMC of the first pair (Bi), a slight hyperpolarization corresponding to a coupling coefficient (cc) of 0.018 is evoked in cell 2 of the second pair (Di). Hyperpolarization (or lack thereof) is accentuated in smoothed traces (box algorithm, 100 points) (Bi, Dii).](image)

In a third set of experiments activity was not evoked in either cell of the recorded pair. Here, potential synaptic coupling was assessed while simply monitoring spontaneous synaptic activity in one AMC, while the second AMC was spontaneously active. Again, postsynaptic signals were systematically explored by averaging both pre- and postsynaptic signals after aligning both signals with reference to spikes in the active neuron.
Results

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Table 3.3 Numbers of AMC pairs tested for coupling while spontaneously active.

From all pairs recorded, one pair was found to oscillate in a common rhythm (Figure 3.26 A, B). Both neurons in this experiment were identified as entrained AMCs by means of hyperpolarizing current injection (see section 3.3). Here, spontaneous APs in the first neuron (eAMC 1) did not elicit any postsynaptic activity in the second neuron (eAMC 2; Figure 3.26 C). Crosscorrelation of the recordings from both AMCs (depicted in Figure 3.26 B) illustrates the high degree of correlation between the pair (Figure 3.26 D). There was, however a 365 ms lag between the two (red arrow in Figure 3.26 D) supporting the lack of a direct synaptic connection. Upon reciprocal testing for electric coupling, no response was detectable in the respective postsynaptic neuron (Figure 3.26 E, F). Post hoc staining of the biocytin-filled pair with Alexa 488-conjugated streptavidin and subsequent confocal microscopy revealed close proximity but no direct contact between both eAMCs (Figure 3.26 All, iii).

Figure 3.26 Paired recording from two “synchronized” eAMCs. Aii, Epifluorescence photomicrograph for Alexa 488 depicting the recording pipettes attached to two eAMCs. Aiii, Maximum projection of a confocal z-stack of biocytin-filled eAMCs (see Aii) stained post hoc with Alexa 488-conjugated streptavidin. Aiii, Expanded view of area denoted by rectangle in Aii. Note how neurite from eAMC 1 bypasses some of eAMC 2 without physical contact. Bi, Paired whole-cell continuous current-clamp recording from eAMC 1 (black trace) and eAMC 2 (red trace). Section of recording in dashed rectangle depicted in an expanded.
Results

Time scale in Bii. Note how bursting in eAMC 1 coincides with subthreshold depolarization in eAMC 2. Spontaneous action potentials in eAMC 1 were peak-aligned and averaged (Ci). No postsynaptic potentials (PSPs) are evident in average of corresponding sections recorded from eAMC 2 (Ci). D, Normalized crosscorrelation of recordings in Bi. eAMC 1 and eAMC 2 are highly correlated but phase-shifted by 365 ms (red arrow indicates shifted maximum). A current-induced hyperpolarization in either eAMC 1 (Eii, black) or eAMC 2 (Fii, red) did not result in an attenuated voltage response in the other neuron [Eii, Fii; smoothed traces (box algorithm, 100 points) in Eii, Fii].

3.11 Calcium imaging of rhythmic activity in the AOB

In addition to electrophysiological recordings from AOB mitral cells we took an imaging approach to study AMC oscillations in the acute slice preparation. Imaging calcium transients can serve as a surrogate for neuronal spiking activity and has been widely used to study activity in (interconnected) neuronal populations (Garaschuk et al., 2000; Peterlin et al., 2000). Monitoring of calcium activity on the single-cell level, on the other hand, is widely used to assess basic mechanisms of calcium signaling in neurons as well as for functional analysis of dendrites and spines (A. G. Carter & Sabatini, 2004; Bywalez et al., 2015; Eilers et al., 1996).

First, we sought to perform combined electrophysiological and fluorometric calcium recordings in single AMCs in order to correlate measured calcium transients to spike output (Figure 3.27). Initially, we used Fluo-4 pentapotassium (10, 5, or 3 μM in I4) and diffusion-loaded neurons via the ruptured membrane (Figure 3.27 A). We found calcium transients to be concomitant with bursting activity in oscillating AMCs (Figure 3.27 B). In all recordings, however, AP firing seized approximately one to four minutes into the recording (data not shown). While depolarizing current injections occasionally elicited just one spike at this stage, subthreshold oscillation of the membrane potential in eAMCs persisted. These findings suggest the dye’s buffer capacity to change intracellular calcium dynamics in such a way that action potential firing becomes impossible. Progressive lowering of the dye concentration from 10 to 3 μM did not positively affect the ability to spike. In a second set of experiments we used fura-2 pentapotassium which has a lower affinity for calcium and might therefore contribute to the total amount of intracellular calcium buffer molecules to a lesser extent than Fluo-4 [Kd: 140 vs 345 nM; (Grienberger & Konnerth, 2012)]. In total, recordings were attempted in over 100 AMCs. Here, in the vast majority of experiments no gigaseal could be established. In the few cases where a gigaseal was formed, the process took unusually long (several minutes compared to few seconds in regular recordings), and mostly resulted in failure to rupture the plasma membrane and establish a whole-cell configuration. Progressive lowering of the dye concentration (100, 30, 10 μM), again, did not increase the success rate. Successful whole-cell recordings were thus only performed in a handful of cases. Figure 3.27 C shows an exemplary recording from an AMC loaded with fura-2. Here, the high signal-to-noise ratio allowed for resolution of single action potentials.

Figure 3.27 Combined electrophysiological and fluorometric calcium recordings in AMCs. A, Photomicrograph of widefield fluorescence image from an eAMC loaded with Fluo-4 pentapotassium salt via the attached patch pipette. B, Representative whole-cell continuous recording of spontaneous activity of the neuron depicted in A (black trace). Green trace depicts

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So far, in our experiments monitoring of oscillatory AMC activity was restricted to the single-cell level \textit{in vitro}. To address questions regarding rhythmic heterogeneity and synchronization in the local network we took a population imaging approach. Therefore, we either bulk-loaded acute AOB slices from wildtype animals with Fluo-4 AM (see section 2.2.4), or prepared slices from Tbet::Cre GCaMP6f animals which express the genetically encoded calcium indicator GCaMP6f in mitral cells of the AOB and MOB (see section 2.2.1). Activity was monitored using either widefield or confocal microscopy.

Imaging neuronal activity in GCaMP6f-expressing animals revealed large-scale oscillatory calcium waves that extended throughout the mitral cell layer of the AOB (Figure 3.28 A, B). In widefield experiments these global oscillations were observed in eight of nine slices (four animals). In three slices (one animal) large-scale oscillations gradually seized during the recording (Figure 3.28 C). Moreover, during a prolonged recording in one slice large-scale oscillations initially seized (Figure 3.28 Di, ii), remained absent for some time (>15 min; Figure 3.28 Di, ii), and eventually reappear even more pronounced (Figure 3.28 Div). The frequency of large-scale oscillations varied considerably, ranging from 0.012 to 0.113 Hz (0.052 ± 0.025 Hz; mean ± SD). This variability is similar to our observations on the single-cell level in whole cell patch-clamp recordings where frequencies ranged from 0.024 to 0.132 Hz in iAMCs (0.066 ± 0.031 Hz; mean ± SD), and from 0.031 to 0.176 Hz in eAMCs (0.064 ± 0.040 Hz; mean ± SD) (see section 3.5).
In addition to the global rhythmicity observed throughout the AOB mitral cell layer in widefield experiments with GCaMP6f-expressing animals, calcium transients in AMC somata distinct from the global rhythm were clearly discernible to the naked eye. During offline analysis, however, global activity dominated the average pixel intensity within user-selected regions of interest (ROIs) encompassing these somata. Therefore, we performed a local background correction for each ROI to increase the signal-to-noise ratio (see section 2.2.5). For each cell two ROIs were specified: one encompassing the soma, and a second that encompasses the adjacent neuropil (Figure 3.29 Ai, ii).
average pixel intensity of the surround ROI was subtracted from that of the somatic ROI. Using pairwise analysis of signal cross-covariance at zero lag as a measure of synchrony, our recordings revealed a high degree of correlation between all uncorrected somatic ROI pairs [including large ROI I (red in Figure 3.29 Ai); Figure 3.29 Bi]. Performing the same analysis for surround ROIs demonstrated an even higher degree of correlation (Figure 3.29 Bii) that reflects the synchrony of large-scale calcium waves throughout the AOB mitral cell layer. Local background correction strongly reduced zero-lag covariance in all but one pair (Figure 3.29 C). Distinct peaks in the power spectral densities (PSDs) of background-corrected calcium transients measured from six AMC somata (Figure 3.29 D) underscore this finding: the dominant frequencies for individual neurons clearly differ from each other. Additionally, this is evident in the (background-corrected) traces depicting changes in fluorescence intensity over time in the six individual somatic ROIs (Figure 3.29 E; black traces). Importantly, none of these neurons oscillates in a rhythm that is similar to the simultaneously observed global rhythm (red trace). These data argue against a global synchronization of neuronal activity in the AOB mitral cell layer.
Figure 3.29 Rhythmic activity of individual AMCs is highly variable despite “global” oscillation. A, Widefield fluorescence photomicrograph depicting GCaMP6f signal in the MCL of an acute parasagittal section of the AOB. Figure 3.28 B shows data from the same slice at a later stage during the recording. Big ROI (i) indicated in red, somatic ROIs (1, 3, 5, 7, 9, 11) in black. Area in white rectangle (shown enlarged in Aii) illustrates “surround” ROI (3’), drawn to compute local background correction (see Materials and Methods), encompassing somatic ROI (3). B, Heat map showing pairwise analysis of zero-lag covariance for somatic (center) and peripheral (surround) ROIs. Diagonal elements have a value of 1 by definition. C, Heat map showing pairwise analysis of zero-lag covariance between AMCs and large ROI (red in Aii) after background correction (subtracted). D, Individual power spectra depicting strength of the variations (energy) as a function of frequency for somatic ROIs in Aii. ROI numbers are indicated. E, Traces depicting changes in fluorescence intensity over time in large ROI (red) and individual somatic ROIs (black).
Results

Rhythmic diversity, however, does not imply a complete absence of synchrony between AMCs. In several experiments, we occasionally found synchronization of oscillatory mitral cell pairs (data not shown; bachelor thesis of Kira Gerhold, 2015). Figure 3.30 depicts an experiment where larger ensembles of putative AMCs exhibited synchronized activity. Here, monitoring of large-scale activity in the mitral cell layer of the pAOB (ROI I) and aAOB (ROI II) (Figure 3.30 Ai) revealed a large-scale calcium wave that was more strongly pronounced in the pAOB when the local network was pharmacologically unperturbed (Figure 3.30 B). Inhibition of glutamatergic fast synaptic transmission (100 µM AP5 + 10 µM NBQX) dramatically altered the observed pattern, by decreasing the oscillation frequency. Here, this slower rhythm became more clearly pronounced in the aAOB. Large-scale rhythmic activity in this Fluo-4 AM-loaded slice, however, was less evident compared to slices from GCaMP6f-expressing animals. Analysis of the activity of single neurons (each corrected for its respective local background) exposed two distinct oscillatory microcircuits, each of which was characterized by a unique dominant rhythm. A group of nine neurons, all but one located in the pAOB (white ROIs in Figure 3.30 Aii), exhibited synchronized oscillatory activity that strongly resembled the large-scale rhythm observed in the pAOB under control conditions (microcircuit 1; Figure 3.30 C). Upon pharmacological inhibition of AMPA / Kainate and NMDA receptor-mediated transmission rhythmic activity was abolished in six of nine neurons (ROIs 1, 3, 5, 11, 17, 19) indicating that these belonged to the glut.-sensitive eAMC population. Oscillation frequencies in the remaining three neurons were not obviously altered (ROIs 7, 9, 15), and thus likely belonged to either the iAMC or glut.-insensitive eAMC subpopulation. In the aAOB, on the other hand, a group of 5 neurons (red ROIs in Figure 3.30 Aii) displayed synchronized rhythmic activity on a slower time scale that persisted under incubation with AP5 + NBQX (microcircuit 2; Figure 3.30 C). This rhythm was similar to the large-scale oscillation that emerged in the aAOB upon application of pharmacological agents.
Results

Figure 3.30 Oscillatory activity of individual AMCs can be synchronized to form microcircuits that are distinguished by their individual rhythmic patterns. A, Widefield fluorescence photomicrographs depicting an acute parasagittal slice of the AOB.
Results

Loaded with Fluo-4 AM. Two large ROIs were drawn to monitor large-scale activity in the MCL (Ai). Ai depicts ROIs of oscillating MCs that were assigned to one of two synchronized microcircuits: microcircuit 1 (9 neurons, white), or microcircuit 2 (5 neurons, red). B. Changes in fluorescence intensity in ROI I and II indicate large-scale oscillation that is more dominantly pronounced in ROI I. Blocking fast excitatory synaptic transmission [AP5 (100µM) + NBQX (10 µM)] reveals a slower oscillation that is more strongly pronounced in ROI II. C. Traces depicting activity in AMCs that have been assigned to microcircuit 1 (white ROIs in Ai) due to the high degree of similarity in their oscillation patterns. Blocking fast excitatory synaptic transmission abolishes this oscillation in 6 of 9 cells. D. Activity in AMCs assigned to microcircuit 2 (red ROIs in Ai) due to high degree of similarity in their respective oscillatory activity patterns. Here, application of AP5 (100 µM) + NBQX (10 µM) does not affect oscillations.

Pairwise analysis of cross-covariance at zero lag under control conditions (Figure 3.31 A) shows a high degree of correlation between all surround ROIs that do not contain any somata (central panel in Figure 3.31 A). In this experiment, intensities measured in somatic ROIs that have not been background-corrected (left matrix in Figure 3.31 A) were correlated to a much lesser extent. Here, microcircuits 1 and 2 (described in Figure 3.30) are denoted by black rectangles that highlight an overall higher degree of correlation at zero lag within the individual groups. Background correction (right panel in Figure 3.31 A) especially accentuates the boundaries of microcircuit 1 (denoted by large black rectangle) by decreasing cross-covariance between its member cells and AMCs from microcircuit 2 (small black rectangle). Moreover, background-correction decreased the correlation between individual neurons and large ROIs (I and II). Block of fast glutamatergic transmission (100 µM AP5 + 10 µM NBQX) led to an overall increase in zero-lag covariance between non-corrected measurements from AMC somata. This was concomitant with, and likely resulted from an increase in synchrony of background activity (center and surround matrices in Figure 3.31 B vs A). For background-corrected activity, the same analysis revealed a strong overall decrease in synchrony within microcircuit 1 (Figure 3.31 B), whereas zero-lag covariance between neurons in microcircuit 2 was not as strongly affected by ionotropic glutamate receptor antagonists. These results correspond well to the observations made in Figure 3.30, and thus confirm that cross-covariance at zero-lag is well suited to assess the degree of synchrony in a neuronal population. In this experiment, oscillatory activity in three neurons of microcircuit 1 was not sensitive to AP5 + NBQX (ROIs 7, 9, 15), but was abolished in the remaining six cells (ROIs 1, 3, 5, 11, 17; Figure 3.30 C). Here, pairwise analysis of cross-covariance shows that activity indeed remains positively correlated (green rectangles; right panel in Figure 3.31 B). Figure 3.31 D shows example crosscorrelograms for a pair where glutamatergic block reduced correlation, and a pair where glutamatergic block did not affect this parameter (Figure 3.31 Di and Dii, respectively; see corresponding blue rectangles in rightmost panels of Figure 3.31 A, B). Figure 3.31 C shows the power spectral densities for neurons in microcircuits 1 and 2 grouped with the respective large ROIs (I and II) in which these cells reside under control conditions (black traces) and block of excitatory fast synaptic transmission (red traces; Figure 3.31 Ci and Cii, respectively). A peak at 1.56*10^-3 Hz in all power spectra likely represents the DC component in the fluorescence intensity signals. Before blocker application, the global wave’s dominant frequency in the pAOB is at 0.023 Hz (ROI I; indicated by vertical dashed lines in Figure 3.31 Ci). All member neurons of microcircuit 1 show a pronounced peak at this frequency under control conditions (Figure 3.31 Ci). Additionally, the majority of cells exhibited signal variations at 0.045 Hz (e.g. ROI 5). In neurons where periodicity was not sensitive to glutamatergic block (ROIs 7, 9, 15) the dominant peak remained unchanged at 0.023 Hz. Although large-scale rhythmic activity was evident in the aAOB, especially when glutamatergic fast synaptic transmission was blocked, no peaks were evident in the respective power spectra (ROI II in Figure 3.31 Cii). Neurons in microcircuit 2 that resided within this large ROI all exhibited the strongest variations at 0.01 Hz (ROIs 1, 3, 5, 7, 9; vertical dashed lines in Figure 3.31 Cii). Compliant with the fact that member neurons of microcircuit 1 cluster in the pAOB, whereas member neurons of microcircuit 2 cluster in the aAOB (see Figure 3.30 Ai, ii), a weak negative correlation between physical distance and signal correlation was found under control conditions (p = -0.335; Figure 3.31 Ei). This correlation was lost upon block of AMPA / Kainate and NMDA receptors (p = -0.038; Figure 3.31 Eii).
Results

Figure 3.31 Quantification of experiment depicted in Figure 3.30. A, B. Heat maps showing pairwise analysis of zero-lag covariance under control conditions (A: no pharmacological perturbation of network activity) and during incubation with blockers of fast synaptic excitation [B, AP5 (100 μM) + NBQX (10 μM)] for raw somatic (Ai, Bi; center), peripheral (Aii, Bii; surround), and background-corrected (Aiii, Biii; subtracted) ROIs. Pairs assigned to either microcircuit 1 or 2 are encompassed.
Results

by a smaller and larger rectangle (Ai-ii, Bi-ii), respectively. C, PSDs for each somatic, background-corrected ROI assigned to a given microcircuit (microcircuit 1 in Ci, microcircuit 2 in Cii), and for the corresponding large ROI that encompasses the majority of these somata (ROI I and II in Ci and Cii, respectively). Black traces depict PSDs under control conditions. Red traces show PSDs during incubation with AP5 (100 µM) + NBQX (10 µM). ROI numbers as indicated. Di-ii, Crosscorrelograms depicting covariance for two pairs indicated by blue rectangles in Aiii, Biii for either control conditions (black traces; see Aiii), or under incubation with AP5 + NBQX (red traces; see Biii). E, Pairwise correlation analyses of signal correlation (zero-lag covariance) and physical distance for control (Ei) and blocker conditions (Eii). Linear regression indicates weak negative correlation of both parameters under control conditions (Ei; Pearson’s correlation coefficient, \(\rho = -0.335\)) that is lost upon block of AMPA / Kainate and NMDA receptor-mediated transmission (Eii; Pearson’s correlation coefficient, \(\rho = -0.038\)).

In the experiment presented in Figure 3.29 rhythmicity in AMCs was highly variable and distinct from the observed global rhythm. In contrast, in the following experiment (Figure 3.30) large-scale activity was synced to either one or another microcircuit of individual synchronized neurons. In widefield experiments, individual AMC somata were often not well discernible due to out-of-focus light. Especially in GCaMP6f-expressing animals, identification of somata was further hampered by pronounced global rhythms. Figure 3.32 depicts an experiment in which a relatively large number of neurons were detected in the focal plane. Here, large-scale rhythmic activity that appeared synchronized throughout the GCaMP6f-expressing mitral cell layer of the AOB was evident once again (Figure 3.32 Ai, B). Nevertheless, a total of 21 AMC somata were identified (Figure 3.32 Aii). Of these, 18 exhibited highly variable rhythmic activity (Figure 3.32 C). Most importantly, however, the summed activity of all oscillating cells, as depicted by the average of all traces (green trace; Figure 3.32 C), resulted in a periodic signal that is highly similar to the observed global activity (Figure 3.32 D).
Further analysis again illustrates the impact of local background correction in widefield calcium imaging of neuronal activity in slices of the AOB (Figure 3.33 Ai-iii). Summed activity from apparent neuropil regions [likely including 'out-of-focus' somata (Figure 3.33 Aii)] masks calcium transients on the single-cell level (Figure 3.33 Ai). Subtraction of local background activity presents an easy way to unmask the activity of individual AMCs for further analysis (Figure 3.33 Aiii). Here, neuronal activity in the majority of neurons was not well correlated, as illustrated by the power spectra and crosscorrelograms of two example pairs: ROIs 19, 29 and ROIs 11, 35 (light blue and black traces in Figure 3.33 C). Other pairs, such as ROIs 31 and 39, however, were better correlated and shared the same peaks in their individual power spectra (light red traces in Figure 3.33 C). Pairwise analysis of zero-lag covariance across the oscillating population indicates that synchronization was not as strongly pronounced as a first glance might suggest (Figure 3.33 Aiii vs Ai). Comparison of individual power spectra further supports this notion. Figure 3.33 B shows the power spectra of large ROIs (ROIs I-VI; red) grouped with PSDs from single oscillating (black) and non-oscillating (grey) AMCs that reside within the respective large ROI. While all PSDs from large ROIs have a clear peak at 0.022 Hz (denoted
Results

by dashed vertical lines in all PSDs), PSDs of single neurons within a given ROI show considerable variation not only from large-scale PSDs, but also from each other, exhibiting the strongest signal variations at distinct frequencies. Here, no correlation between a given AMC pair’s cross-covariance and its physical distance was evident (Figure 3.33 D). Oscillation frequencies of rhythmic AMCs, as calculated by IPI analysis (see section 2.2.5), ranged from 0.033 to 0.112 Hz (0.074 ± 0.023 Hz; mean ± SD; Figure 3.33 E). The mean frequency of large-scale calcium waves was calculated by IPI analysis of the averaged signal in all big ROIs and was within the range in which single-cell oscillatory activity occurred (0.056 Hz; green dot in Figure 3.33 E).
Figure 3.33 Quantification of experiment depicted in Figure 3.32. A. Heat maps showing pairwise analysis of zero-lag covariance for somatic (Ai, center), peripheral (Aii, surround), and background-corrected ROIs (Aiii, subtracted). Large ROIs (I-VI, see Figure 3.32 Aii) are included in Ai and Aiii. Aiii, Green rectangles denote which oscillatory neuron lies within which large ROI. ROI numbers of non-oscillating neurons are denoted in gray (Ai, Aiii). B. PSDs for all large and somatic, background-corrected ROIs. Diagrams are grouped by large ROIs (Bi-Vi; I-VI, red traces). Somatic ROIs (black traces) are assigned to large ROI they lie encompassed in. PSDs from non-oscillating ROIs (15, 25, and 41) depicted in grey. Dashed vertical lines indicate peak location of respective big ROI’s PSD. C. Overlaid PSDs (top) and crosscorrelograms (bottom) for three pairs indicated by blue rectangles in Aiii as examples of for pairs with slight negative correlation (light blue), no correlation (black), or positive correlation (salmon) at zero lag. ROI numbers indicated within PSDs and above crosscorrelograms. Dashed vertical lines in PSDs denote maxima for each ROI. D. Pairwise correlation analyses of signal correlation (zero-lag covariance) and physical distance. Linear regression indicates no correlation of both parameters (Pearson’s correlation coefficient, $p = 0.044$). E, Scatter
In order to better resolve single-cell calcium transients in GCaMP6f-expressing AOB mitral cells we turned towards confocal microscopy for further imaging experiments. Here, a total of eight slices from four animals were imaged. Large-scale oscillatory calcium waves were less prevalent and were observed in three slices from two animals. In one of these slices no rhythmic cells lay in the focal plane, whereas in the second slice eight of eleven neurons in the focal plane were rhythmically active. Finally, in the third slice that displayed large-scale oscillations, activity was periodic in 23 of 42 neurons (Figure 3.34). Here, large-scale calcium waves spanned throughout the mitral cell layer (Figure 3.34 Ai, Bi). Similar to our findings in widefield experiments, rhythmicity was highly variable across the oscillating population (Figure 3.34 C). Again, the summed signal from all oscillating AMCs produced a periodic waveform (green trace in Figure 3.34 C) that is similar to the averaged large-scale oscillation (Figure 3.34 D) in its time course.

Figure 3.34 Confocal calcium imaging experiment in which polyrhythmic activity in individual AMCs interferes to produce large-scale oscillation. A. Maximum projection of a confocal z-stack depicting GCaMP6 signal in AMCs of an acute parasagittal section of the AOB. Large ROIs (Ai; red, I-VI) extend across MCL in the anterior-posterior direction. Oscillating (white, numbered; n = 23) and non-oscillating (grey, not numbered; n = 19) ROIs indicated in Ai. B. Large-scale oscillation is evident in the changes of GCaMP6f fluorescence intensity in all three large ROIs (I-III). C. Traces (black) from individual oscillatory AMCs (white in Ai) illustrating rhythmic variability between neurons. Averaged signal from all oscillating cells (bottom, right;
green) gives rise to oscillatory pattern that is distinct in its rhythm when compared to single cell oscillatory activity. D, Overlay of summed signal in C (green) with average global oscillation [mean (ROI I-VI), red].

Pairwise correlation analysis, as measured by the cross-covariance at zero lag, shows a lack of pronounced synchronization in the neuronal population within the focal plane (Figure 3.35 Ai); see example pairs in Figure 3.35 C). At the same time, background activity was more strongly correlated (Figure 3.35 Aii). Even in confocal microscopy this decreased the signal-to-noise ratio in calcium transients on the single-cell level (Figure 3.35 Ai), although to a lesser extent compared to widefield experiments. Large-scale calcium transients in all three large ROIs (Figure 3.34 Ai) exhibited the strongest variations at 0.016 Hz (red traces in Figure 3.35 Bi, ii, iii). Three of four oscillating AMCs that lay within ROI I had dominant frequencies <0.016 Hz at 0.009 (ROI 9), 0.013 (ROI 11), and 0.014 Hz (ROI 5) while only ROI 3 had its peak at 0.016 Hz (Figure 3.35 Bi). Within ROI II, ROIs 15, 19, 21, 33; 41 and 43 all shared their dominant peak at the background frequency of 0.016 Hz, while two neurons (ROIs 45 and 47) had distinct peaks at 0.020 and 0.008 Hz, respectively (Figure 3.35 Bii). The higher similarity in dominant frequencies in AMCs residing within ROI II was accompanied by higher cross-covariances at zero lag compared to neurons within ROI I (Figure 3.35 Aiii). Rhythmic variability within ROI III was increased with neurons experiencing the strongest variations at frequencies ranging from 0.005 to 0.022 Hz (Figure 3.35 Biii). Again, no pronounced correlation between signal cross-covariance at zero lag and physical distance of AMC pairs was observed (Figure 3.35 D). Rhythmicity within the slice, as estimated by IPI analysis, was highly variable (see also Figure 3.34 C) and ranged from 0.011 to 0.057 Hz (0.041 ± 0.011 Hz; mean ± SD; Figure 3.35 E). Similar to the widefield experiment where individual oscillations on the single-cell level summed to produce a wave similar to the large-scale wave (Figure 3.32 D, Figure 3.33 E), the average frequency of large-scale oscillatory activity at 0.042 Hz in this experiment (green dot in Figure 3.35 E) was nearly identical to the oscillating population’s mean.
Figure 3.35 Quantification of experiment depicted in Figure 3.34. A, Heat maps showing pairwise analysis of zero-lag covariance for somatic (Ai, center), peripheral (Aii, surround), and background-corrected ROIs (Aiii, subtracted). Large ROIs (I-III, see Figure 3.34 Al) are included in Ai and Aii. Aiii, Green rectangles denote which oscillatory neuron lies within which large
ROI. ROI numbers of non-oscillating neurons are denoted in gray (Ai, Aiii). PSDs for all large and oscillating somatic, background-corrected ROIs. Diagrams are grouped by large ROIs (Bi-iii, I-III, red traces). Somatic ROIs (black traces) are assigned to large ROI they lie encompassed in. PSDs from non-oscillating ROIs not shown. Dashed vertical lines indicate peak location of respective big ROI’s PSD. C, Overlaid PSDs (top) and crosscorrelograms (bottom) for three pairs indicated by blue rectangles in Aiii as examples of oscillating pairs with slight negative correlation (light blue), no correlation (black), or positive correlation (red) at zero lag. ROI numbers indicated within PSDs and above crosscorrelograms. Dashed vertical lines in PSDs denote maxima for each ROI. D, Pairwise correlation analyses of signal correlation (zero-lag covariance) and physical distance. Linear regression indicates no correlation of both parameters (Pearson’s correlation coefficient, $p = 0.27$). E, Scatter dot plot depicting the distribution of oscillation frequencies in single neurons (black dots) recorded in this experiment. Average value is shown as mean ± SD (red; 0.041 ± 0.011 Hz). Frequency of averaged large ROIs (see Figure 3.34 D, red trace) is 0.042 Hz (green dot).

In total, we observed global oscillations in the AOB mitral cell layer of GCaMP6f-expressing animals in 11 of 17 slices (8 animals). Interference of single-AMC periodic neuronal activity resulting in formation of a wave that oscillates at the same frequency as large-scale background activity was observed in three slices from three animals (one widefield, two confocal experiments). In these experiments, 18, 10, and 23 rhythmic AMCs, respectively, lay in the focal plane. In cases where large-scale calcium waves were evident but summed activity of periodic single-neuron calcium transients did not produce a periodic signal (9 experiments; 7 widefield, 2 confocal), the number of oscillating AMCs per experiment ranged from three to eight. These data indicate that oscillations in AMCs, while variable in their respective rhythm, may interfere to produce the observed large-scale waves that may extend throughout the AOB mitral cell layer. The fact that summed single-cell rhythmic activity in some slices where global waves were observed was not periodic may simply be attributed to the reduced number of oscillating neurons in the focal plane of these experiments. These results are summarized in Table 3.4.

<table>
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<th>Global oscillation</th>
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Table 3.4 Large-scale calcium waves and numbers of rhythmic AMCs per experiment. Global oscillations may arise from constructive interference of single cell oscillatory activity as indicated by experiments with large numbers of oscillatory AMCs within the focal plane.
Discussion

The detection of chemical cues in the environment is critical to the survival of every organism. Not surprisingly, animals have evolved highly sophisticated olfactory systems to master the task of broad chemical recognition. The AOS plays a major role in the detection of semiochemicals that convey information about the emitter’s identity, gender, social rank, and sexual state, and thus regulation of social behavior. This sensory system was initially believed to have evolved in tetrapods during the transition from water to a terrestrial environment (M Halpern, 1987). Today, accumulated evidence rather contests this assumption. Modern amphibians and amniotes, as well as two families of fully aquatic, nonmetamorphosing salamanders, also possess vomeronasal organs, implying that the emergence of the AOS preceded that of terrestrial life (Gliem et al., 2013; H. L. Eisthen, 1997; Sansone et al., 2014; Syed et al., 2016). Although an extensive body of scientific literature has been dedicated to the study of the AOS, many basic physiological principles underlying sensory processing within this system remain poorly understood. Here, the AOB is the first stage of information processing and its principal neurons, AMCs, represent a direct neural link between vomeronasal sensory input and limbic output. Early investigators had thought of the AOB as a cortical variation of the MOB and therefore not the superior olfactory center (Cajal 1901). Ever since, the AOB has “suffered” from this premise and functional analogies to the MOB remaining mostly speculative (C Dulac & Wagner, 2006). Striking morphological differences between the two processing centers, however, indicate that sensory computation in the AOB is fundamentally different compared to the MOB (Catherine Dulac & Torello, 2003). Today, important insights into the organizational principles of connectivity, sensory input, and integration in the AOB have been gained. Nonetheless, central aspects of AOB physiology remain largely unexplored.

Recently, we have shown that a group of AOB mitral cells is intrinsically rhythmogenic and exhibits spontaneous infraslow stereotypical rhythmic discharge (Gorin, Tsitoura et al., 2016). A phenotypically identical phenomenon was simultaneously reported in AMCs of the adult rat (Vargas-Barroso et al., 2015). The conservation of AOB infraslow oscillations across species suggests that they are functionally relevant. To what extent intrinsic rhythmogenesis in AMCs shapes information processing and sensory coding in the AOB, however, remains unexplored. Therefore, the primary aim of my dissertational work was to gain deeper understanding of the impact of iAMC oscillatory discharge on the intact AOB network.

4.1 Spontaneous AOB activity in vivo and in vitro

In vivo vomeronasal stimulation of the VNO, combined with multisite recordings of neuronal activity in the AOB, has revealed that the AOB can encode both conspecific and allospecific cues, as well as strain and reproductive-state (Ben-Shaul et al., 2010; Kahan & Ben-Shaul, 2016; Tolokh et al., 2013). Brain circuit computations, however, are not exclusively determined by sensory input. Intrinsically generated spatiotemporal structured patterns of spontaneous activity additionally shape these computations (Romano et al., 2015). As information about spontaneous AOB activity is lacking, we performed in vivo recordings from the AOB mitral cell layer of anesthetized mice in a collaborative effort with Yoram Ben-Shaul and Anat Kahan (Department of Medical Neurobiology, The Hebrew University of Jerusalem), omitting vomeronasal stimulation. We found spontaneous activity in putative AMCs to be highly heterogeneous, ranging from apparently random to more regular firing. A substantial fraction of neurons (17%) was characterized by highly rhythmic spontaneous discharge. The temporal features of periodic bursting were heterogeneous across the population indicating the absence of a single characteristic rhythm governing AOB oscillations. In these neurons, spiking activity was markedly increased compared with previously observed low baseline rates of irregularly firing.
neurons (Ben-Shaul et al., 2010; Luo et al., 2003). Further analyses assessing “burstiness” and regularity indicate that oscillating AOB neurons form a distinct group and do not represent the extreme of a continuous, normally distributed neuronal population in vivo.

Similar to our findings in vivo, AMCs display two characteristic firing patterns in vitro: neurons fire action potentials either irregularly with no apparent periodicity, or rhythmically with alternating periods of activity and silence. Moreover, \( V_{\text{mem}} \) in rhythmic AMCs is bistable and oscillates between recurring up and down states. When mitral cell firing was recorded in the “loose-seal” cell-attached configuration, essentially identical patterns of spontaneous activity were observed. Interestingly, periodic AMC bursting already occurred in mice as young as postnatal day 8. Whether the AOS is fully functional in neonates, however, is still subject to debate (Hovis, Ramnath, Dahlen, Romanova, Bier, et al., 2012).

The critical period for mitral cell dendritic refinement and formation of well-defined glomeruli, for instance, occurs after VSN axonal coalescence during the first four to six postnatal days (Hovis, Ramnath, Dahlen, Romanova, LaRocca, et al., 2012; Salazar & Brennan, 2001). Further assessment of infraslow rhythmicity in the juvenile AOB might thus provide useful insights into the development of the AOS.

Sensory input to the AOB is topographically organized: while apical, V1R-expressing VSNs project to the aAOB, basal V2R-expressing sensory neurons project to the pAOB (Del Punta et al., 2002; L Belluscio et al., 1999). Although AMCs possess several apical dendrites and innervate several glomeruli, they are implicated to only receive sensory input from glomeruli in the homonymous AOB half (Jia & Halpern, 1997; Larriva-Sahd, 2008; Mori et al., 2000). Furthermore, the anterior and posterior AOB are reported to contribute differently to AOS-mediated social and sexual behaviors (Chamero et al., 2007, 2011; Dudley & Moss, 1999; Oboti et al., 2014; Pérez-Gómez et al., 2014; Tirindelli et al., 2009). Recently, a population of cholinergic interneurons that exhibits a highly dichotomized distribution along the anterior-posterior axis in the AOB has been identified, suggesting that AMCs in the two halves might be subject to differential cholinergic modulation (Marking et al., 2017). Additionally, while projections from anterior and posterior AMCs are largely convergent, there are specific areas, both within the amygdala and hypothalamus that receive segregated input from either the anterior or posterior division of the AOB (Martínez-Marcos & Halpern, 1999; Mohedano-Moriano et al., 2007, 2008). We recorded spontaneous activity from AMCs with somata residing in both the pAOB and aAOB, and found no apparent differences between the two regions with respect to their spontaneous activity. This suggests that, functionally, AMC rhythmicity might not relate to receptor identity, serving a more fundamental role in information routing within the AOS instead. Alternatively, effects of infraslow oscillatory activity on sensory processing in limbic circuits are to be found in areas where AOB inputs converge.

Overall, the prevalence of slow oscillatory discharge was larger in vitro than observed in vivo. While only about a fifth of AOB neurons exhibited periodic activity in vivo, more than half of the AMC population oscillated in vitro. Several factors could account for this apparent discrepancy. Experimental in vitro conditions might favor oscillatory discharge. While our extracellular loose-patch recordings exclude dialysis of intracellular components to promote oscillatory behavior, extracellular ionic concentrations might not exactly mirror endogenous ionic concentrations. The extracellular \( \text{Ca}^{2+} \) concentration, for example, exerts profound effects on intrinsic rhythmogenesis (Gorin, Tsitoura et al., 2016). Additionally, AOB slices are isolated from both peripheral sensory input and top-down modulation. In vivo, either factor could substantially alter a given AMC’s output. Either way, infraslow rhythmicity in AOB projection neurons will likely have a physiological impact on downstream processing.
4.2 A distinct electrophysiological phenotype confers autorhythmicity on iAMCs

Intrinsic ionic conductances bestow iAMCs with $V_{\text{mem}}$ bistability that manifests in periodically recurring up and down states. Previously, we have shown that cyclic activation of three interdependent ionic conductances drives this phenomenon. Low threshold $I_{\text{NaP}}$ functions as the major excitatory element that drives iAMC transition from $V_d$ to $V_u$. R-type $\text{Ca}_V$ channels play a significant role in oscillation maintenance and shape, while the resulting increase in cytoplasmic $\text{Ca}^{2+}$ is coupled to progressive activation of BK channels which, in concert with slow voltage-dependent $I_{\text{NaP}}$ inactivation (Jasinski et al., 2013), causes burst termination (section 1.4; Gorin, Tsitoura et al., 2016).

Here, we tested whether or not $I_{\text{NaP}}$, $I_R$, and $I_{\text{BK}}$ are unique properties of intrinsically rhythmogenic AMCs. We therefore compared electrophysiological profiles of these currents in iAMCs to the remaining AMC population. All three currents were found across the entire AMC population. However, in contrast to $I_R$, both $I_{\text{NaP}}$ and $I_{\text{BK}}$ were differentially expressed between the two groups. The activation threshold for $I_{\text{NaP}}$ was significantly lower in iAMCs than in non-rhythmogenic neurons, and amplitudes were significantly increased. Recent studies report that $I_{\text{NaP}}$ can activate at more hyperpolarized voltages than previously appreciated (B. C. Carter et al., 2012; Huang & Trussell, 2008). Our discovery that $I_{\text{NaP}}$ in iAMCs $\sim -80$ mV, compared to $\sim -75$ mV in non-rhythmogenic AMCs, supports these findings. The pacemaker-current can thus be engaged by small shifts in net current at the downstate $V_{\text{mem}}$ range (Yamada-Hanff & Bean, 2013). Additionally, we observed a pronounced left-shift in the $V_{\text{mem}}$ dependence of $I_{\text{BK}}$ activation in iAMCs compared to non-rhythmogenic neurons ($\Delta V_{1/2} = 20.9$ mV). BK channels exhibit a large unitary conductance, are cooperatively gated by membrane depolarization and intracellular $\text{Ca}^{2+}$ (Sugawara & Nikaido, 2014), and were found to promote intrinsic bursting in TCs of the MOB (Liu & Shipley, 2008). The distinct combination of R-type $\text{Ca}_V$ and BK channels in rhythmogenic AMCs supports relatively prolonged bursting: R-type channels are the only high-voltage-activated $\text{Ca}^{2+}$ channels that do not associate with BK channels in $\text{Ca}^{2+}$ nanodomains (Fakler & Adelman, 2008). Thus, a slower, more global increase in cytoplasmic $\text{Ca}^{2+}$ is required to activate substantial $I_{\text{BK}}$. Together with the pronounced left shift in the $V_{\text{mem}}$ dependence of $I_{\text{BK}}$ activation that we observed here, the specific $I_R$–$I_{\text{BK}}$ combination appears ideally suited to shape autorhythmicity in these neurons. Even though identical firing patterns can be achieved by multiple current combinations (Eve Marder & Goaillard, 2006), we confirmed that cyclic activation of the aforementioned conductances is sufficient to drive autorhythmicity in model-based $V_{\text{mem}}$ simulations that we carried out in collaboration with Simon O’Connor (Biocomputation Group, University of Hertfordshire).

Our results thus illustrate the profound effects that biophysical diversity can exert on a seemingly homogeneous neuronal population. In summary, although not exclusively expressed in the intrinsically oscillating mitral cell population, $I_{\text{NaP}}$, $I_R$, and $I_{\text{BK}}$ confer a distinct electrophysiological phenotype on iAMCs.

4.3 Oscillatory AMC subpopulations in the AOB

Similar to intrinsic oscillators described in other circuits (Blethyn et al., 2006; Hayar et al., 2004), rhythmogenic AMCs display a positive causal correlation between oscillation frequency and “baseline”
Discussion

V_{\text{mem}} (Crunelli & Hughes, 2010; Gorin et al., 2016): oscillation frequency changes as a function of depolarizing or hyperpolarizing current injection. Whereas hyperpolarization increases IBIs, depolarization reduces IBIs. Additionally, iAMCs exhibit a characteristic V_{\text{mem}} threshold below which V_{\text{mem}} bistability is abolished and oscillations persist in isolation from fast synaptic drive.

Here, we identified a second group of oscillating AMCs in which oscillation frequencies are not affected by negative current injection and subthreshold oscillations persist under hyperpolarized conditions, indicating the observed phenomenon to be brought about by a network-dependent mechanism. We show that, in the majority of oscillating AMCs (~61%), periodic activity emerges from changes in network activity. Furthermore, in experiments where we combined pharmacology with negative DC current injections, we have shown that the newly identified population of eAMCs comprises two distinct subpopulations: glut.-sensitive and glut.-insensitive eAMCs, constituting 44% and 56% of the entrained population, respectively. In glut.-sensitive eAMCs, rhythmic bursting is sensitive to block of AMPA/Kainate and NMDA receptor-mediated transmission, whereas periodicity in glut.-insensitive eAMCs is not affected. Extrapolating, we conclude that 22% of AMCs are intrinsically rhythmogenic. In 15% of AMCs, fast excitatory synaptic transmission is necessary and sufficient to entrain rhythmic activity, whereas 19% of AMCs are entrained by unknown network dynamics.

In most neurons, recurring up and down states emerge from changes in network activity that produces synaptic barrages of excitation and inhibition (Crunelli & Hughes, 2010; Krahe & Gabbiani, 2004). Network dynamics, in turn, depend on the complex interaction between intrinsic membrane properties of individual neurons and the synaptic strengths and time courses between them (E Marder et al., 1996). Moreover, neuronal activity itself modifies not only synaptic efficacy but also the intrinsic membrane properties of neurons. With regard to intrinsic properties, both passive and active membrane properties affect information processing and temporal filtering in single neurons (Angelo & Margrie, 2011; Fortune & Rose, 1997; Magee & Cook, 2000; Yamashita et al., 2013). Therefore, we first sought to assess for variations in basic intrinsic properties of all AMC subpopulations and compared membrane capacitance, membrane time constant, and input resistance in whole-cell patch-clamp recordings. Comparing irregular AMCs to the oscillating population as a whole revealed that irregular neurons are leakier than their rhythmic counterparts. A depolarizing current of fixed size will therefore likely result in a stronger depolarization, thus making oscillating AMCs more excitable compared to their arrhythmic counterparts. Next, differentiating between autorhythmic and entrained AMCs, and between glut.-sensitive and glut.-insensitive eAMCs, we found no significant differences in either parameter. We did however observe a tendency toward increased t_{\text{mem}} and R_{\text{input}} in glut.-insensitive eAMCs. Here, the relatively small sample size, however, does not allow for definite conclusions, and future experiments will need to further address potential differences between both iAMCs and eAMCs as well as between glut.-sensitive and glut.-insensitive eAMCs.

Addressing AMC active neuronal properties next, we compared mean instantaneous spike frequencies as a function of stationary current input. While f-I curves for irregular and entrained AMCs were essentially indistinguishable with both populations accommodating spike rates of up to 21 Hz, iAMCs displayed evoked action potentials at significantly higher frequencies across the entire stimulation range. Here, maximal firing rates were ~35 Hz. These findings contradict previous findings in our lab, where no differences in the response to depolarizing current injections were found between iAMCs and irregular AMCs (Gorin, Tsitoura et al., 2016). This discrepancy likely stems from differences in data analysis: while we initially plotted spike frequencies against the absolute injected current, we later reasoned that it might be more reasonable to take the respective holding current in each neuron into account. Thus, we now observed how instantaneous spike frequency changes in response to a given current injection relative to a given holding current. Careful inspection of the recorded f-I curves
reveals that spike rate, especially in iAMCs, is not yet saturated, in agreement with findings by Zibman et al. where maximal firing rates in AMCs were evoked at current injections of ≥150 pA (Zibman et al., 2011). Neuronal firing patterns are determined by the repertoire, density, and distribution of voltage-activated ion channels (Mircea Steriade, 2004; Schulz et al., 2006; Stemmler & Koch, 1999; Yamashita et al., 2013), in addition to leak conductances that determine $R_{\text{input}}$. iAMCs exhibit both increased input resistance and higher spike frequencies to a given depolarization compared to non-rhythmogenic AMCs. Together with the aforementioned particular features of $I_{\text{NaP}}$ (higher amplitudes, activation at more hyperpolarized $V_{\text{mem}}$), iAMCs are ideally equipped for robust burst generation.

Similar to our in vivo findings, parameters describing the temporal patterns of activity and silence varied considerably across the intrinsically rhythmogenic population (Gorin, Tsitoura et al., 2016). We thus sought to compare parameters such as IBIs, burst durations, and the resulting oscillation frequencies, as well as within-burst firing rates, and up- and downstate $V_{\text{mem}}$ across all oscillating populations. No differences were found, either between iAMCs and eAMCs, or between glut.-sensitive and glut.-insensitive eAMCs with regard to parameters describing the temporal pattern of activity. Moreover, rhythmic variability is similarly diverse in all oscillating AMC subpopulations. While iAMCs and eAMCs did not differ in their downstate membrane potential, $V_u$ was significantly more depolarized in iAMCs ($\Delta V_u \sim 4$ mV) and $V_d$ was significantly more depolarized in glut.-sensitive compared to glut.-insensitive eAMCs ($\Delta V_d > 7$ mV). Within-burst firing rates were relatively low, yet highly diverse across all groups. Although the observed rhythms clustered at the lower end of the bandwidth scale of neuronal oscillators (Tonetti et al., 1976), the wide spectrum within this infraslow range argues against a distinct prevalent AOB rhythm. Due to the similarities in distribution of burst durations, IBIs, and $f_{\text{burst}}$, however, we hypothesized that intrinsically generated oscillations in iAMCs might entrain rhythmic activity in eAMCs via a hitherto undescribed excitatory connection between the two classes of AOB projection neurons.

In summary, both passive and active properties mediate robust burst generation in intrinsically rhythmogenic neurons. Burst firing, in turn, may increase the reliability of communication with postsynaptic partners (Lisman, 1997; Pena & Konishi, 2002). The observed relatively low spike rates within bursts might seem to contradict this hypothesis because it is often assumed that a shorter interspike interval within the burst will result in larger combined postsynaptic potential (PSP). More recent studies, however, propose that bursting may additionally provide effective mechanisms for selective communication between neurons (Izhikevich et al., 2003). According to this complementary hypothesis, bursts with specific resonant interspike frequencies are more likely to cause a postsynaptic cell to fire than are bursts with higher or lower frequencies.

Consequently, we asked whether iAMCs and eAMCs differ in synaptic connectivity and analyzed spontaneous synaptic input to both populations. In the majority of eAMCs, baseline oscillations became evident during the recording, whereas the majority of iAMCs lacked any obvious baseline oscillations. We found that synaptic drive in eAMCs is markedly increased compared to iAMCs. Other parameters, such as amplitude, charge transfer, or rise and decay kinetics of average synaptic events, did not differ between both groups. Importantly, besides being markedly increased, synaptic input to entrained AMCs is periodic. While amplitudes of average PSCs are slightly reduced in IBIs compared to bursts, $f_{\text{PSC}}$ is lowered by ~50%. In contrast, the relatively sparse synaptic activity in intrinsically rhythmogenic neurons is uncorrelated. Hence, rhythmogenic and non-rhythmogenic neurons do not merely differ in some intrinsic biophysical properties, but are, in addition, differentially wired within the local AOB network. Moreover, our findings underpin the assumption that spontaneous correlated neuronal activity drives periodic bursting in eAMCs.
4.4 Oscillatory entrainment of AMCs

To identify the nature of periodic synaptic input to eAMCs, we undertook a pharmacological experimental approach. First, we sought to study whether inhibition plays a role in infraslow oscillatory network activity. Similar to the MOB, granule cells in the AOB mediate lateral and recurrent inhibition of mitral cells. Recurrent inhibition at dendrodendritic synapses in the MOB has been found to tune γ-oscillations (Halabisky & Strowbridge, 2003; Lagier et al., 2007; Neville & Haberly, 2003; Schoppa, 2006) and mediate mitral cell synchrony (Schoppa, 2006). Additionally, inhibition plays a key role in rhythmic neural activity in other parts of the brain (Bartos et al., 2007; Buzsáki & Chrobak, 1995; Traub et al., 1996; Whittington et al., 2000). We found that AMCs, across populations, receive extensive inhibitory drive. Spontaneous IPSCs are sensitive to gabazine and exhibit faster kinetics compared to PSCs that are insensitive to GABAergic block. Most notably, inhibitory synaptic drive in AMCs is non-rhythmic—at least not on an infraslow timescale. Consequently, inhibitory fast synaptic transmission does not seem to play a role in oscillatory entrainment in the AOB. In Gorin et al. (2016), however, we have shown that incubation with gabazine can alter some oscillation parameters, indicating that inhibition might serve to modulate rhythmicity at the temporal level.

Next, we analyzed spontaneous excitatory postsynaptic activity in both glut.-sensitive and glut.-insensitive eAMCs. We found that EPSC frequencies in both populations are markedly increased during bursts and were almost absent during IBIs. Across the entrained population, block of NMDA receptors reduced average within-burst \( f_{\text{EPSC}} \) by \(~20\%\), while inhibition of AMPA/Kainate receptors resulted in a stronger decrease by \(~50\%). Blocking both AMPA/Kainate and NMDA receptor-mediated transmission simultaneously did not result in an additive effect on \( f_{\text{EPSC}} \) reduction. This is not surprising, however, since NMDA receptors act as molecular coincidence detectors: NMDA receptor-mediated currents depend on membrane depolarization to relieve powerful voltage-dependent NMDAR channel block by external magnesium (Clarke et al., 2013). Blocking AMPA/Kainate receptors might already decrease postsynaptic depolarization far enough to prevent relieve of magnesium block in NMDA receptors. In addition to the decreased \( f_{\text{EPSC}} \) in IBIs, averaged EPSCs during bursts tend to transfer an increased charge compared to EPSCs in IBIs. Entrained oscillatory activity in eAMCs is thus driven by periodic surges of excitatory synaptic transmission that is mediated, at least in part, by AMPA/Kainate and NMDA receptors. The increase in excitatory input to eAMCs manifests mainly as an increase in spontaneous EPSC frequency, and to a lesser degree as an increase in charge carried by individual events. In glut.-sensitive eAMCs, block of fast glutamatergic transmission reduced frequency of spontaneous EPSCs more strongly compared to glut.-insensitive eAMCs, indicating that excitatory fast synaptic drive is more pronounced in glut.-sensitive eAMCs. In glut.-insensitive eAMCs, rhythmicity in synaptic drive was maintained upon isolation from fast synaptic transmission indicating a different mechanistic basis for oscillatory entrainment in this subpopulation. Future experiments should therefore address the identity of transmitters and postsynaptic receptors involved.

In 13% of eAMCs, EPSC amplitude distribution was multimodal. Inverse correlation between EPSC amplitudes and kinetics indicates that EPSC populations with smaller amplitudes might stem from synapses that are located more distally, whereas EPSCs with larger amplitudes might stem from more proximally located synapses because dendritic filtering slows the kinetics of postsynaptic currents from distant synapses measured at the soma (Rall, 1967). Similar to MOB mitral cells (Bischofberger & Jonas, 1997; Margrie et al., 2001), AMC dendrites support unattenuated backpropagation of APs into the glomerulus (J. Ma & Lowe, 2004). Future experiments combining electrophysiological experiments with morphological reconstructions could assess for a potential correlation between number of modes in the amplitude distribution of EPSCs and number of glomerular tufts. Such a positive correlation could suggest AMC entrainment to occur in the glomerular layer of the AOB.
Thus far, our experiments show that periodic entrainment of AMCs is mediated by excitatory synapses in the AOB. We hypothesize that iAMCs might entrain rhythmic activity in eAMCs via a hitherto undescribed excitatory connection between the two classes of projection neurons. In the MOB, excitatory coupling between sister mitral cells that project to the same glomerulus has been shown to be mediated both via chemical synapses and gap junctions (Pimentel & Margrie, 2008; Schoppa & Westbrook, 2001; Urban & Sakmann, 2002). The only way to unequivocally demonstrate mono- or polysynaptic excitatory connectivity between AMCs is to simultaneously record neuronal activity from two or more candidate neurons. Therefore, we have established an experimental framework utilizing paired whole-cell patch clamp recordings as the only method that guarantees triggering of a presynaptic AP (Debanne et al., 2008), and have begun performing experiments to assess for chemical and electrical coupling of AMCs. So far, we have not demonstrated chemical synaptic transmission between any pair tested. Electrical coupling was evident in one pair of non-oscillating AMCs. Moreover, from all pairs recorded, only one pair oscillated in a common rhythm. Our findings are not surprising, however, since the present sample size is still small and, depending on the connectivity within a given network, large sample sizes are likely required to demonstrate connectivity or the lack thereof.

4.5 Rhythmic heterogeneity, synchronization, and interference

On the population level, we found rhythmic activity in AOB mitral cells to be highly heterogeneous. In neurons that either loaded with fluo-4/AM or expressed the genetically encoded indicator GCaMP6f, calcium transients show that rhythmic variability, previously only demonstrated on the single-cell level in patch clamp recordings, can exist in parallel at any given moment. AMCs usually exhibit distinct PSDs and little covariance. A few pairs or small ensembles, however, are usually found to oscillate synchronously. In these experiments, overall, no correlation was evident between the degree of synchronization and the physical distance between two synchronous neurons. In one experiment, however, larger ensembles exhibited synchronized activity and formed two distinct microcircuits, each of which was characterized by a unique dominant rhythm. Here, neurons that shared a distinct rhythm tended to cluster closer together. By temporally linking AOB neurons into functional assemblies, synchronous firing could facilitate synaptic plasticity and input selection (Buzsáki & Draguhn, 2004). Despite the pronounced rhythmic heterogeneity observed in most cases, imaging neuronal activity in GCaMP6f-expressing animals revealed concurrent large-scale oscillatory calcium waves that extended throughout the AOB mitral cell layer in ~65 % of slices. In ~30% of such cases, these seized during the recording and, in one case, reappeared even more pronounced after an extended period (>15 min). Similar large-scale oscillatory calcium waves have been observed in the immature cortex (Garaschuk et al., 2000). Here, these calcium waves were proposed to regulate long-distance wiring in the immature cortex and were associated with field-potential changes. Moreover, similar to our findings in the AOB, they required the activation of AMPA and NMDA receptors, while GABA<sub>A</sub> receptors were not involved in wave initiation. The developmental transition of GABAergic transmission from depolarizing to hyperpolarizing around postnatal day 7, however, stopped this oscillatory activity. Large-scale calcium waves throughout the AOB mitral cell layer occur independent of the dominant frequencies displayed by individual neurons. This finding argues against a global synchronization of neuronal oscillatory activity, a potential indicator of seizure activity (Huguenard & McCormick, 2007; McCormick & Contreras, 2001; Mircea Steriade, 2005; Van Drongelen et al., 2003). Strikingly, in slices that contain many oscillatory AMCs, the summed activity from all rhythmic neurons results in an ‘arithmetic’ wave that oscillates at a frequency similar to the observed large-scale background activity. Oscillations in AMCs, while variable in their respective rhythm, may thus interfere to produce the observed global waves that extend throughout the AOB mitral cell layer. If so, these waves would rather represent an epiphenomenon than a physiologically relevant process. Further imaging experiments will need to address the question whether and, if so, how single-cell oscillatory waves interact.
activity changes when global waves emerge or seize. Simultaneous field potential recordings from the AOB mitral cell layer using multielectrode arrays might give useful insights into how heterogeneous rhythmicity on the single-neuron level generates large-scale oscillations. Unfortunately, progress in understanding the AOS has long been impeded by its relative inaccessibility to standard physiological approaches (Meeks & Holy, 2009). Using a chronically implanted glass microprism in vivo could provide optical access with cellular or even subcellular resolution (Andermann et al., 2013; Low et al., 2014) of GCaMP6f-expressing AOB neurons. This approach could, thus, provide a better understanding of how spontaneous rhythmicity in the AOB emerges and changes over time. Moreover, simultaneous stimulus delivery to the VNO could give insight on how sensory input affects rhythmicity and, therefore, how information is processed and coded by the AOB.

So far, we can only speculate on the functional role that infraslow AOB oscillations might serve. Rhythmic cycles between high and low postsynaptic excitability states add a temporal dimension to a given circuit’s sensory coding space (Schroeder & Lakatos, 2009). This might seem to be of particular significance in a sensory system that detects a plethora of multidimensional chemosignals, bypasses cortical circuits, and yet evokes stereotypic behavioral responses. Similar to intrinsic theta frequency oscillations that entrain to the sniffing cycle in ET cells of the MOB (Hayar et al., 2004, 2005), operation of the vomeronasal pump (M Meredith & O’Connell, 1979) could entrain iAMC oscillations. In the hamster vomeronasal organ, peristaltic vasoconstriction cycles of 0.2–0.5 Hz were recorded in vivo (Michael Meredith, 1994). In other brain areas, oscillations with higher frequencies are usually confined to a small neuronal space, whereas slow oscillations are described to recruit very large networks (Csicsvari et al., 2003; M. Steriade, 2001). Consequently, downstream processing modules for vomeronasal stimuli could present targets of infraslow oscillatory activity in the AOB. Notably, these include several nuclei that mediate pulsatile neuroendocrine release by synchronized slow rhythmic bursting of, for example, gonadotropin-releasing hormone neurons (Chu et al., 2012; Michael Meredith, 1998; Schauer et al., 2015) or vasopressin magnocellular neurosecretory cells (Brown, 2004; Israel et al., 2010; Sabatier et al., 2004).
Summary

5 Summary

Most vertebrates have evolved multiple olfactory subsystems that serve a broad behavioral context, ranging from the detection of foods to social behaviors and predator avoidance. Among these subsystems, the AOS plays a major role in semiochemical detection. The AOS bypasses the thalamocortical axis. Hence, AMCs represent a direct neural link between vomeronasal sensory input and limbic output. Recently, we described a group of intrinsically rhythmogenic AMCs that exhibits spontaneous infraslow rhythmic discharge. To what extent intrinsic AMC rhythmogenesis shapes information processing and sensory coding, however, remained unexplored. Therefore, the primary aim of my dissertational work was to gain a deeper understanding of the impact of iAMC rhythmogenesis on the intact AOB network.

In a collaborative effort with Yoram Ben-Shaul’s group, I identified a group of AOB neurons that oscillate in vivo. These neurons form a distinct group with respect to their spontaneous activity and do not represent the extreme of a continuous, normally distributed neuronal population. Similar to in vitro observations the temporal features of periodic bursting are heterogeneous. This indicates the absence of a single characteristic rhythm governing AOB oscillations. In vitro, I observed periodic AMC bursting in mice as young as postnatal day 8, indicating a potential role in AOS development.

In iAMCs, intrinsic rhythmogenesis is driven by cyclic activation of three interdependent ionic conductances: $I_{NaP}$, $I_R$, and $I_{BK}$. Here, I show that these conductances are not unique to rhythmogenic neurons but are found across the AMC population. However, two of these conductances are differentially expressed in iAMCs. Here, $I_{NaP}$ activates at more hyperpolarized potentials and has larger amplitudes, whereas $I_{BK}$ activation is left-shifted. Thus, these conductances confer a distinct electrophysiological phenotype on iAMCs making them ideally equipped for robust burst generation. Performing membrane potential simulations in collaboration with Simon O’Connor, we show that the interplay of $I_{NaP}$, $I_R$, and $I_{BK}$ is sufficient to drive autorhythmicity in a model AMC.

Importantly, I identify a second group of oscillating AMCs, in which rhythmicity is entrained by network-dependent mechanisms. These eAMCs, in turn, comprise two distinct subpopulations: glut.-sensitive eAMCs, in which fast excitatory synaptic transmission is necessary and sufficient to entrain rhythmic activity, and glut.-insensitive eAMCs, in which fast glutamatergic transmission contributes to, but is not sufficient to sustain oscillatory activity. Comparing intrinsically rhythogenic and entrained neurons, I show that they do not merely differ in some intrinsic biophysical properties, but, in addition, are differentially wired within the AOB network. Synaptic drive in eAMCs is markedly increased and periodic, whereas iAMCs receive sparse, irregular synaptic input. In pharmacological experiments, I show that entrained oscillatory activity in eAMCs is driven by periodic surges of excitatory synaptic transmission that is mediated, at least in part, by AMPA / Kainate and NMDA receptors. The increase in excitatory input to eAMCs manifests mainly as an increase in spontaneous EPSC frequency, and to a lesser degree as an increase in charge carried by individual events. In contrast, AMCs receive extensive inhibitory drive that is not periodic. Consequently, inhibitory fast synaptic transmission does not seem to play a role in oscillatory entrainment in the AOB.

Furthermore, hypothesizing that iAMCs entrain oscillatory activity in eAMCs, I established an experimental framework using paired patch-clamp experiments to assess for chemical and synaptic coupling between neurons.

Finally, I demonstrate the existence of concurrent rhythmic heterogeneity between individual neurons on the population level. Ensembles of rhythmic neurons synchronize to form distinct microcircuits characterized by a unique dominant rhythm. Moreover, I show that diverse rhythmic activity from
Summary

Single cells may interfere to produce large-scale calcium waves that extend throughout the AOB mitral cell layer.

Together, the data I obtained in this thesis provide novel insights into functional AOB connectivity and demonstrate that intrinsic rhythmogenesis exerts profound effects on the local network via a hitherto undescribed excitatory connection. These effects manifest in periodic activity \textit{in vivo}, and may thus influence sensory processing and, accordingly, the animal’s endocrine status.
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7 Appendices

7.1 Changes in extracellular pH regulate excitability of vomeronasal sensory neurons under physiological conditions

In mice, the AOS plays a critical role in social communication with vomeronasal stimuli typically being secreted in various bodily fluids. Rodent urine is a rich source of semiochemicals and contains cues that report a female’s cycle stage (Achiraman et al., 2010; Haga-Yamanaka et al., 2014; J. He et al., 2008; Johnston & Bronson, 1982; Lydell & Doty, 1972; Nielsen et al., 2011) and, consequently, sexual receptivity (Aron, 1979; Davies & Bellamy, 1974). In her dissertational work, Annika Cichy reported estrus-specific drops in mouse urinary pH that were exclusively observed in sexually experienced females (Cichy et al., 2015). We therefore hypothesized that, while sampling various stimulatory semiochemicals dissolved in the intraluminal mucus, VSNs might also be affected by the general physicochemical properties of the “solvent”.

In Cichy et al. (2015) we have shown that VSNs reliably detect extracellular acidosis, and that acid-evoked responses share the biophysical and pharmacological hallmarks of the hyperpolarization activated current $I_h$. A pH-induced shift in the voltage-dependence of $I_h$ activation causes the opening of HCN channels at rest, thereby increasing VSN excitability. Moreover, in behavioral experiments and in vivo AOB recordings we demonstrated that stimulus pH plays a modulatory role and thus emerges as a novel dimension in the AOS coding space.

Contributing to this study, my aim was to analyze whether extracellular acidification evokes VSN firing at physiological/unperturbed resting membrane potentials. Therefore, I recorded VSN neuronal activity in a loose-seal cell-attached configuration upon exposure to relatively mild acidification (pH 6.75 to pH 6.0) or elevated K+ (Figure 7.1). Spike recordings from 40 VSNs revealed dose-dependent recruitment of pH-sensitive neurons (Figure 7.1 B) that responded to increasing extracellular proton concentrations with time-locked trains of action potentials (Figure 7.1 C, D), indicating that intact VSNs can generate substantial output when challenged with relatively subtle changes in extracellular pH.
Together, our results identified extracellular acidification as a potent activator of vomeronasal $I_h$ and suggest HCN channel-dependent vomeronasal gain control of social chemosignaling. Hereby, an estrus-dependent drop in pH could readily result in a general subthreshold increase in VSN excitability, and thus raise the recipient’s “alert level”.

Figure 7.1 Subtle changes in extracellular pH induce vomeronasal responses under physiological conditions. A, Original representative extracellular loose-patch recording from a single neuron challenged successively with elevated $K^+$ (50 mM) and increasing extracellular proton concentrations (pH 6.75–pH 6.0). Horizontal black bars indicate stimulation ($K^+$, 1 s; pH, 5 s). B, Bar chart depicting response rate versus proton concentration. Data are normalized to the $K^+$-sensitive VSN population. C, Spike raster plots of 40 VSNs stimulated as in A. Stimulus exposure is indicated by the horizontal blue bars and gray columnar shading. G, Peristimulus time histogram (PSTH) illustrating $K^+$-pH-dependent changes in spike frequency over time. Individual data points in a given PSTH depict the average firing rates of all tested VSNs (means ± SEM; 1 s bin width; n = 40). Stimulus-evoked mean firing rates up to 6.2 ± 0.9 Hz were recorded (pH 6.0). (Cichy et al., 2015)
## 7.2 Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>aAOB</td>
<td>Anterior accessory olfactory bulb</td>
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<tr>
<td>ACH</td>
<td>Autocorrelation histogram</td>
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<td>AOB</td>
<td>Accessory olfactory bulb</td>
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<td>AON</td>
<td>Anterior olfactory nucleus</td>
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<td>AOS</td>
<td>Accessory olfactory system</td>
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<tr>
<td>AP</td>
<td>Action potential</td>
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<tr>
<td>BK</td>
<td>Big conductance potassium current</td>
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<td>BNST</td>
<td>Bed nucleus of <em>stria terminalis</em></td>
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<tr>
<td>Cav</td>
<td>Voltage-gated calcium channel</td>
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<tr>
<td>$C_{\text{mem}}$</td>
<td>Membrane capacitance</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CoA</td>
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<td>EPSC</td>
<td>Excitatory postsynaptic potential</td>
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<td>$f_{\text{AP}}$</td>
<td>Within-burst spike frequency</td>
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</tr>
<tr>
<td>$f_{\text{IPSC}}$</td>
<td>IPSC frequency</td>
</tr>
<tr>
<td>FPR</td>
<td>Formyl peptide receptor</td>
</tr>
<tr>
<td>$f_{\text{PSC}}$</td>
<td>PSC frequency</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GC</td>
<td>Granule cell</td>
</tr>
<tr>
<td>GG</td>
<td>Grueneberg ganglion</td>
</tr>
<tr>
<td>GL</td>
<td>Glomerular layer</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamic-pituitary-gonadal axis</td>
</tr>
<tr>
<td>iAMC</td>
<td>Intrinsically oscillating AOB mitral cell</td>
</tr>
<tr>
<td>$I_h$</td>
<td>Hyperpolarization-activated cyclic nucleotide-gated current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>$I_{\text{NaP}}$</td>
<td>Persistent sodium current</td>
</tr>
<tr>
<td>IPI</td>
<td>Interpeak interval</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
</tr>
<tr>
<td>ISI</td>
<td>Interspike interval</td>
</tr>
<tr>
<td>$I_T$</td>
<td>T-type calcium current</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LA</td>
<td>Lateral amygdala</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LOT</td>
<td>Lateral olfactory tract</td>
</tr>
<tr>
<td>M/TC</td>
<td>Mitral and tufted cell</td>
</tr>
<tr>
<td>MC</td>
<td>Mitral cell</td>
</tr>
<tr>
<td>MeA</td>
<td>Anterior division of the medial amygdala</td>
</tr>
<tr>
<td>MeP</td>
<td>Posterior division of the medial amygdala</td>
</tr>
<tr>
<td>MOB</td>
<td>Main olfactory bulb</td>
</tr>
<tr>
<td>MOE</td>
<td>Main olfactory epithelium</td>
</tr>
<tr>
<td>MPOA</td>
<td>Medial preoptic area</td>
</tr>
<tr>
<td>NAOT</td>
<td>Nucleus of the accessory olfactory tract</td>
</tr>
<tr>
<td>OR</td>
<td>Olfactory receptor</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory sensory neuron</td>
</tr>
<tr>
<td>OT</td>
<td>Olfactory tubercle</td>
</tr>
<tr>
<td>pAOB</td>
<td>posterior accessory olfactory bulb</td>
</tr>
<tr>
<td>PGC</td>
<td>Periglomerular cell</td>
</tr>
<tr>
<td>PIR</td>
<td>Piriform cortex</td>
</tr>
<tr>
<td>PMCN</td>
<td>Posteromedial cortical amygdaloid nucleus</td>
</tr>
<tr>
<td>PSC</td>
<td>Postsynaptic current</td>
</tr>
<tr>
<td>PSD</td>
<td>Power spectral density</td>
</tr>
<tr>
<td>$R_{\text{input}}$</td>
<td>Input resistance</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SK</td>
<td>Small conductance potassium current</td>
</tr>
<tr>
<td>SO</td>
<td>Septal organ of Masera</td>
</tr>
<tr>
<td>TAAR</td>
<td>Trace amine-associated receptor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TC</td>
<td>Tufted cell</td>
</tr>
<tr>
<td>V1R</td>
<td>Vomeronasal receptor type 1</td>
</tr>
<tr>
<td>V2R</td>
<td>Vomeronasal receptor type 2</td>
</tr>
<tr>
<td>V_d</td>
<td>Downstate membrane potential</td>
</tr>
<tr>
<td>V_{DF}</td>
<td>Driving force</td>
</tr>
<tr>
<td>V_{mem}</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>VNO</td>
<td>Vomeronasal organ</td>
</tr>
<tr>
<td>V_{rest}</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>V_u</td>
<td>Upstate membrane potential</td>
</tr>
<tr>
<td>τ_{fast}</td>
<td>Fast component of decay time constant</td>
</tr>
<tr>
<td>τ_{mem}</td>
<td>Membrane time constant</td>
</tr>
</tbody>
</table>
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