Presynaptic Muscarinic Receptors Enhance Glutamate Release at the Mitral/Tufted to Granule Cell Dendrodendritic Synapse in the Rat Main Olfactory Bulb

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Ghatpande AS, Gelperin A. Presynaptic muscarinic receptors enhance glutamate release at the mitral/tufted to granule cell dendrodendritic synapse in the rat main olfactory bulb. J Neurophysiol 101: 2052–2061, 2009. First published February 18, 2008; doi:10.1152/jn.90734.2008. The mammalian olfactory bulb receives multiple modulatory inputs, including a cholinergic input from the basal forebrain. Understanding the functional roles played by the cholinergic input requires an understanding of the cellular mechanisms it modulates. In an in vitro olfactory bulb slice preparation we demonstrate cholinergic muscarinic modulation of glutamate release onto granule cells that results in γ-aminobutyric acid (GABA) release onto mitral/tufted cells. We demonstrate that the broad-spectrum cholinergic agonist carbachol triggers glutamate release from mitral/tufted cells that activates both AMPA and NMDA receptors on granule cells. Activation of the granule cell glutamate receptors leads to calcium influx through voltage-gated calcium channels, resulting in spike-independent, asynchronous GABA release at reciprocal dendrodendritic synapses that granule cells form with mitral/tufted cells. This cholinergic modulation of glutamate release persists through much of postnatal bulbar development, suggesting a functional role for cholinergic inputs from the basal forebrain in bulbar processing of olfactory inputs and possibly in postnatal development of the olfactory bulb.

INTRODUCTION

The olfactory bulb is an obligatory bottleneck in the flow of information from the nose to the brain. Millions of olfactory receptor neurons send their axons into the main olfactory bulb (MOB) and synapse with close to 150,000 relay neurons called mitral/tufted cells that, in turn, innervate many neurons in multiple cortical components of the olfactory system (Shepherd and Greer 1998; Wilson and Mainen 2006; Wilson et al. 2006). Thus the olfactory bulb is an appropriate target for neuromodulatory systems that may carry contextual information from higher cortical centers to influence bulbar information processing (Kay and Sherman 2007). Consistent with this view of bulbar circuitry acting like a thalamic relay, the bulb receives multiple “centrifugal” inputs from olfactory cortical areas as well as from neuromodulatory centers in the basal forebrain (Luskin and Price 1983; Shipley and Ennis 1996).

Among these centrifugal inputs, the bulb receives projections from cholinergic neurons in the horizontal limb of the diagonal band of Broca (Luskin and Price 1983; Macrides et al. 1981; Zaborszky et al. 1986). This cholinergic input innervates all layers of the bulb that, in turn, express multiple types of cholinergic receptors (Kasa et al. 1995; Le Jeune et al. 1995). It is thus likely that the cholinergic input has more than one function in the bulb. In accord with this expectation, chemical ablation or pharmacological blockade of the cholinergic input to the bulb has multiple effects on odor-guided behavior in rodents (Doty et al. 1999; Linster et al. 2001; Ravel et al. 1994; Wilson et al. 2004).

Other work suggests a distinct developmental role for bulbar cholinergic inputs, as also seen extensively in the cortex (Bear and Singer 1986; Kaneko et al. 2006; Lauder and Schambra 1999; Pallera et al. 1994). Understanding the various cellular mechanisms modulated by cholinergic inputs and activated cholinergic receptors is necessary to appreciate the multiple roles played by cholinergic inputs in olfactory bulb function and development.

A critical part of bulbar synaptic processing is carried out by inhibitory interneurons found at two distinct levels in the mammalian olfactory bulb (Shepherd and Greer 1998; Wachowiak and Shipley 2006). Of these, granule cells are the largest population of GABAergic inhibitory interneurons in the bulb. Granule cell dendritic spines form synapses with the aspiny, glutamatergic mitral/tufted cell dendrites and these dendrodendritic reciprocal synapses are a key feature of synaptic interactions in the bulb (Jahr and Nicoll 1982; Rall et al. 1966). The reciprocal mitral/tufted–granule synapses are thought to be involved in generating odor-evoked oscillations in the olfactory bulb in vivo (Gelperin 2006; Lagier et al. 2004; Rinberg and Gelperin 2006). The same synapses are known to contribute to mitral/tufted and granule cell synchrony in vitro (Friedman and Strowbridge 2003; Schoppa 2006a,b). These synapses are known targets of the cholinergic input from the basal forebrain (Shepherd and Greer 1998).

Two in vitro studies have addressed how cholinergic mechanisms modulate synaptic transmission at reciprocal synapses by modulating γ-aminobutyric acid (GABA) release via presynaptic mechanisms on granule cells (Castillo et al. 1999; Ghatpande et al. 2006). A recent study has also investigated postsynaptic actions of acetylcholine (ACh) modulating the intrinsic membrane properties of granule cells by activating somatic muscarinic receptors (Pressler et al. 2007).

There is preliminary evidence for carbachol (CCh)-triggered glutamate release onto granule cells (Ghatpande et al. 2006) that suggest the presence of cholinergic receptors on mitral/tufted cells, especially in postnatal day 7 and younger animals.

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The experiments reported here follow up on those preliminary findings, chiefly through whole cell recordings from granule cells, to investigate the mechanisms postsynaptic to CCh-triggered glutamate release. Our results indicate that CCh activates muscarinic receptors that trigger glutamate release onto granule cells, in turn leading to activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-D-aspartate receptors (NMDARs) on granule cells. Carbachol application simultaneously increases GABA release onto mitral/tufted and granule cells. Based on the experiments reported here and the earlier work, we propose that the cholinergic input to the bulb plays a role in processing olfactory input to the bulb and perhaps in its postnatal development.

METHODS

Slice preparation

Olfactory bulbs from postnatal day 4 (P4) to P17 Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were removed into ice-cold, oxygenated (equilibrated with 95% O2-5% CO2) high-Mg2+-artificial cerebrospinal fluid (aCSF) containing (in mM) 120 NaCl, 3.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 10 glucose, 2.5 MgCl2, and 2.5 CaCl2. Horizontal slices (350 µm) at 1–2 ml/min with oxygenated, standard aCSF at RT containing (in volume 1 ml) with a glass coverslip bottom, submerged, and perfused each slice was transferred to a custom-made Plexiglas chamber (512–1,028 data points) for every individual data file. The thresholds for amplitude and PSC area were set to 5σp and 20- to 50σp, respectively, for each file. These criteria were previously determined to give the best detection of PSCs by analyzing sample data files while varying these two parameters and evaluating the detection results visually. After automatic detection of events by the program, we manually reviewed the recording and deleted those. The program was used to generate event lists and statistics in epochs of 120 or 300 s. Several such files were recorded in each experimental condition. Each file was exported to the Mini Analysis program (ver. 6.0, Synaptosoft, Decatur, GA). The program detects postsynaptic currents (PSCs) using several parameters to describe unitary PSCs, of which we modified the amplitude and area thresholds, keeping the other parameters at their default values. The SD of the noise (σn) was estimated using a suitable epoch of eventless data (512–1,028 data points) for every individual data file. The thresholds for amplitude and PSC area were set to 5σp and 20- to 50σp, respectively, for each file. These criteria were previously determined to give the best detection of PSCs by analyzing sample data files while varying these two parameters and evaluating the detection results visually. After automatic detection of events by the program, we routinely rescanned the detected events for obvious detection mistakes and deleted those. The program was used to generate event lists and cumulative frequency histograms of event amplitude and interevent intervals. Statistical significance of differences between these distributions in a single cell subjected to different experimental treatments was determined using the nonparametric Kolmogorov–Smirnov test (KS test) within the Mini Analysis program. Data (frequency, amplitude, etc.) from many cells were evaluated using paired/unpaired Student’s t-test in SigmaPlot. Final plots and figures were made in SigmaPlot 2001 (SPSS, California State University, Fullerton).

Voltage- and current-clamp recordings

Whole cell patch recordings were made from mitral/tufted or granule cells. Patch pipettes were pulled from borosilicate glass to a resistance of 1.5–3 MΩ using a Sutter P2000 horizontal puller.

Postsynaptic currents from mitral/tufted or granule cells were recorded with a pipette solution containing (in mM) 130 CsCl, 5 KCl, 10 EGTA, 2 ATP (Mg2+ salt), 0.2 GTP (Na+ salt), and 10 HEPES (Na+ salt), pH 7.2 adjusted with KOH (EC1 = 0.85 mV). Some experiments used a pipette solution with equimolar K+ gluconate instead of CsCl (EC1 = −827 mV). The membrane was voltage-clamped to a holding potential of −70 mV. Currents were amplified and recorded using a List EPC7 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany), filtered at 1–2 kHz using the built-in three-pole Bessel filter of the amplifier, and digitized at 5 kHz using a Digidata 1322A A/D board. Data were acquired and stored on a Pentium PC using WinEDR or WinWCP (ver. 2.5.8 or 2.5.9) software (freely available from John Dempster, Strathclyde Institute of Pharmacy and Biomedical Sciences). Access resistance (Ra) was monitored periodically and recordings were terminated if Ra was >50 MΩ (Ra range 10–50 MΩ).

Drug solutions were prepared in base extracellular solution and bath-applied by switching the perfusion reservoirs of a homemade, gravity-driven perfusion system. Effective concentrations in the bulk solution bathing the slice were achieved within about 2 min of the start of perfusion.

Carbachol (CCh), picrotoxin (PTX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonovaleric acid (APV), and pirenzepine (Pir) were from Sigma (St. Louis, MO).

Data and statistical analysis

Data were acquired using WinEDR (ver. 2.5.8/9) in gap-free mode in epochs of 120 or 300 s. Several such files were recorded in each experimental condition. Each file was exported to the Mini Analysis program (ver. 6.0, Synaptosoft, Decatur, GA). The program detects postsynaptic currents (PSCs) using several parameters to describe unitary PSCs, of which we modified the amplitude and area thresholds, keeping the other parameters at their default values. The SD of the noise (σn) was estimated using a suitable epoch of eventless data (512–1,028 data points) for every individual data file. The thresholds for amplitude and PSC area were set to 5σp and 20- to 50σp, respectively, for each file. These criteria were previously determined to give the best detection of PSCs by analyzing sample data files while varying these two parameters and evaluating the detection results visually. After automatic detection of events by the program, we routinely rescanned the detected events for obvious detection mistakes and deleted those. The program was used to generate event lists and cumulative frequency histograms of event amplitude and interevent intervals. Statistical significance of differences between these distributions in a single cell subjected to different experimental treatments was determined using the nonparametric Kolmogorov–Smirnov test (KS test) within the Mini Analysis program. Data (frequency, amplitude, etc.) from many cells were evaluated using paired/unpaired Student’s t-test in SigmaPlot. Final plots and figures were made in SigmaPlot 2001 (SPSS, California State University, Fullerton).

Intracellular dye fills

To determine the morphology of the cell from which we recorded, we included 100 µM to 1 mM Lucifer yellow dye (Sigma) or 40 µM Alexa Fluor (AF-488) dye in the patch pipette in some experiments. The dye was directly dissolved in the pipette solution described earlier.
Activation of M1 muscarinic receptors enhances spike-independent, glutamate receptor–dependent IPSCs in mitral/tufted cells in P6–P8 MOB slices

We recorded whole cell synaptic activity from mitral/tufted cells (Fig. 1) in bulb slices prepared from P6–P8 rats. These recordings were carried out with a pipette solution containing 130 mM chloride, which increases the amplitude of chloride currents through GABA$_A$ receptors. The bath contained magnesium (1 mM) and calcium (2.5 mM) in the external recording solution and also contained TTX (1 µM) to block spiking. As reported earlier (Fig. 1; Ghatpande et al. 2006), applying CCh through the bath increases the frequency of inhibitory postsynaptic currents (IPSCs) recorded in mitral/tufted cells. This effect of CCh on mitral/tufted cells showed a dose dependence over 10–500 µM CCh (Fig. 1A). The synaptic currents increased in frequency but not in amplitude over the same concentration range (Fig. 1A; median amplitudes were 30, 35, 25, and 35 pA in 0, 5, 50, and 500 µM CCh, whereas the median frequencies were 2, 1.7, 2.5, and 4.4 Hz in the respective CCh concentrations). A majority of these synaptic currents are GABA$_A$-receptor–mediated chloride currents based on their kinetics and their susceptibility to block by picrotoxin (Fig. 1B, n = 3 cells). This effect of CCh is likely mediated by M1 muscarinic receptors since it is blocked by 10 µM pirenzepine (Fig. 1C; Pir, 1.4 Hz), and finally washout of Pir with 50 µM CCh (4 Hz). D: the 2 current traces are from a mitral/tufted cell bathed in 1 µM TTX + 50 µM CCh and subsequent addition of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) + 50 µM 2-amino-5-phosphonovaleric acid (APV). E: box plots of fold-change in IPSC frequency in 50 µM CCh normalized to frequency in TTX and subsequent addition of either 10 µM CNQX + 50 µM APV or 10 µM CNQX normalized to frequency in TTX (horizontal dashed line at 1). Data recorded from mitral/tufted cells of P6–P8 (~3-fold median increase in CCh and ~0.8 in CCh + GluR blockers; P = 0.009, paired t-test, n = 7 cells) and P13–P15 (~2-fold median increase in CCh with 2.2-fold increase in CCh + GluR blockers; P = 0.6, paired t-test, n = 5 cells) MOB slices, respectively.

FIG. 1. M1 muscarinic receptor activation leads to increase in GABAergic synaptic activity sensitive to glutamate receptor (GluR) blockade in mitral/tufted cells of postnatal day 6 (P6) to P8 main olfactory bulb (MOB) slices. A: whole cell synaptic activity recorded from a mitral/tufted cell held at −70 mV. The bath contained 1 µM tetrodotoxin (TTX) to which was added increasing concentrations of carbachol (CCh). Middle panel shows 3 cumulative amplitude distributions of synaptic activity recorded at 0 (median amplitude 30 pA), 5 (35 pA), 50 (25 pA), and 500 µM CCh (35 pA). Right panel shows cumulative distributions of interevent intervals of the same recordings with median frequencies of 2, 1.7, 2.5, and 4.4 Hz, respectively. B: whole cell current traces from a mitral/tufted cell recorded in TTX and subsequent addition of 50 µM CCh and 100 µM picrotoxin (PTX). C: cumulative distributions of interevent intervals of inhibitory postsynaptic currents (IPSCs) from a mitral/tufted cell bathed in TTX (median frequency, 1.4 Hz), subsequent additions of 50 µM CCh (6 Hz) and 10 µM pirenzepine (Pir, 1.4 Hz), and finally washout of Pir with 50 µM CCh (4 Hz). D: the 2 current traces are from a mitral/tufted cell bathed in 1 µM TTX + 50 µM CCh and subsequent addition of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) + 50 µM 2-amino-5-phosphonovaleric acid (APV). E: box plots of fold-change in IPSC frequency in 50 µM CCh normalized to frequency in TTX and subsequent addition of either 10 µM CNQX + 50 µM APV or 10 µM CNQX normalized to frequency in TTX (horizontal dashed line at 1). Data recorded from mitral/tufted cells of P6–P8 (~3-fold median increase in CCh and ~0.8 in CCh + GluR blockers; P = 0.009, paired t-test, n = 7 cells) and P13–P15 (~2.3-fold median increase in CCh with 2.2-fold increase in CCh + GluR blockers; P = 0.6, paired t-test, n = 5 cells) MOB slices, respectively.

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This CCh-stimulated increase in IPSC frequency was not detected when ionotropic glutamate receptor (GluR) blockers (CNQX and APV) were applied in P6–P8 slices (Fig. 1D; threefold median change in CCh and ∼0.8 in CCh + GluR blockers; P = 0.009, paired t-test, n = 7 cells). In contrast, the CCh-triggered IPSC frequency increase persisted in mitral/tufted cells from P13–P15 MOB slices in the presence of GluR blockers (Fig. 1D; ∼2.3-fold median change in CCh with 2.2-fold in CCh + GluR blockers; P = 0.6, paired t-test, n = 5 cells). These results suggest receptors for CCh/ACH are present on GABAergic cells forming synapses with mitral/tufted cells in animals older than P13, whereas P7 and younger animals apparently possess presynaptic GluRs that trigger GABA release onto mitral/tufted cells. The mechanisms and consequences of M1 receptor activation on granule cells at later ages (postnatal 2nd week and later) have recently been explored (Ghatpande et al. 2006; Pressler et al. 2007). In this study, we focused on the mechanisms involved in the CCh effect in 1-wk-old animals.

**CCH-stimulated IPSCs in neonatal mitral/tufted cells requires AMPA/kainate GluRs and depends on NMDARs as well as calcium channels**

We investigated the role played by the different types of ionotropic GluRs and calcium channels underlying the CCh-triggered IPSCs in mitral/tufted cells using different pharmacological reagents. The CCh-triggered increase in IPSC frequency in mitral/tufted cells was blocked entirely by bath-application of 10 μM CNQX, a known AMPA/kainate receptor antagonist. Figure 2A shows cumulative distributions of IPSC interevent intervals from a mitral/tufted cell with sequential superfusion of TTX, CCh, and finally 10 μM CNQX. The median frequency of IPSCs increased nearly fivefold with CCh for the cell shown (5 Hz in CCh; 0.8–0.9 Hz in TTX), but dropped slightly below the control (frequency in CCh + CNQX 0.6 Hz). As shown in the accompanying bar chart, the ratio of the average IPSC frequency in TTX to that in CCh was 0.32 ± 0.06 (paired t-test, P = 0.0003; n = 5 cells) and dropped to 0.26 ± 0.08 (comparison between +CCh and +CCh + CNQX; paired t-test, P = 0.0008; n = 5 cells) when CNQX was applied subsequent to CCh. The average IPSC frequencies in TTX and CNQX were not significantly different from one another. Thus AMPA/kainate receptors play an obligatory role in CCh-stimulated IPSCs in mitral/tufted cells.

Next, we tested the role played by NMDARs by applying APV, an NMDAR antagonist. Surprisingly the IPSC frequency after APV application was intermediate to that of CCh and baseline (median frequencies: TTX, 1.6 Hz, CCh + TTX, 6.6 Hz; CCh + TTX + APV, 4 Hz; P < 10−6, KS test). Average IPSC frequencies recorded for 2 min in each condition were in the ratio 0.16 ± 0.044, and 0.39 ± 0.1 in TTX and CCh + TTX + APV, respectively (P = 0.01 between CCh and APV and P = 0.04 between TTX and APV, paired t-test, n = 5 cells). This suggests that even with TTX present, GABAergic synapses terminating on mitral/tufted cells are sufficiently depolarized to unblock NMDARs, which then contribute to CCh-stimulated IPSCs in mitral/tufted cells.

We then examined the role of voltage-gated calcium channels (VGCCs) in CCh-stimulated inhibition by applying 100 μM Cd2+, an inorganic calcium channel blocker. Figure 2C shows the cumulative distributions of IPSCs in CCh (median frequency, 2 Hz), followed by Cd2+ application (median frequency, 1.2 Hz), and finally a washout of the cadmium with CCh (median frequency, 3 Hz; similar data in two other cells). Thus cadmium application reduced the CCh-mediated increase in IPSC frequency. In accord with data reported in Ghatpande et al. (2006), the TTX-resistant, CCh-stimulated inhibition in mitral/tufted cells is Cd2+ sensitive, again suggesting that the GABAergic terminals depolarize sufficiently to open VGCCs, which then contribute to GABA release onto mitral/tufted cells.
The largest population of GABAergic cells with synapses on mitral/tufted cells is the granule cell population in the MOB, although a subpopulation of periglomerular cells is also known to be GABAergic (Shepherd and Greer 1998). Granule cells form dendrodendritic synapses with mitral/tufted cell secondary dendrites and inhibition mediated through these synapses is known to be TTX resistant and dependent on NMDARs and VGCCs (Chen et al. 2000; Isaacson 2001; Isaacson and Strowbridge 1998; Schoppa et al. 1998). AMPA/kainate receptors are also thought to be present at these synapses, but are thought to play a facilitatory role, at least in conditions not involving high-frequency stimulation (Isaacson 2001; Schoppa 2006a). Calcium-imaging studies suggest that spontaneous glutamate release onto granule cell spines can lead to local depolarizations and opening of NMDARs and VGCCs (Egger et al. 2003, 2005; Zelles et al. 2006). Given the similarity of mechanisms inferred from the results described so far in earlier reports, we hypothesized that CCh stimulates release of glutamate from mitral/tufted cell secondary dendrites onto granule cell dendritic spines, activating AMPA/kainate and NMDA receptors along with VGCCs, leading to calcium influx that in turn triggers GABA release in P6–P8 animals.

M1-receptor activation enhances excitatory GluR-mediated synaptic currents in granule cells

If the aforementioned hypothesis is true, whole cell recordings from granule cells (GCs) should show an increase in glutamatergic synaptic currents in the P6–P8 age group but not in the P13–P15 age group. We recorded spontaneous whole cell synaptic activity from GC soma within the granule cell layer (GCL) of MOB slices from rats aged 4–17 days. To ensure recordings from mature, stage 5 GCs, we chose cells with spontaneous synaptic activity of ≥0.3 Hz (Carleton et al. 2003). Fluorescent dye (either Lucifer yellow or AF-488) was included in the high-chloride pipette solution to observe cell morphology (Fig. 3A) in some recordings.

We identified four distinct types of synaptic currents in GCs (Fig. 3A). The first, most frequent event (type III) had a fast onset and a slow decay phase. These events were large amplitude and blocked by picrotoxin (PTX), suggesting they were GABA<sub>A</sub>-receptor–mediated currents. This GABAAergic input to granule cells may arise from either Blanes cells or deep, short axon cells (Eyre et al. 2008; Pressler and Strowbridge 2006). Two other smaller-amplitude currents were observed; type I events had a fast onset and a relatively rapid decay. Type II events were less frequent and had a slower onset and decay than those of type I events. We also recorded a fourth type of current (type IV) at depolarized holding potentials (+50 mV) with very slow decay rates, as shown in Fig. 3A. Type I, II, and IV events persisted in picrotoxin and were blocked by CNQX, suggesting they were carried by glutamate receptors. Type IV events were also absent when APV alone was superfused through the bath (data not shown). Thus we confirmed that the GCs from which we recorded had functional synapses and possessed the full complement of synaptic currents recorded from these cells by others (Belluzzi et al. 2003; Carleton et al. 2003).

Bath perfusion of TTX (1 µM) and PTX (100 µM) substantially reduced synaptic activity (current traces in Fig. 3B; short dashed line in the cumulative distribution shown below the raw data; in this example: median frequency, 0.16 Hz). When CCh was subsequently applied, slightly more than half of these cells responded with increased synaptic activity (Fig. 3B; +CCh current trace and dash-double dotted line in graph; for the example shown: median frequency, 3.3 Hz; 27 of 48 granule cells recorded responded to CCh). Surprisingly, granule cells from animals as old as P17 responded to CCh. Of 27 responsive cells, 17 were from P6–P8 and 10 from P9–P17 age groups, suggesting that cholinergic modulation of glutamate release onto granule cells persists in older animals.

This increased excitatory synaptic activity was blocked by GluR antagonists CNQX + APV (Fig. 3C; +CCh median frequency, 10 Hz; +CNQX + APV median frequency, 0.2 Hz; and median frequency after washout of GluR blockers was 3.3 Hz).

The increased glutamate release onto granule cells gave rise to depolarizing excitatory postsynaptic potentials (EPSPs) at the soma that were completely blocked by the M1 muscarinic receptor antagonist pirenzepine (Fig. 3D; TTX + PTX median frequency, 0.8 Hz; +CCh median frequency, 20 Hz; and with pirenzepine the median frequency was 0.4 Hz, respectively). Pirenzepine application restored the EPSP frequency to baseline in three of three cells recorded for this experiment. This same concentration of pirenzepine also blocked GABAAergic currents in mitral/tufted cells, as shown earlier (Fig. 1C).

CCh-stimulated glutamate release activates AMPA/kainate GluRs and NMDARs on granule cells

The CCh-stimulated miniature excitatory postsynaptic currents (mEPSCs) in granule cells were blocked by CNQX and APV applied together. We also explored the role of AMPA/kainate GluRs and NMDARs separately as described in this section.

Figure 4A shows excitatory synaptic activity recorded from a granule cell in CCh and subsequent addition of CNQX to the bath. The cumulative distribution of EPSC intervals shows CNQX reduced median frequency of EPSCs back to baseline (median frequency was 0.1 Hz in TTX, 3.3 Hz in CCh, and 0.1 Hz in CNQX for the cell shown). This effect was reliable; mean frequencies of EPSCs in the three recording conditions are shown in the accompanying bar chart. This confirms that CNQX-sensitive AMPA/kainate receptors are activated during CCh-stimulated excitatory activity in granule cells.

Next we tested whether a fraction of the CCh-stimulated activity was due to currents through NMDARs. As shown in the top panel of Fig. 4B, we superfused the bath with APV, an NMDAR blocker, along with CCh. Interestingly, APV reduced but did not eliminate the CCh-stimulated increase in EPSC frequency, as seen in the raw data as well as in the distribution of EPSC intervals, suggesting that some fraction of the EPSCs were carried by NMDARs. We then applied APV first to the cell and then subsequently applied CCh along with APV. CCh application subsequent to APV application resulted in a clear increase in EPSC frequency, as seen in the bottom panel of Fig. 4B. This demonstrates that NMDARs are not essential for CCh-stimulated increase in EPSC frequency in granule cells. In contrast, the CNQX susceptibility of the stimulatory CCh effect argues for an obligatory role for AMPA/kainate receptors in CCh-stimulated excitatory activity in granule cells.
The results illustrated in Fig. 2C indicate a role for VGCCs in CCh-stimulated GABA release onto mitral/tufted cells. We tested the role of these channels in the CCh-stimulated excitatory activity in granule cells by applying 100 μM Cd²⁺ along with CCh while recording from voltage-clamped granule cells. The raw current traces from a voltage-clamped granule cell in TTX with picrotoxin, added CCh, and after subsequent addition of 100 μM cadmium are shown in Fig. 4C. The CCh-stimulated excitatory activity is sensitive to cadmium. The interevent distributions of EPSCs in the three bath conditions are shown in the adjacent plot. On CCh addition, the median frequency of EPSCs went up severalfold (1.7 Hz from 0.12 Hz) and returned to baseline (0.15 Hz) with subsequent addition of 100 μM cadmium (qualitatively similar data were obtained in two other granule cells). This result suggests a role for VGCCs in glutamate release onto granule cells subsequent to activation of the muscarinic receptor.

**Discussion**

The MOB receives extensive cholinergic inputs from the basal forebrain. A number of different types of cholinergic receptors are expressed in the MOB. Thus very likely the cholinergic input modulates multiple aspects of olfactory information processing in the bulb, ranging from odor discrimination to olfactory memories.

Blockade of nicotinic receptors abolishes, whereas muscarinic receptor blockade reduces, spontaneous discrimination
between chemically similar odorants (Mandairon et al. 2006). Disruption of the cholinergic input leads to increased generalization of chemically similar odorants, an ability thought to depend on perceptual learning (Linster et al. 2001; Wilson et al. 2004). Injection of physostigmine, an acetylcholinesterase inhibitor, has been shown to enhance olfactory discrimination in rats (Chaudhury et al. 2009; Doty et al. 1999). Short-term olfactory memory is degraded when scopolamine, a muscarinic receptor antagonist, is injected into the olfactory bulb of rats carrying out olfactory tasks (Ravel et al. 1994). Multiple electrophysiological effects resulting from activation of different types and differentially localized cholinergic receptors have been shown on bulbar neurons in anesthetized rats and in bulb slices (Castillo et al. 1999; Elaagouby et al. 1991; Ghatpande et al. 2006; Pignatelli and Belluzzi 2008; Pressler et al. 2007; Tsuno et al. 2008).

The dendrodendritic synapses between mitral/tufted and granule cells are likely to be an important target of this cholinergic input since they are believed to be involved in not only odor discrimination but also one locus of olfactory memories. Our data suggest that dendrodendritic synaptic transmission can indeed be modulated by ACh.

**Cellular mechanism of the muscarinic effect leading to increased IPSC frequency in neonatal mitral/tufted cells**

Our results show that CCh stimulates glutamate release onto granule cells while also stimulating GABA release onto mitral/tufted cells. Furthermore, this GABAergic input is dependent on the same cellular mechanisms that are activated by CCh on granule cells, implying that the GABA release is occurring from granule cells. Granule cells receive glutamatergic input from mitral/tufted cells as well as from centrifugal fibers originating in the olfactory cortex. Our data indicate that CCh stimulates the glutamatergic input from mitral/tufted cell axon collaterals as well as from centrifugal fibers originating in the olfactory cortex.
ondary dendrites rather than other inputs for the following reasons.

1) All our data have been recorded in the presence of TTX, effectively decoupling excitatory somatodendritic inputs from GABA release sites on granule cells. Thus the muscarinic receptors as well as the presynaptic GluRs need to be at or near GABA release sites. The presence of GluRs presynaptic to GABA release is well known at dendrodendritic synapses between mitral/tufted and granule cells.

2) The unusual dependence of GABA release on glutamate release and subsequent activation of AMPARs combined with NMDARs and VGCCs is, again, a well-known feature of dendrodendritic inhibition.

Our results suggest that quantal, TTX-resistant, CCh-stimulated glutamate release is sufficient to open AMPA/kainate receptors that sufficiently depolarize the granule cell spine to unblock NMDARs and probably activate VGCCs. Recent calcium-imaging studies have suggested that spontaneous glutamate release onto granule cell spines is sufficient to cause calcium influx through NMDARs and calcium channels (Egger et al. 2003, 2005). Furthermore, quantal glutamate release has been shown to be sufficient to depolarize granule cell spines to cause local spiking activity in the frog (Zelles et al. 2006). These reports suggest that CCh-stimulated quantal glutamate release might indeed be sufficient to cause GABA feedback back onto the mitral/tufted cell. The physiological significance of such localized feedback is under active investigation.

**Apparent age dependence of muscarinic modulation of glutamate release from mitral/tufted cell dendrites**

Our data are consistent with increased release of glutamate from mitral/tufted cell lateral dendrites on activation of muscarinic receptors. These receptors appear to be localized on the mitral/tufted cell dendrites. In an earlier publication, the bulk of the results suggested that the muscarinic receptors on granule cells triggered an increase in GABA release, although preliminary data also suggested that in rats <10 days old muscarinic receptors modulated glutamate release from mitral/tufted cell dendrites (Ghatpande et al. 2006). Thus there appears to be an apparent age-dependent switch in localization or transduction machinery coupled to muscarinic receptors at these synapses. On closer examination of the results presented here, a somewhat different picture emerges. Our granule cell recordings show CCh-stimulated increase in frequency of EPSCs or EPSPs as late as P17. We do not currently understand why only half of the granule cells from which we recorded respond to the CCh-triggered glutamate release from mitral/tufted cell dendrites. There is some physiological evidence for granule cell subtypes that differentially express metabotropic receptors (Heinbockel et al. 2007). Within the last few years it has become clear that olfactory bulb interneurons are phenotypically and developmentally diverse (Lledo et al. 2008). These phenotypically diverse GC subtypes are thought to have distinct localization and connectivities within the bulb layers. Since our sample of granule cell recordings must include many of these differentially connected subtypes this might explain why approximately half the granule cells “responded” to CCh. This needs to be systematically investigated.

Apparently in contradiction to our granule cell recordings, the mitral/tufted cell data in Fig. 1D clearly show that the majority of GABAergic feedback is GluR dependent in the first postnatal week and becomes independent of GluRs in the next week.

Our interpretation of these results is shown in Fig. 5. We suggest that either the cholinergic input to granule cell dendritic spines or the muscarinic receptors on the spines might develop largely after the first postnatal week. This is supported by reports in the literature of a striking correlation between development of a presynaptic cholinergic marker (i.e., choline acetyltransferase) and postsynaptic muscarinic receptors during the first 4 wk of postnatal development (Large et al. 1986). Furthermore, IP3-sensitive calcium stores are also thought to appear only after the first postnatal week in rats (Slawecki et al. 1997). The olfactory bulb granule cell population increases and reaches a plateau over this time period (Bayer 1983; Rosselli-Austin and Altman 1979). This correlation between granule cell population increase, molecular markers of cholinergic synapse development, and functional changes in cholinergic modulation of transmitter release shown here may indicate an underly-...
ing causative role for cholinergic input in bulbar, especially granule cell development and ongoing apoptosis seen in the bulb.

Furthermore, our data suggest cholinergic modulation of spike-independent, spontaneous dendrodendritic transmitter release must be important for bulbar function at the earliest postnatal or embryonic stages when the bulb has formed. Spike-independent, spontaneous GABA release might be important in controlling the extent of backpropagation of action potentials along the mitral/tufted cell dendrites, thereby controlling the extent of lateral inhibition (Lowe 2002, 2003). The functional distinction between cholinergic modulation of glutamate release at the mitral/tufted cell dendrite or GABA release at the granule cell dendritic spine remains to be resolved. It is likely that this distinction will arise due to the host of other mechanisms that also modulate glutamate or GABA release at these synapses.

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REFERENCES


