Store Calcium Mediates Cholinergic Effects on mIPSCs in the Rat Main Olfactory Bulb

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Ghatpande, Ambarish S., Kartik Sivaraaman, and Sukumar Vijayaraghavan. Store calcium mediates cholinergic effects on mIPSCs in the rat main olfactory bulb. J Neurophysiol 95: 1345–1355, 2006. The significance of endoplasmic reticulum (ER) store calcium in modulating transmitter release is slowly gaining recognition. One transmitter system that might play an important role in store calcium modulation of transmitter release in the CNS is acetylcholine (ACh). The main olfactory bulb (OB) receives rich cholinergic innervation from the horizontal limb of the diagonal band of Broca and blocking cholinergic signaling in the bulb inhibits the ability of animals to discriminate between closely related odors. Here we show that exposing OB slices to carbamylcholine (CCh), a hydrolysis-resistant analog of ACh, increases γ-aminobutyric acid (GABA) release at dendrodendritic synapses onto the mitral cells. This increase in transmitter release is mediated by the activation of the M1 class of muscarinic receptors and requires the mobilization of calcium from the ER. The site of action of CCh for this effect is developmentally regulated. In animals younger than postnatal day 10, the major action of CCh appears to be on mitral cells, enhancing GABA release by reciprocal signaling resulting from increased glutamate release from mitral cells. In animals older than postnatal day 10, CCh appears to modulate transmitter release from dendrites of the interneurons themselves. Our results point to modulation of inhibition as an important role for cholinergic signaling in the OB. Our data also strengthen the emerging idea of a role for store calcium in modulating transmitter release at CNS synapses.

INTRODUCTION

Recent studies have suggested that action potential–independent transmitter release in the form of miniature excitatory and inhibitory currents (mEPSCs and mIPSCs, respectively), long considered oddities of electrophysiological recordings of synaptic transmission, might be physiologically relevant in their own right. These events might mediate changes in synaptic plasticity and presynaptic action potential–independent forms of synaptic transmission (Sharma and Vijayaraghavan 2003; Shigetomi and Kato 2004; Sutton et al. 2004). This viewpoint is further strengthened by the finding that miniature currents and action potential–evoked responses might be differentially, often oppositely, regulated (Mackler and Reist 2001; Schoch et al. 2001; Sugita et al. 2001; Washbourne et al. 2002). Thus there is a strong rationale for examining physiological control of mEPSCs and mIPSCs.

One mechanism by which miniature release at terminals can be modulated is by the release of endoplasmic reticulum (ER) store calcium. The presence of ER on presynaptic terminals, its proximity to release sites, and the existence of calcium release machinery have been documented (Bouchard et al. 2003; Verkhratsky 2005). The importance of this mechanism is slowly gaining acceptance (Collin et al. 2005). Calcium-induced calcium release (CICR) by the activation of ryanodine receptors has been shown to regulate mIPSC amplitudes in cerebellar purkinje neurons (Llano et al. 2000) and both mEPSC frequency and amplitude are changed by the activation of nicotinic acetylcholine receptors (nAChRs) on mossy fiber terminals of the rat hippocampus (Sharma and Vijayaraghavan 2003). Although the existence of G-protein–coupled receptors at presynaptic terminals is known, reported instances of transmitter release being modulated by an inositol 1,4,5-triphosphate receptor (IP3R)–mediated store calcium release are rarer (Li et al. 1998). On the other hand, there is no convincing demonstration of a role for ER calcium release in directly modulating responses in CNS neurons evoked by action potentials (Carter et al. 2002; Galante and Marty 2003).

Action potential–independent synaptic communication has been demonstrated and might be physiologically relevant at the dendrodendritic synapses between mitral cells and granule cells in the olfactory bulb (OB) (Isaacson and Strowbridge 1998; Nicoll and Jahr 1982; Schoppa et al. 1998). At these synapses, depolarization of mitral cells in the presence of tetrodotoxin (TTX) can lead to glutamate release, which in turn results in γ-aminobutyric acid (GABA) release from granule cells mediated by N-methyl-D-aspartate receptors (NMDARs) and voltage-gated calcium channels (VGCCs) on dendritic spines. This form of communication is thought to be important for self-inhibition of mitral cells as well as lateral inhibition of nearby principal neurons by spread of depolarization across dendritic spines (Isaacson and Strowbridge 1998). The possibility that ER store calcium might contribute to signaling at these synapses, where action potential–independent transmission has been well documented, remains to be investigated.

In rodents, ACh plays an important role in olfactory system function and plasticity, and in the modulation of olfactory perceptual learning (for review see Wilson et al. 2004). The existence of extrinsic cholinergic innervation of the OB from the basal forebrain has been documented (Macrides et al. 1981). Immunocytochemical studies demonstrate that cholinergic afferents innervate both the glomerular layer and the...
external plexiform layer, leading to the postulation of complex roles for cholinergic transmission in the OB (Castillo et al. 1999).

In this study, we ask whether release of ER store calcium plays a role in cholinergic modulation of transmitter release in the rat main OB. We demonstrate that carbamylcholine (CCh) enhances the frequency of mIPSCs recorded from mitral cells in bulb slices. The action of CCh is mainly at dendrodendritic synapses in the bulb. This effect is by the activation of muscarinic ACh receptors (mACHRs) and is mediated by the release of ER store calcium. We further show that the effect of CCh changes during development from enhancing glutamate release from mitral cell dendrites, leading to a glutamate receptor–mediated increase in GABA release, to the direct enhancement of GABA release from interneurons.

M E T H O D S

Slice preparation

Main olfactory bulbs from 9- to 17-day-old 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 CaCl₂, and 2.5 MgCl₂. Horizontal slices (350 μM thick) were cut in the same solution by sticking the ventral surface of each separated bulb to the stage of a Leica VT 1000S slicer (Leica, Cambridge, MA) or an EMS 4500 slicer (EMS, Hatfield, PA) in front of a rectangular block of 2% agarose with the outer lateral side of the bulb facing the slicer blade. The slices were incubated submerged, or at an air–aCSF interface, in a chamber containing oxygenated high Mg²⁺ aCSF at 32°C for approximately 30 min, after which the chamber was transferred to room temperature (RT, 20–24°C). For recording, slices were perfused at 1–2 ml/min with oxygenated, standard aCSF at RT containing (in mM) 120 NaCl, 3.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 2.5 CaCl₂, and 1 MgCl₂ along with 0.5–1 μM TTX. This was our base extracellular solution used to prepare all other extracellular solutions for recording purposes. Cells were visualized on a Zeiss axioskop FS or an Olympus BX50WI microscope, using a 40× water immersion objective and differential interference contrast optics. Mitral and granule cells were identified based on morphology and location. All experiments were done at room temperature.

Voltage-clamp recordings

Whole cell voltage-clamp recordings were made from mitral and granule cells using methods described in the literature (Hamill et al. 1981) and from our lab (Sharma and Vijayaraghavan 2001, 2003). Patch pipettes were pulled from borosilicate glass capillaries (G150-4, 1.981) and from our lab (Sharma and Vijayaraghavan 2001, 2003). Voltage-clamp recordings were acquired and stored on a Pentium PC using PClamp 7 (Molecular Devices). Access resistance (Rₐ) was monitored periodically to evaluate the quality of the recordings. Series resistance was not compensated. Drug solutions prepared in base extracellular solution were bath-applied by switching the perfusion reservoirs of a homemade, gravity-driven perfusion system.

Thapsigargin (TG) was obtained from Molecular Probes (Eugene, OR), TTX was obtained from EMD Biosciences (San Diego, CA) or Alomone Labs (Jerusalem, Israel). All other reagents were from Sigma (St. Louis, MO).

Data and statistical analysis

Data were acquired in gap-free mode for 120–300 s. Several such files were recorded for each condition. The data were then exported to the Mini-analysis program (Synaptosoft). The SD of noise (σᵥ) was estimated using a suitable eventless stretch of data (512–1,028 data points). The program detects mIPSCs using several parameters of a unitary IPSC of which we modified the amplitude and area thresholds, keeping the other default parameters of γ-aminobutyric acid type A (GABA_A)-mediated fast IPSCs in the routine. The amplitude threshold was set to 5σᵥ and the area threshold was set to 53σᵥ. After automatic detection of events by the program, we rescanned the event files for obvious detection errors and deleted those. The program was used to generate event lists and frequency histograms of event amplitude and interevent intervals, which were further processed, and final plots were made in SigmaPlot 2001 (SPSS, Davis, CA) or MicroCal Origin software (MicroCal, Northampton, MA). All averages are expressed as means ± SE. Statistical significance of data averages was determined using Student’s t-test. All distributions were compared using the nonparametric Kolmogorov–Smirnoff (KS) test.

Intracellular fills with Lucifer yellow

To determine the ratio of mitral cells with intact versus cut glo- meral tufts on primary dendrites in our experiments, we filled a few mitral cells with 0.25 or 0.5% Lucifer yellow dye through the patch pipette. The dye was dissolved in the CsCl pipette solution and diluted to its final concentration using a hypotonic pipette solution containing 100 mM CsCl. The cells were filled for a minimum of 20 min and the live slices were subsequently imaged using Zeiss Axioskop 2 FS fitted with a 40× water immersion objective. A 150-W xenon arc lamp was used for the excitation light. Excitation wavelength was obtained with a 485-nm filter (Chroma) and emission was collected using a 505-nm LP filter (Chroma). Images were collected using a Cooke Sensicam pelitter-cooled camera (PCO, Kelheim, Germany). Images were acquired and analyzed using SLIDEBOOK software (Intelligent Imaging Innovations, Denver, CO). A series of z-sections were acquired at 0.5-μm intervals and were compressed to a two-dimensional projection image.

R E S U L T S

CCh causes a prolonged increase in the frequency of GABA_A-mediated mIPSCs in mitral cells

We performed whole cell voltage-clamp recordings of mIPSCs from mitral cells in 350-μm-thick horizontal OB slices during bath perfusion of CCh in aCSF containing 0.5–1 μM TTX. CCh (5–50 μM) increased mIPSC frequency in mitral cells held at −70 mV using a pipette filled with a CsCl-based internal solution (see METHODS, representative experiment in Fig. 1A). Cumulative interevent interval distributions for each recorded cell showed consistent shifts to the left in the presence of CCh (Fig. 1B, P < 10⁻⁴; no P value was
The frequency increase reversed on washing with control aCSF for approximately 15 min (Fig. 1E) and could be reelicited from the same cell on a subsequent second application of CCh (data not shown). No events were observed when CCh was applied along with 20 μM bicuculline (Fig. 1A) and the currents reversed at the chloride-reversal potential (see Methods for \( E_{\text{Cl}} \)). This indicated all the events observed were GABA\(\text{A} \)-mediated mIPSCs. The mIPSC frequency increased on CCh application in mitral cells held at \(-50 \text{ mV} \) with pipettes containing potassium gluconate–based internal solution (see Methods). These currents were outward in accord with the chloride reversal potential (Fig. 1F).

On continuous application of CCh, mIPSC frequency remained elevated for a considerable period. From five cells where recordings were continued \( \approx 1 \text{ h} \), the response decayed slowly with a 1/2 of \( 35 \pm 6 \text{ min} \) (\( n = 5 \); Fig. 2, A and B), giving an approximate decay time constant of 24 min. This observation conveniently allowed us to make statistically significant comparisons of mIPSC frequency changes produced in single cells by various experimental manipulations within one time constant.

**CCh increases mIPSC frequency by activating M1 mACHRs**

The rat main OB expresses ionotropic nACHRs along with G-protein–coupled mACHRs (Le Jeune et al. 1995, 1996) in all the laminae at varying densities. To identify the AChR type responsible for the increased mIPSC frequency we applied mACH and nACH blockers to the slice along with CCh. The mACHR antagonist atropine (10 μM), applied along with CCh, shifted the interevent interval distribution significantly to the right (\( P < 0.0001 \), CCh vs. CCh + atropine, \( n = 2 \) cells; Fig. 3D). Pirenzepine (10 μM), an M1 subtype-selective mACH antagonist, also blocked the increase in mean mIPSC frequency from 6.6 ± 0.3 times control in CCh alone to 1.5 ± 0.3 times (Fig. 3A, bottom trace, PZ + CCh, B and D, all \( P < 0.0001 \), KS test, three cells). On the other hand, 50 μM mecamylamine (mec, a nonspecific nAChR antagonist at this concentration) applied along with CCh showed no significant effect on the ability of CCh to increase mIPSC frequency (3.4 ± 1.5 vs. 3.9 ± 2.0, \( n = 3 \) cells, CCh vs. CCh + mec; \( P > 0.4 \); Fig. 3, C and D). No transient inward current attributable to the activation of nAChRs on mitral cells was observed, presumably because of the low affinity of CCh for nAChRs or desensitization on slow bath application of the agonist. These results suggest that the action of CCh on GABA release is mediated by M1 mACHRs.

**The effects of CCh involve the release of calcium from ER stores**

Because a common transduction mechanism of M1 mACHRs is the activation of phospholipase C, generating diacylglycerol and IP\(\text{3} \) as second messengers, we asked whether actions of CCh might involve release of store calcium through the activation of IP\(\text{3} \)Rs.

The effects of CCh persisted when external calcium was removed. In the absence of external calcium (calcium in aCSF substituted with 0.5 mM EGTA), there was a significant shift to the left of the interevent histogram (\( P < 0.0001 \) in three
cells, \( P = 0.02 \) in the fourth) demonstrating that the changes in mIPSC frequency were independent of calcium flux from the external medium. In the absence of external calcium, CCh increased mean mIPSC frequency by 4.9 ± 2.2-fold (\( n = 4 \); compare with 3.9 ± 0.6-fold increase seen with CCh in 2 mM calcium, \( n = 12 \)). Prolonged exposure of slices to CCh in zero calcium was not attempted, precluding accurate determinations of the duration of the frequency increase under these conditions. What our results clearly indicate is that calcium influx is not required for CCh to enhance GABA release, consistent with a role for mAChRs (Rathouz et al. 1995) and also consistent with data from Fig. 3 showing no discernible role for the ionotropic nAChRs.

We then asked whether the increase in mIPSC frequency was dependent on ER calcium. We depleted calcium from the ER either by coapplication of 5 \( \mu \)M thapsigargin (TG) and 50 \( \mu \)M CCh or by prior incubation of the slice with 5 \( \mu \)M TG for 30 min. Results are shown in Fig. 4. Coapplication of CCh with TG significantly shifted the mini-interinterval distribution to the right (\( n = 3 \) cells, \( P < 0.0001 \) in two cells, \( P = 0.002 \) in the third; Fig. 4, A, B, and D, TG1). Preincubation of the slices with TG completely abolished CCh-mediated mIPSC increases (0.75 ± 0.04-fold of the control, \( P > 0.1, n = 3 \) cells; Fig. 4, C and D, TG2). If calcium released from stores modulates release probabilities then TG would transiently increase mIPSC frequency that would return to baseline on store depletion, as shown in our previous work (Sharma and Vijayaraghavan 2003). On the other hand, if release is modulated by capacitative calcium entry on store depletion, one would expect to see a sustained increase in basal mIPSC frequency on TG treatment (Emptage et al. 2001). Application of TG caused short-lived bursts of mIPSCs (Fig. 4, E and F), which died over time, arguing for direct action of calcium release from ER on mIPSC frequencies. Although the overall mean frequency in TG showed no significant change, there was a threefold increase in the peak frequency (defined as the maximal frequency seen in any one bin, \( P < 0.03 \); Fig. 4G). Arguing against a direct involvement of capacitative calcium entry is the finding that in slices preincubated with TG, mIPSCs were observed...

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**FIG. 2.** mIPSC frequency remains elevated in response to long duration of CCh application. A: whole cell current traces at −70 mV from a mitral cell before and at increasing time intervals during bath application of 50 \( \mu \)M CCh. B: plot of mIPSC frequency against time, data from the cell in A. Thick line above shows duration of CCh application and the thin line superimposed on the decaying phase of the plot is a fit to a single exponential function with a \( t_{1/2} \) of 42.6 min and a calculated \( t \) of 25.5 min.

**FIG. 3.** CCh increases mIPSC frequency by activating muscarinic acetylcholine receptors (mAChRs). A: current traces recorded from a mitral cell in aCSF (top; control), 50 \( \mu \)M CCh (middle; +CCh), and 50 \( \mu \)M CCh with 10 \( \mu \)M pirenzepine (bottom; PZ + CCh). B: cumulative interevent distributions from the same cell in aCSF (solid line), 50 \( \mu \)M CCh (dashed line), and 50 \( \mu \)M CCh + 10 \( \mu \)M pirenzepine (dotted line); the interevent intervals at 0.5 probability were 1.6 s (0.6 Hz), <0.3 s (3.3 Hz at 0.57 probability), and 1.8 s (0.5 Hz), respectively. C: current traces recorded from another mitral cell in the presence of 50 \( \mu \)M CCh (top) and 50 \( \mu \)M mecamylamine with 50 \( \mu \)M CCh (bottom). D: mean fold change in mIPSC frequency in the conditions given above. Fold change was calculated as a ratio of number of events in each condition to the appropriate control. Asterisk indicates statistical significance (\( P = 0.01 \), paired t-test).
with an average frequency of \(0.6 \pm 0.07\) Hz, comparable to that seen in control conditions \(0.8 \pm 0.1\) Hz.

**CCh increases mIPSC frequency onto lateral dendrites of mitral cells**

Mitral cells receive GABAergic input at their primary (glomerular tufts), as well as at their lateral dendrites. We tried localizing the CCh effect, and consequently the AChRs responsible, to either one or both of these loci by taking advantage of the fact that mitral cells sometimes lose their glomerular tufts during the slicing process. Thus we recorded from mitral cells with pipettes containing 0.25–0.5% Lucifer yellow in the CsCl intracellular solution and imaged the fluorescent cell to determine subsequently whether it had an intact glomerular tuft.

Because the position of the glomerular layer was easily identifiable from DIC images, this approach allowed us to obtain recordings from mitral cells that had intact primary dendritic tufts and from cells that did not. Both types of mitral cells showed increased mIPSC frequency in response to bath perfusion of 50 \(\mu\)M CCh (Fig. 5, A and B; \(P < 0.0001\) for 5A and \(P < 0.002\) for 5B). Although cells with intact glomerular tufts appeared to respond more strongly (mean mIPSC frequency in CCh was 4.8 \pm 1.3 times that in control, \(n = 4\)), cells with cut glomeruli also responded in a distinct manner to CCh (mean mIPSC frequency in CCh was 1.7 \pm 0.1 times that in control, \(n = 3\)). The difference in the magnitude of CCh response between cells with cut and uncut primary dendrites did not reach statistical significance \(P = 0.09\).

**FIG. 4.** Thapsigargin (TG) prevents increase in mIPSC frequency arising from CCh. \(A\): current traces recorded from a mitral cell in standard aCSF (top), 50 \(\mu\)M CCh (middle), and 5 \(\mu\)M TG (TG) applied along with 50 \(\mu\)M CCh (bottom). \(B\): interevent interval distributions from the same cell in aCSF (thick line), 50 \(\mu\)M CCh (dashed line), and 5 \(\mu\)M TG with 50 \(\mu\)M CCh (dotted line). Interevent intervals at 0.5 probability were 0.9 s (1 Hz), <0.3 s (3.3 Hz at 0.53 probability), and <0.8 s (1.2 Hz at 0.52 probability), respectively. \(C\): interevent interval distributions from another mitral cell preincubated in 5 \(\mu\)M TG for 30 min. Interevent interval at 0.5 probability was 1.3 s (0.7Hz) in standard aCSF (solid line) and 1.5 s (0.6 Hz) in 50 \(\mu\)M CCh. \(D\): mean fold change in mIPSC frequency in 3 cells each in the conditions described in \(A\). TG1 = TG coapplied with 50 \(\mu\)M CCh. TG2 = data from cells preincubated for 30 min in TG. \(E\): current traces recorded from a cell before (top trace) and during (bottom trace) bath application of 5 \(\mu\)M TG (+TG). Application of TG caused short-lived burst of mIPSCs. \(F\): frequency histogram for a cell during TG application. Note transient spikes of high-frequency events. \(G\): peak frequency in control and during TG application. TG treatment showed periods of up to 3-fold higher frequencies compared with control (\(n = 3\) cells; \(P < 0.03\)).
in the magnitude of CCh effects did not permit any conclusion regarding the relative contributions from the two sites. A parsimonious conclusion that can be made with confidence is that one site of CCh-mediated increase in mIPSC frequency is the dendrodendritic synapse between the granule cells and the mitral cell lateral dendrites.

The effects of CCh show a developmental shift in locus of action

Our data thus far suggest that CCh modulates GABA release in a manner consistent with M1 mAChR-dependent release of ER calcium. In the mitral cell–granule cell reciprocal synapse, GABA release can be modulated two ways. In the first case, M1 mAChRs, located on mitral cell dendrites, may increase glutamate release, which in turn could enhance GABA release by acting on glutamate receptors (GluRs) on granule cell spines (Isaacson and Strowbridge 1998; Nicoll and Jahr 1982; Schoppa et al. 1998). The second possibility is the direct action of M1 mAChRs, located on granule cell spines, on GABA release. We argued that if the first possibility was correct, then the effect of CCh should be attenuated in the presence of NMDAR and /H9251-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) blockers. The effects of CCh on mIPSC frequency were examined, in bulbs from 11- to 15-day-old animals, in the presence of 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 50 μM 2-amino-5-phosphonovaleric acid (APV) to block the AMPARs and NMDARs, respectively. The frequency changes induced by CCh in mitral cells with cut (3 cells) and intact (4 cells) primary dendrite. In both cases there was a significant increase in frequency over control (P < 0.01; paired t-test).
was 4.1 15-day-old rats, the average fold increase on CCh application tent finding. For example, in other experiments from 11- to effects was larger in the young animals, this was not a consis-
mitral cells only in younger animals. Although in these experiments the magnitude of CCh and the control being ceded to the interneurons in older the cholinergic effect being the mitral cell in younger animals are developmentally controlled, with the primary mediator of CCh effects attenuated CCh-mediated increases in mIPSC mEPSC frequency compared to 9-day-old rats, CCh increased mEPSC frequency compared DNQX-sensitive mEPSCs (data not shown). In slices from 7-

However, when we performed the same experiment with younger (7- to 9-day-old) animals, blocking ionotropic glutamate receptors attenuated CCh-mediated increases in mIPSC frequency (Fig. 6, A and C, n = 7, P = 0.005, CCh vs. CCh + APV + DNQX). These results suggest that the effects of CCh are developmentally controlled, with the primary mediator of the cholinergic effect being the mitral cell in younger animals and the control being ceded to the interneurons in older animals. Although in these experiments the magnitude of CCh effects was larger in the young animals, this was not a consistent finding. For example, in other experiments from 11- to 15-day-old rats, the average fold increase on CCh application was 4.1 ± 0.7-fold (range 1.3- to 9.6-fold, n = 15 cells).

Recordings from individual granule cells in slices bathed in 1 μM TTX and 100 μM picrotoxin revealed small APV + DNQX-sensitive mEPSCs (data not shown). In slices from 7- to 9-day-old rats, CCh increased mEPSC frequency compared with control (three out of three cells, P < 0.0001, KS test; Fig. 6D). In contrast, from animals aged 11–17 days only one out of four cells showed a significant leftward shift in the interevent interval distribution. In the other three cells, no change in the distribution was observed (P values between 0.3 and 0.9, KS test; Fig. 6E). These data are consistent with our conclusion that CCh mediates its actions by the release of glutamate from mitral cells only in younger animals.

Further evidence for a mechanistic shift during development comes from the examination of the effects of cadmium. The presence of 100 μM cadmium in the aCSF had no effect on the ability of CCh to mediate increases in mIPSC frequencies in 11- to 14-day-old animals (Fig. 7B; control vs. CCh P < 0.001, n = 4), consistent with our zero calcium data and a previously published report (Castillo et al. 1999). However, in 7- to 9-day-old animals, the presence of cadmium completely attenuated the response to CCh (Fig. 7A, control vs. CCh P = 0.69; n = 7) arguing for the importance of VGCCs in mediating the effects of CCh in younger animals, but not in older ones.

Similarly, the presence of 1 mg/ml heparin in the pipette to block IP3Rs attenuated CCh response from younger animals
DISCUSSION

Cholinergic actions on the OB are likely to be complex: nAChRs excite both mitral cells and periglomerular (PG) interneurons, whereas mAChRs inhibit the firing of granule cells and, at the same time, positively modulating GABA release from these neurons onto mitral cells (Castillo et al. 1999). Thus examining the mechanistic underpinnings of each of these cholinergic effects is an essential prerequisite to the understanding of the role cholinergic inputs into the bulb play in modulating its functions. Here we examine mechanisms underlying one effect of AChR activation: the increased action potential–independent release of GABA onto mitral cell dendrites. In this study, we demonstrate the following: 1) CCh causes a long-lasting increase in mIPSC frequency in mitral cells mediated by the activation of mAChRs. 2) This increase in mIPSC frequency persists in the absence of external calcium and is abolished by the depletion of ER store calcium. 3) The effects of CCh are primarily on mitral cells in young animals, whereas results from older animals are consistent with direct action of the agonist on granule cell dendritic spines.

CCh induced an increase in mIPSC frequency without a consistent change in amplitude. Leftward, rightward, or no shift in cumulative amplitude distribution was observed identical to results obtained by others (Castillo et al. 1999). As in that study, our results argue against a specific subpopulation of CCh-sensitive GABAergic synapses.

AChRs are widespread throughout the OB, suggesting complex roles in odorant detection and processing. Expression of mRNAs for M1, M3, and M4 mAChRs have been reported in the rodent OB (Buckley et al. 1988) as well as those for α3, α4, α5, α7, β2, and β4 nAChR subunits (Keiger and Walker 2000). Quantitative autoradiographic studies demonstrate the presence of M1 and M2 mAChR subtypes essentially in all layers of the bulb (Le Jeune et al. 1996). Localization studies show that among nAChRs, the α7-containing receptor subtypes are concentrated in the glomerulus (Hunt and Schmidt 1979; Le Jeune et al. 1996), whereas the α4β2 subtype was more widespread with a few glomeruli in the caudal bulb showing high expression (Le Jeune et al. 1996). There is functional evidence for the existence of α4β2 nAChRs on mitral cells (Castillo et al. 1999) and M1 mAChRs in the bulb (Olianas and Onali 1993).

One ultrastructural study showed the M2 subtype of mAChRs widely expressed on granule cell dendrites (Crespo et al. 2000). Although this finding might implicate M2 mAChRs in the CCh-mediated increase in GABA release, it seems unlikely that these receptors mediate the effect reported in this study. First reported presynaptic actions of M2 mAChRs point to a major inhibitory role in transmitter release for this subtype (Li et al. 2004; Trendelenburg et al. 2003; Zhang et al. 2005). Second, both pharmacological evidence and the underlying signal transduction mechanism are consistent with mediation by M1 receptors. CCh-mediated increase in mIPSC frequency was blocked by pirenzepine, an antagonist that convincingly differentiates M1 and M2 receptor subtypes. The effect on mIPSCs was further blocked by depleting ER store calcium, and by blocking IP₃Rs with intracellular heparin, consistent with an IP₃-mediated M1 mAChR effect rather than a Gₛ-coupled M2 mAChR action.
Most reported mAChR effects on synaptic transmission are inhibitory, presumably by their action on presynaptic voltage-gated calcium channels (Boehm and Huck 1997; Nicoll et al. 1990). Indeed, mechanisms underlying presynaptic metabotropic receptor-mediated increases in transmitter release, in general, are not very clear. The mediated M1 mAChRs that increased in mIPSC frequency could potentially be explained by two possible mechanisms: 1) by inhibition of M-currents (Lechner et al. 2003) and 2) by release of store calcium by an IP3,R mechanism. The involvement of store calcium in CCh-mediated increase in mIPSC frequency was demonstrated by both the independence of the effect from external calcium and cadmium, as well as our finding that depleting store calcium with TG completely abolished CCh-mediated increase in mIPSC frequency. Although the presence of cesium in the recording pipette and the lack of a role for VGCCs in older animals would argue against an M-current–mediated mechanism responsible for changes in mIPSC frequencies, whether there is a contribution from these channels under more physiological conditions needs to be investigated further. This is especially relevant in light of recent studies implicating phospholipase C activation in the modulation of M-currents (Horowitz et al. 2005; Suh and Hille 2002).

The sustained increase in mIPSC frequency by CCh could be attributable to long-lived calcium oscillations. Because the fraction of ER calcium released in a single calcium transient is very small, one could postulate that sustained effects could be obtained even in the absence of calcium influx (Inoue et al. 2004). However, the alternate possibility—that calcium release from ER activates some downstream cascades responsible for the duration of CCh effects—cannot be ruled out at present. Calcium imaging studies currently under way will help resolve this issue.

The role of ER store calcium in modulating synaptic transmission is far from clear, although the presence of ER at presynaptic terminals is known (Bouchard et al. 2003; Padua et al. 1996). Although some studies have suggested a role for store calcium in modulating synaptic plasticity (Emppte et al. 2001; Lauri et al. 2003; Narita et al. 2000), others have called these conclusions to question (Carter et al. 2002). Inconsistent reports from the literature show that normal evoked transmission requires contribution from ER calcium stores (Collin et al. 2005). On the other hand, dramatic effects of store calcium manipulation have been observed on mEPSC frequencies (Sharma and Vijayaraghavan 2003; Smith and Cunnane 1996) and store-mediated calcium release causes synchronization of transmitter release leading to large multivesicular mIPSCs (Llano et al. 2000) and mEPSCs (Sharma and Vijayaraghavan 2003). These findings, along with others showing differential regulation of mIPSCs and evoked responses (Mackler and Reist 2001; Schoch et al. 2001; Sugita et al. 2001; Washbourne et al. 2002), make it tempting to speculate that ER calcium might be a selective mechanism for control of action potential–independent transmitter release. There has recently been considerable excitement about the possibility that synchronization of spontaneous release might represent an action potential–independent form of transmission across CNS synapses (Sharma and Vijayaraghavan 2003; Shigetomi and Kato 2004).

Most studies implicate presynaptic ryanodine receptors and CICR in modulating transmitter release. Much less is known about signaling involving presynaptic IP3,Rs (Collin et al. 2005), mainly because of the absence of specific pharmacological tools to examine this pathway. Most agents inhibiting IP3,Rs also affect other calcium signaling pathways (Ehrlich et al. 1994). Heparin, a drug commonly used as a blocker of IP3,Rs (Sezikawa and Bonham 2005; Wang et al. 2005), when added in the patch pipette, blocks CCh-mediated mIPSC frequency changes in young animals. This result suggests that IP3,Rs are involved in mediating CCh effects at least in animals before postnatal day 10. The existence of presynaptic metabotropic receptors known to signal by phospholipase C and studies like this one showing modulation of transmitter release by these receptors clearly provide evidence implicating IP3,Rs. Whether calcium release by IP3,Rs becomes further amplified as a result of CICR in this preparation remains to be seen.

The control of spontaneous release by store calcium opens up some interesting avenues for investigation. First, the relative location of the stores with respect to release sites and the spread of released calcium, controlled by diffusion and buffering, need to be examined in better detail. Second, the question whether the population of vesicles released by store calcium is the same as that released by an invading action potential needs to be resolved. Recent findings suggesting that proximity to release sites might not be the determinant of whether a vesicle can be released (Rizzoli and Betz 2004, 2005) and the possible existence of a pool of vesicles that drives spontaneous transmission (Sara et al. 2005) lends credence to the idea that different sources of calcium might release different population of vesicles.

Evidence presented in our study demonstrates that the effects of CCh on mIPSC frequencies are mediated by its action on the dendrodendritic synapses between mitral cells and interneurons. Although our results also suggest the involvement of GABAergic PG cells in the muscarinic effects on mIPSCs, the extent of their involvement is not clear. If CCh-mediated changes in inhibitory drive at the glomerulus are significant, then together with the proposed existence of AChRs on dopaminergic PG cells (Nickell and Shipley 1988), the net consequence of muscarinic modulation in the glomerular layer might be to reduce the strength of the olfactory input (Hsia et al. 1999).

At the lateral dendrites of the mitral cells, M1 mAChRs might modulate the strength of self-inhibition mediated by the mitral cell–granule cell synapse. In other words, cholinergic input, by mAChRs, might be involved in setting the gain of this circuit. Combined with possible reduction in the strength of the olfactory input, such an arrangement might serve to lower detection sensitivity to increase specificity. The sustained nature of the inhibitory effects mediated by mAChRs favors this idea of global thresholds, although—until the exact nature of the endogenous signaling is revealed—such ideas remain speculative.

The reciprocal nature of this synapse suggested two possible sites of action for CCh, the first possibility being a direct action of CCh on ER stores located on granule cell spines. Most of the data presented in this work speak to this possibility. However, a surprising finding from our experiments using AMPA and NMDA receptor blockers was that early in development (between postnatal days 7 and 9) most of the CCh-mediated increases in mIPSC frequencies could be blocked by glutamate receptor antagonists. This difference in mAChR signaling during development was also demonstrated in other ways. In
young animals, CCh effects were abolished in the presence of intracellular heparin to block IP$_{3}$Rs or extracellular cadmium to block VGCCs. In addition, recording from granule cells at these ages showed a CCh-mediated increase in glutamatergic mEPSC frequencies. Taken together, these results imply that the increase in mIPSC frequencies in young animals arises from an increased glutamate release from mitral cell dendrites, resulting from mACHR- and IP$_{3}$R-mediated calcium release from ER stores. The released glutamate, in turn, enhances GABA release at granule cell spines in a GluR- and VGCC-dependent fashion. In this context, it has been shown that VGCCs play a significant role in reciprocal signaling at these synapses (Halabisky et al. 2000; Isaacson 2001; Isaacson and Strowbridge 1998). Whether there is an age-dependent change in the relative contributions of NMDARs and VGCCs to this reciprocal signaling remains to be investigated.

Data from the older animals (i.e., insensitivity to GluR blockers, removal of external calcium, heparin dialysis of mitral cells, and to VGCC blockade) suggest that at these ages the primary determinant of CCh effects on mIPSCs is store calcium release from granule cell spines.

Whether this developmental switch for cholinergic control of GABA release from the OB around day 10 of postnatal development implies redistribution of M1 mACHRs or a switch in the transduction pathway remains to be seen. Distribution studies of [H]$^{3}$]pirenzepine binding show a dramatic increase in binding during the first few postnatal days, peaking at day 10. However, this developmental profile appears to be more or less uniform throughout the bulb (Le Jeune et al. 1996). On the other hand, distribution of IP$_{3}$Rs in the developing OB is consistent with our findings on the effect of CCh. Using polyclonal IP$_{3}$R antibodies it has been shown that in the first postnatal week, IP$_{3}$Rs are almost exclusively expressed in mitral cell soma and dendrites. By the second week, there is a dramatic decline of the receptor in mitral cells with a large concomitant increase of the receptor expression in granule cells and PG cells. By the end of the third postnatal week expression is almost exclusively in these interneurons (Slawek et al. 1997). From our results, it appears that control of CCh-mediated mIPSC increase is ceded to the interneurons from mitral cells by the second postnatal week, making it tempting to speculate that the location of IP$_{3}$R signaling might determine the site of mACHR action in controlling GABA release from granule cell spines. This explanation would be true whether the developmental changes are a result of the disappearance of CCh effects from mitral cells and its appearance on granule cells at 11–15 days of postnatal development or that effects on mitral cells predominate at earlier stages, shutting down later.

Interestingly, this developmental switch coincides with the end of the sensitive period for odor-dependent imprinting in neonatal rats. Although norepinephrine is the main transmitter implicated in this switch (Moriceau and Sullivan 2004), it is likely that the cholinergic system plays a role as well.

Cholinergic projections to the olfactory bulb are important in some forms of odor discrimination. It has been shown that inhibition of bulb mACHRs results in the loss of perceptual learning, i.e., the ability of animals to learn to discriminate closely related odors (Wilson et al. 2004). A consequence of the mACHR-mediated increase in GABA release needs to be worked out. Dendrodendritic GABAergic signaling is the key determinant of olfactory nerve evoked γ oscilations in the bulb (Lagier et al. 2004). These oscillations have been shown to be important for odor discrimination (Stopfer et al. 1997) and because increased GABA release would increase the amplitude of these oscillations, such mechanisms might play a role in cholinergic effects on perceptual learning. It remains to be demonstrated that mACHR modulation of GABA release alters the strength of this oscillation, as has been suggested (Hall and Delaney 2001). Our findings reveal the mechanistic bases of one of the effects of a transmitter system likely to have important consequences for signal processing in mammalian olfaction. Further, our results suggest an important role for store calcium in mediating these effects.

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