Neuron, Vol. 37, 639–647, February 20, 2003, Copyright ©2003 by Cell Press

Presynaptic Cyclic Nucleotide-Gated Ion Channels Modulate Neurotransmission in the Mammalian Olfactory Bulb

Gabe J. Murphy and Jeffry S. Isaacson*
Neuroscience Graduate Program and
Department of Neuroscience
University of California, San Diego
School of Medicine
La Jolla, California 92093

Summary

Cyclic nucleotide-gated channels (CNGCs) on the dendritic cilia of olfactory receptor neurons (ORNs) are critical for sensory transduction in the olfactory system. Do CNGCs also play a role in the axons and/or nerve terminals of ORNs? We find that the cyclic nucleotides cAMP and cGMP can both facilitate and depress synaptic transmission between olfactory nerve fibers and their targets in olfactory bulb glomeruli. Cyclic nucleotides increase intracellular Ca^{2+} in ORN terminals and enhance spontaneous transmitter release; at higher concentrations, cyclic nucleotides depress evoked transmission by altering olfactory nerve excitability. Cyclic nucleotides have no effect on transmission or nerve excitability, however, in mice lacking olfactory CNGCs. Taken together, our results identify a novel role for presynaptic CNGCs in modulating neurotransmission.

Introduction

Cyclic nucleotide-gated channels (CNGCs) play a critical role in the transduction of visual and olfactory stimuli. Light-mediated decreases in intracellular cGMP lead to membrane hyperpolarization in photoreceptors by reducing CNGC activity (Yau and Baylor, 1989). In olfactory receptor neurons (ORNs), stimulation of G protein-coupled odorant receptors leads to production of cAMP by adenylate cyclase. The subsequent increase in cAMP depolarizes ORNs via CNGC activation (Nakamura and Gold, 1987).

In addition to governing sensory transduction, CNGCs may also play a broader role in the central nervous system. In the inner segment of cone photoreceptors, a cGMP-gated current can influence vesicle release (Rieke and Schwartz, 1994). Olfactory CNGCs are thought to be expressed in the hippocampus, and cyclic nucleotides activate channels in inside-out patches from cultured embryonic hippocampal neurons that exhibit CNGC-like properties (Bradley et al., 1997). CNGCs have also been suggested to contribute to plateau potentials induced by muscarininc receptor activation in hippocampal pyramidal cells (Kuzmiski and MacVicar, 2001).

Recent studies suggest that CNGCs (Matsuzaki et al., 1999) and odorant receptors (Mombaerts et al., 1996; Wang et al., 1998) are also expressed in the nerve terminals of ORNs, where they have been proposed to contribute to the remarkably precise targeting of ORN axons to specific olfactory bulb glomeruli. However, a physiological role for either of these sensory transduction elements in ORN nerve endings has not been established. Here we asked whether CNGCs modulate transmitter release from olfactory nerve terminals in the olfactory bulb. We show that cyclic nucleotides can both depress and facilitate synaptic transmission by activating presynaptic CNGCs.

*Correspondence: jisaacson@ucsd.edu

Results

Olfactory nerve (ON) terminals release glutamate onto the dendrites of principal mitral/tufted (M/T) cells and local interneurons, periglomerular (PG) cells, in olfactory bulb glomeruli. We first examined whether cyclic nucleotides modulate glutamate release from ON terminals. We focused on excitatory postsynaptic currents (EPSCs) in PG neurons because their electrotonic properties permitted better voltage clamp conditions compared to M/T cells. Bath application of forskolin (25–50 μM), an adenylate cyclase activator, largely abolished ON-evoked EPSCs in PG neurons in the presence of the phosphodiesterase inhibitor IBMX (50–100 μM; Figure 1A1). The actions of forskolin on evoked transmission were mimicked by high concentrations of the membrane-permeable cGMP analog 8-Br-cGMP (400–500 μM; Figure 1A2). Dideoxyforskolin (50 μM), an inactive analog that does not stimulate adenylate cyclase, had no effect on ON-evoked EPSCs (data not shown, n = 4). Rp-8-pCPT-cGMP (500 μM), a membrane-permeable cGMP analog that does not activate olfactory CNGCs (Kramer and Tibbs, 1996) was also without affect (data not shown, n = 3). Forskolin and 8-Br-cGMP inhibited ON-evoked EPSCs in tufted cells (data not shown, n = 5), suggesting that the effects of cyclic nucleotides on olfactory nerve-mediated synaptic transmission were independent of the postsynaptic cell type. These data indicate that high concentrations of cAMP and/or cGMP inhibit olfactory nerve-mediated synaptic transmission.

Paired pulse stimulation (75–100 ms interval) of ON fibers produced a marked depression of the amplitude of the second EPSC relative to the first (paired pulse ratio; PPR = 0.39 ± 0.07, n = 11). Manipulations that modulate the PPR typically indicate presynaptic changes in transmitter release probability (Zucker and Regehr, 2002). Neither forskolin nor 8-Br-cGMP altered the PPR of ON-evoked EPSCs (Figure 1A3). In contrast, baclofen (50–100 μM), a $GABA_B$ receptor agonist that reduces the probability of transmitter release from ON fibers (Aroniadou-Anderjaska et al., 2000), dramatically increased the PPR (Figure 1A3). These results suggest that high concentrations of cyclic nucleotides depress ON-evoked transmission through a mechanism distinct from a conventional reduction in release probability.

The cyclic nucleotide-mediated depression of evoked transmitter release was accompanied by a large increase in the frequency of spontaneous EPSCs (sEPSCs; forskolin = 6.9- ± 2.2-fold, n = 5; 8-Br-cGMP = 9.4- ± 4.1-fold, n = 6; Figure 2A). We observed that cyclic

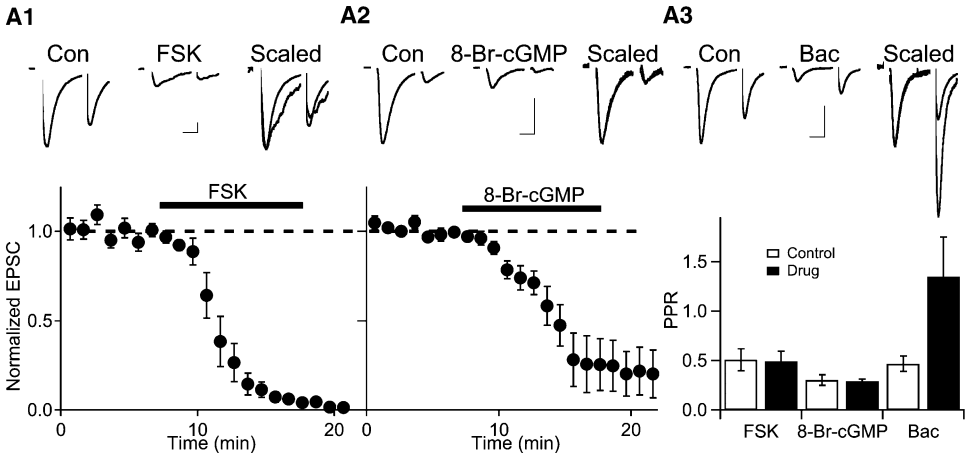

Figure 1. High Concentrations of Cyclic Nucleotides Inhibit Evoked Transmitter Release from Olfactory Nerve Terminals

(A1) Forskolin (50 μM) blocks ON-evoked EPSCs in the presence of IBMX (50–100 μM). Top, one experiment showing paired pulse depression under control conditions (Con) and in forskolin (FSK). Traces were normalized to the amplitude of the first EPSC and superimposed (Scaled). Bottom, summary of the actions of forskolin on the first EPSC (n = 5).

(A2) 8-Br-cGMP (500 μM) inhibits ON-evoked EPSCs. Top, traces from one experiment. Bottom, summary (n = 6).

(A3) Baclofen (Bac, 50–100 μM) inhibits the EPSC and alters the PPR. Top, traces from one cell. Bottom, summary of PPR results (n = 6). Forskolin and 8-Br-cGMP did not change the PPR ($p > 0.5$); baclofen caused a significant increase in the PPR ($p < 0.035$). Baclofen inhibited the amplitude of the first EPSC by 63.7% ± 9.5% (n = 6). Scale bars: 100 pA (vertical), 10 ms (horizontal). Stimulus artifacts and ~50 ms between the paired pulses have been removed.

nucleotide application often increased the sEPSC frequency before the evoked EPSC was blocked (Figure 2A). However, there was no correlation between the relative increase in sEPSC frequency and inhibition of evoked EPSCs in the same cell ($R^2 = 0.096$). The cyclic nucleotide-mediated increase in sEPSC frequency persisted in the presence of tetrodotoxin (TTX; 1 μM) and Cd^{2+} (100 μM, Figure 2B1), indicating that these events were miniature EPSCs (mEPSCs) (Katz, 1969). Although forskolin and 8-Br-cGMP dramatically increased mEPSC frequency ($p < 0.04$ and $p < 0.03$, respectively), cyclic nucleotides did not affect mean mEPSC ampli-

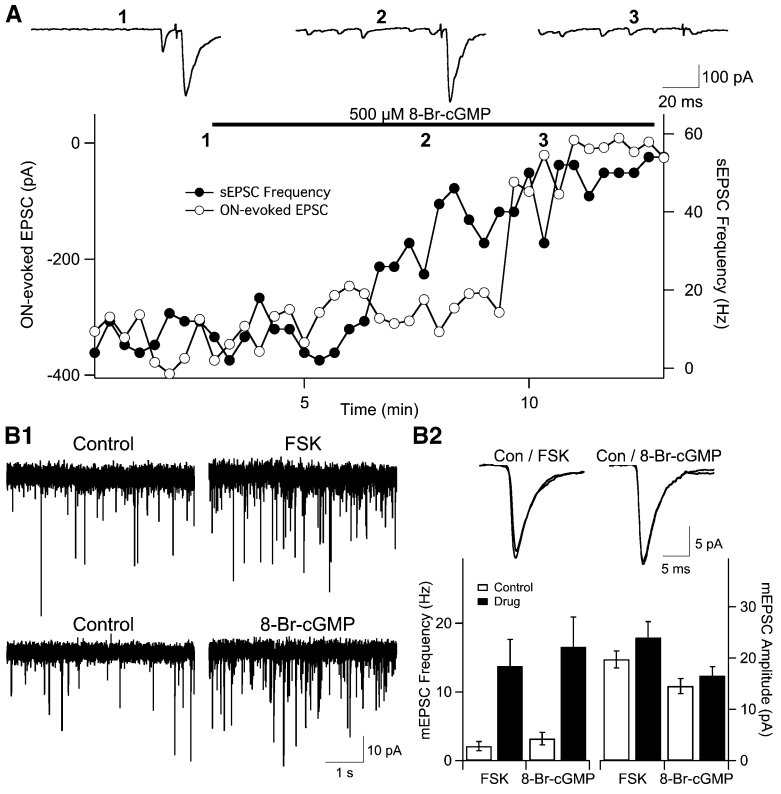

Figure 2. Cyclic Nucleotides Enhance Spontaneous Glutamate Release

(A) Representative experiment illustrating the actions of 8-Br-cGMP on sEPSC frequency (closed circles) and evoked EPSC amplitude (open circles). Top, single traces from time points indicated on plot.

(B) Cyclic nucleotides alter the frequency but not amplitude of mEPSCs recorded in the presence of Cd^{2+} (100 μM) and TTX (1 μM).

(B1) Representative traces before and 5 min after cyclic nucleotide application.

(B2) Top, averaged mEPSCs (>100 events) from two cells before (Con) and after forskolin (left) or 8-Br-cGMP (right) are superimposed. Bottom, summary of the effects of forskolin (n = 7) and 8-Br-cGMP (n = 5) on mEPSC frequency (left) and amplitude (right).

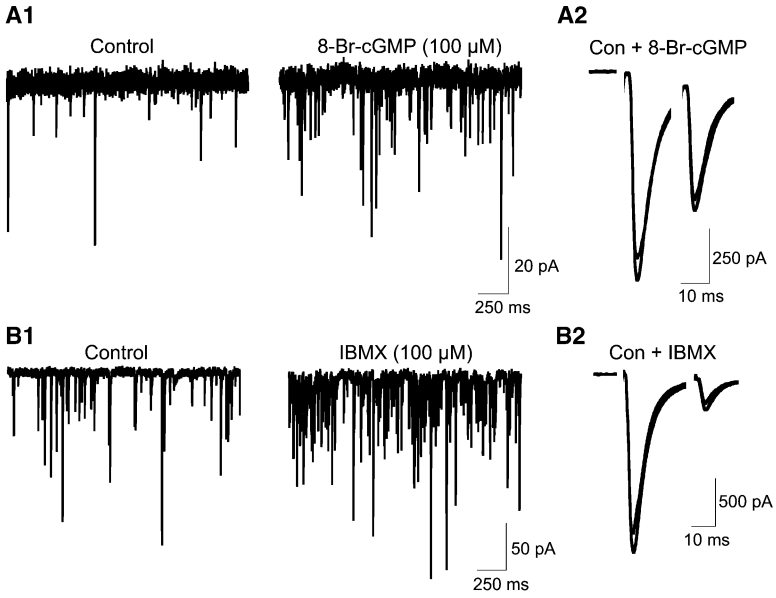

Figure 3. Low Concentrations of Cyclic Nucleotides Enhance Spontaneous Glutamate Release but Do Not Alter Nerve-Evoked Transmission

(A1) 8-Br-cGMP (100 μM) increases the frequency of spontaneous EPSCs. Representative chart records before (Control) and 5 min after 8-Br-cGMP application in one cell.

(A2) At 100 μM, 8-Br-cGMP does not have a significant effect on ON-evoked EPSCs. Paired pulse responses before (Con) and 5 min after 100 μM 8-Br-cGMP application are shown superimposed.

(B1) Representative traces from a different cell showing that application of IBMX (100 μM) alone increases the frequency of spontaneous EPSCs.

(B2) Although IBMX (100 μM) enhances spontaneous release, it does not significantly alter nerve-evoked transmission. Average EPSCs before (Con) and after IBMX application are shown superimposed.

tude (Figure 2B2; $p > 0.2$ and $p > 0.1$, respectively). These data demonstrate that cyclic nucleotides do not alter the sensitivity of postsynaptic glutamate receptors.

We also found that the opposing actions of cyclic nucleotides on evoked and spontaneous transmission were sensitive to cyclic nucleotide concentration. Lower concentrations of 8-Br-cGMP (100–150 μM) also caused a marked increase in sEPSC frequency (6.9- ± 1.9-fold, $n = 7$, Figure 3A1), but unlike higher concentrations, had no significant effect on evoked transmission (9.1% ± 4.1% inhibition, $n = 6$, $p > 0.07$, Figure 3A2). Similarly, elevating endogenous levels of cyclic nucleotides by adding IBMX alone (50–100 μM) increased sEPSC frequency (1.5- ± 0.4-fold, $n = 5$, $p < 0.04$, Figure 3B1) without inhibiting evoked transmission ($n = 4$, $p > 0.1$, Figure 3B2). The effect of IBMX was independent of any action on A1 adenosine receptors, since the A1 receptor antagonist DPCPX (50 μM) did not block the increase in mEPSC frequency produced by IBMX (data not shown, $n = 4$). These results indicate that concentrations of cyclic nucleotides that do not inhibit evoked transmission cause a marked increase in spontaneous transmitter release.

PG cells receive glutamatergic input from ON fibers as well as M/T cell dendrites (Shepherd and Greer, 1998). To confirm that cyclic nucleotides were acting on olfactory nerve terminals, we simultaneously recorded field EPSPs (fEPSPs) in olfactory bulb glomeruli evoked by ON and mitral cell (MC) stimulation. As found for PG cell EPSCs, a high concentration of 8-Br-cGMP (500 μM) caused a marked depression of the ON-evoked fEPSP (Figure 4, $p < 0.01$). In contrast, 8-Br-cGMP had no effect on the MC-evoked response when inhibition of the ON-evoked fEPSP was maximal (Figure 4, $p > 0.5$). Following washout of 8-Br-cGMP, the ON-evoked fEPSP gradually recovered. The MC-evoked fEPSP was slightly facilitated following washout of cGMP; this action was not studied further. Like 8-Br-cGMP, forskolin inhibited ON but not MC fEPSPs (data not shown, $n = 7$). These data indicate that high concentrations of cyclic nucleotides selectively depress ON-mediated transmission.

cAMP and cGMP are nearly equally effective activators of olfactory CNGCs (Zagotta and Siegelbaum,

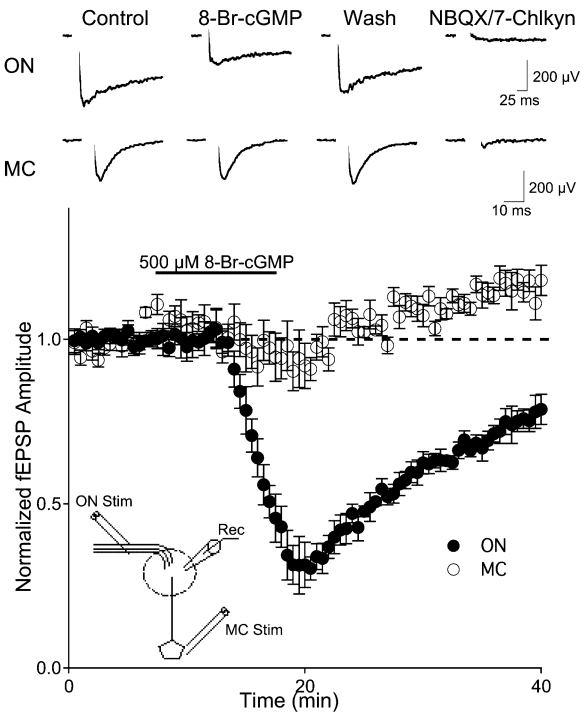

Figure 4. 8-Br-cGMP (500 μM) Decreases the Amplitude of Olfactory Nerve- but Not Mitral Cell-Evoked Glomerular fEPSPs

Top, traces from a representative experiment show ON- and mitral cell (MC)-evoked fEPSPs in the same glomerulus before (Control), during (8-Br-cGMP), and after (Wash) application of the cyclic nucleotide. Application of NBQX (20 μM) and 7-Chlkyn (50 μM) abolished both fEPSPs. Bottom, summary of the actions of 8-Br-cGMP on ON- and mitral cell-evoked fEPSPs ($n = 7$). Inset, schematic drawing of the recording configuration.

1996). The similar actions of cAMP and cGMP on olfactory nerve transmission suggested to us that cyclic nucleotides mediate their effects via activation of olfactory CNGCs. However, cyclic nucleotides can also regulate transmission through a variety of other potential pathways. For example, cyclic nucleotides are potent modulators of hyperpolarization-activated cation channels (I_h) (Pape, 1996), and recent studies suggest that presynaptic I_h channels can influence transmitter release (Beaumont and Zucker, 2000; Mellor et al., 2002; Southan et al., 2000). To address this possibility, we examined the actions of cyclic nucleotides in the presence of the I_h channel antagonist ZD 7288. Pretreatment with ZD 7288 (100 μM, >30 min) did not prevent the inhibition of evoked transmission by 500 μM 8-Br-cGMP (88.7% ± 4.5% inhibition, n = 4), indicating that cyclic nucleotides did not alter transmission by activating I_h channels.

We next considered the possibility that protein kinases underlie the effects of cyclic nucleotides on synaptic transmission. We asked whether incubating slices in the broad-spectrum protein kinase inhibitors staurosporine (2–5 μM), H-89 (20 μM), and H-7 (200 μM) blocked the actions of cyclic nucleotides on ON-mediated synaptic transmission. Incubating slices in this kinase inhibitor cocktail for 3–5 hr did not block the effects of forskolin on evoked EPSC amplitude (76.9% ± 15.3% inhibition, n = 4) or mEPSC frequency (con = 1.3 ± 0.3 Hz, forskolin = 11.3 ± 1.1 Hz, n = 4). These results suggest that protein kinases do not underlie the actions of cyclic nucleotides on olfactory nerve-mediated synaptic transmission.

We next designed experiments to further test the idea that CNGCs mediate the effects of cyclic nucleotides. Since CNGCs are nonselective for monovalent cations and are highly Ca^{2+} permeable (Zagotta and Siegelbaum, 1996), we asked whether cyclic nucleotide-mediated actions on synaptic transmission were associated with changes in presynaptic Ca^{2+}. We selectively labeled olfactory nerve fibers in vivo by injecting Ca^{2+} indicator dye into the nostrils of young rats (Wachowiak and Cohen, 2001). Olfactory bulb slices prepared from these animals exhibited labeling in the olfactory nerve and glomerular layers. 8-Br-cGMP caused a dramatic enhancement in fluorescence of labeled glomeruli, indicating a large increase in presynaptic Ca^{2+} (Figure 5A1). The 8-Br-cGMP-mediated increase in ON-terminal Ca^{2+} occurred in the presence of the glutamate receptor antagonists NBQX (20 μM) and 7-chlorokynurenate (50 μM), and TTX (1 μM), supporting the presynaptic origin of the signal (Figure 5A2). To examine the relationship between cyclic nucleotide-mediated changes in intracellular Ca^{2+} and synaptic transmission, we simultaneously monitored intraterminal Ca^{2+} and ON-evoked fEPSPs in individual glomeruli. The inhibition of ON-evoked fEPSPs by 8-Br-cGMP (500 μM) occurred in parallel with the increase in presynaptic Ca^{2+} (Figure 5B), suggesting that the two processes shared a common mechanism.

Increasing presynaptic Ca^{2+} typically facilitates transmitter release. Indeed, the large cyclic nucleotide-evoked increase in ON-terminal Ca^{2+} is likely to account for the dramatic increase in spontaneous transmitter release. Why then do high concentrations of cyclic nucleotides strongly depress evoked transmission? We considered the possibility that high concentrations of cyclic nucleotides inhibited transmitter release by altering the excitability of ON terminals. To test this idea, we measured nerve excitability by recording the ON fiber volley, a population response reflecting the presynaptic action potential. Forskolin (50 μM) caused a marked reduction in fiber volley amplitude in the presence of IBMX (97.8% ± 1.2% inhibition, n = 3, $p < 0.02$), suggesting that changes in fiber excitability govern the forskolin-mediated inhibition of evoked transmission. We examined the actions of different concentrations of 8-Br-cGMP on the fiber volley to better understand the dose-response relationship between cyclic nucleotides and changes in nerve excitability. 8-Br-cGMP (500 μM) rapidly abolished the ON fiber volley recorded in the presence of glutamate receptor antagonists (Figure 6). This effect was also observed in Ca^{2+}-free aCSF (data not shown, n = 3), ruling out the possibility that changes in ON excitability require extracellular Ca^{2+}. At 200 μM, 8-Br-cGMP blocked the fiber volley to a similar extent but with a slower time course. In contrast, a low concentration of 8-Br-cGMP (100 μM) caused a small increase in the amplitude of the fiber volley (6.5% ± 0.4%, n = 6, $p < 0.01$).

The biphasic effects of 8-Br-cGMP on olfactory nerve excitability are reminiscent of the actions of presynaptic kainate receptors on mossy fiber terminals in the hippocampus (Schmitz et al., 2000). Here, high concentrations of kainate receptor agonists depress fiber excitability, while low concentrations increase excitability. The actions of kainate receptor activation on mossy fibers have been attributed to presynaptic depolarization (Schmitz et al., 2000). Depolarizing ON fibers with a modest increase in extracellular K^+ (5–7.5 mM) blocked the ON fiber volley to a similar extent as high concentrations of 8-Br-cGMP (data not shown, n = 5). Taken together, our findings suggest that cyclic nucleotides alter nerve excitability in a manner similar to depolarization of nerve fibers.

To address directly whether the effects of cyclic nucleotides on olfactory nerve excitability and synaptic transmission were mediated by CNGCs, we studied the actions of 8-Br-cGMP in olfactory bulb slices from mice lacking olfactory CNGCs (Brunet et al., 1996). ON-evoked EPSCs in *CNGC*$^{-/}$ mice were indistinguishable from wild-type (WT) and exhibited a similar degree of paired pulse depression (PPR = 0.26 ± 0.06; n = 4 mice, 12 cells). 8-Br-cGMP had no significant action on olfactory nerve-evoked EPSCs in *CNGC*$^{-/}$ mice ($p > 0.1$; n = 3 mice, 4 cells), although the EPSC could be strongly inhibited by baclofen (Figure 7A). Furthermore, there was no significant difference in the sEPSC frequency before and after cyclic nucleotide application in *CNGC*$^{-/}$ mice (Figure 7B; control = 4.1 ± 1.7 Hz, 8-Br-cGMP = 2.3 ± 1.0 Hz; n = 3 mice, 8 cells, $p > 0.15$). These results indicate that although ON-evoked EPSCs and their modulation by $GABA_B$ receptors appear normal in *CNGC*$^{-/}$ mice, neither evoked nor spontaneous transmitter release is sensitive to cyclic nucleotide application.

As in rats, a high concentration of 8-Br-cGMP (1 mM) largely abolished the ON fiber volley in WT mice (Figure 8). However, 8-Br-cGMP had no effect on the ON fiber volley in slices from *CNGC*$^{-/}$ mutant mice (Figure 8, $p > 0.3$; n = 3 mice, 6 slices), while subsequent application

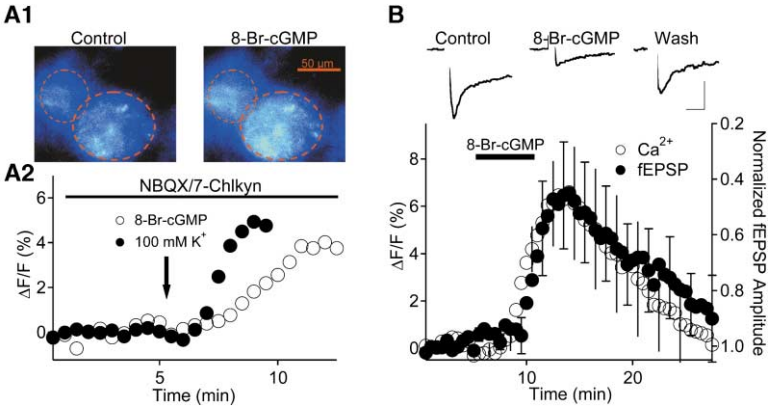

Figure 5. 8-Br-cGMP Increases Intracellular $[Ca^{2+}]$ in ON Terminals

(A1) Pseudocolor images of two adjacent Calcium Green-labeled glomeruli before (left) and after (right) application of 8-Br-cGMP (500 μM) in the presence of TTX (1 μM).

(A2) Change in fluorescence (ΔF/F) in one glomerulus in response to 8-Br-cGMP application (open circles). After washout, subsequent application of high K^+ also produced a large increase in intracellular Ca^{2+} (closed circles). Arrow indicates time at which 8-Br-cGMP or high K^+ were applied.

(B) 8-Br-cGMP simultaneously increases ON-terminal Ca^{2+} and decreases ON-evoked fEPSPs. Top, traces from one experiment before, during, and after washout of 8-Br-cGMP. Bottom, summary of basal ON-terminal Ca^{2+} (open circles, left axis) and ON-evoked fEPSP amplitudes (closed circles, right axis) from the same glomeruli (n = 3). Scale bars: 200μV, 20 ms.

of an elevated K^+ solution or TTX abolished the fiber volley (data not shown, n = 3 mice, 6 slices). Female mice that carried one normal copy of the *CNGC* gene and one mutant copy showed an intermediate phenotype (WT 8-Br-cGMP inhibition = 70.9% ± 3.6%; $CNGC^{-/+}$ 8-Br-cGMP inhibition = 38.2% ± 2.9%). This observation is consistent with the fact that the *CNGC* gene locus is subject to X inactivation (Zhao and Reed, 2001); thus, ~50% of olfactory receptor neurons targeting the same glomerulus will likely express WT CNGCs, while the other half will express mutant channels in a $CNGC^{-/+}$ mouse. Together, these experiments in CNGC mutant mice indicate that all of the actions of cyclic nucleotides on olfactory nerve excitability and transmitter release are due to CNGC activation.

Our data suggest that CNGC activation by high concentrations of cyclic nucleotides increases spontaneous glutamate release, but inhibits evoked transmission via presynaptic depolarization. In contrast, modest activation of CNGCs increases spontaneous transmission without a significant action on nerve-evoked transmission. Increases in mEPSC frequency are typically associated with mechanisms that increase the probability of nerve-evoked transmitter release (P_r) (Zucker and Regehr, 2002). Furthermore, manipulations that increase P_r usually produce a decrease in the PPR. What then can account for the observation that cyclic nucleotides increase the frequency of spontaneous release but do

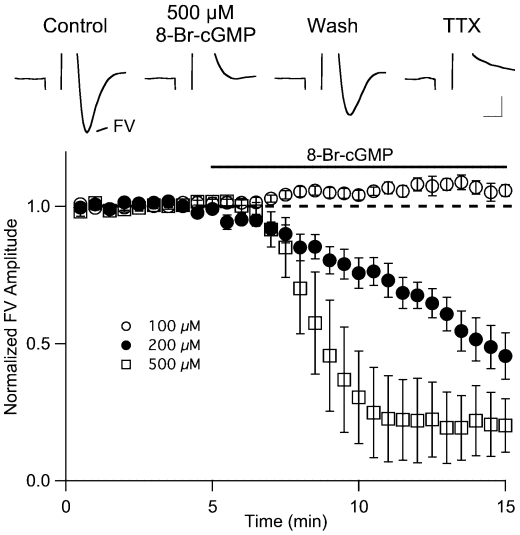

Figure 6. 8-Br-cGMP Blocks the ON Fiber Volley

Top, traces from one representative experiment in which 500 μM 8-Br-cGMP depressed the fiber volley (FV). After washing out the cyclic nucleotide, the FV was abolished by subsequent application of TTX (1 μM). Bottom, summary of the actions of 100, 200, and 500 μM 8-Br-cGMP on FV amplitude (n = 6, 5, and 4, respectively). The stimulus artifact has been truncated in the averaged traces. Scale bars: 200μV, 1 ms.

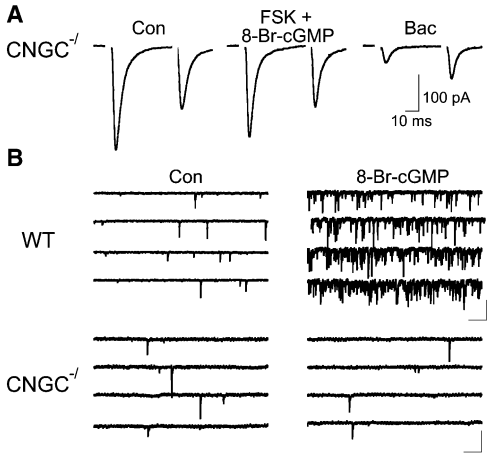

Figure 7. Cyclic Nucleotides Do Not Modulate Synaptic Transmission in Mice Lacking Functional CNGCs

(A) EPSCs evoked by ON paired pulse stimulation are largely insensitive to coapplication of 8-Br-cGMP (500 μM) and forskolin (50 μM). Subsequent application of baclofen (Bac, 50 μM) strongly inhibited the ON-evoked EPSC and altered the PPR.

(B) 8-Br-cGMP produces a large increase in sEPSC frequency in a representative PG cell from a WT mouse (top) but not a $CNGC^{-/-}$ mouse (bottom). Scale bars: 100 pA (top)/50 pA (bottom), 100 ms.

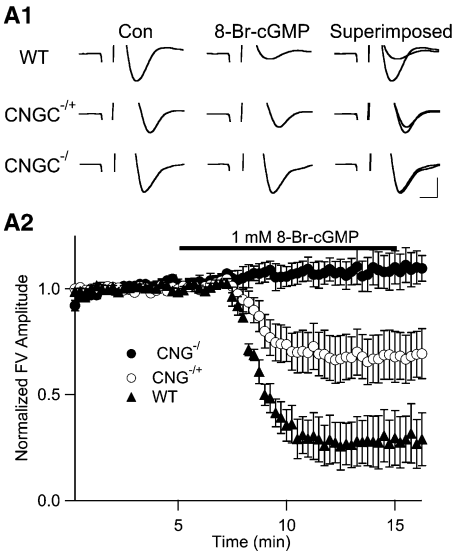

Figure 8. CNGCs Mediate the Effects of 8-Br-cGMP on ON Excitability

(A1) Actions of 8-Br-cGMP on ON fiber volleys in a WT (top), *CNGC*$^{-/+}$ (middle), and *CNGC*$^{-/}$ (bottom) mouse. Scale bars: 500μV, 1 ms.

(A2) Summary of the effects of 8-Br-cGMP on FV amplitude in WT (n = 6 mice), *CNGC*$^{-/+}$ (n = 3 mice), and *CNGC*$^{-/}$ (n = 3 mice).

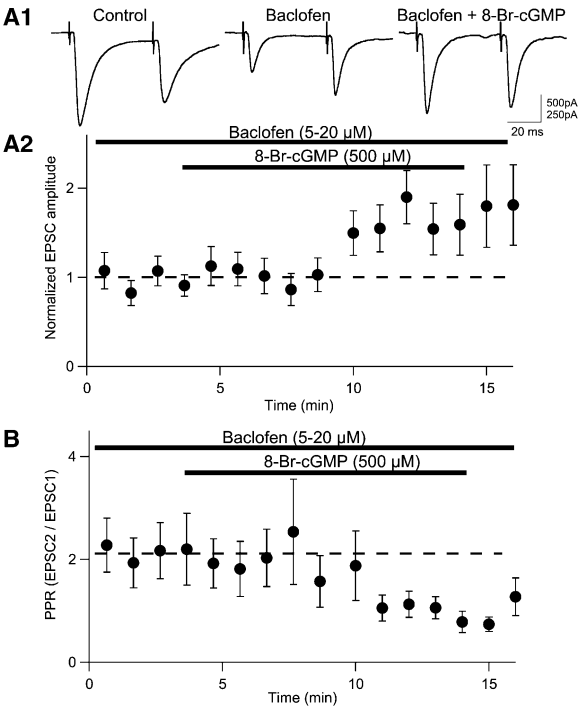

Figure 9. Cyclic Nucleotides Enhance ON-Evoked Transmission When Release Probability Is Low

(A1) Traces from a typical experiment in which activation of presynaptic $GABA_B$ receptors by baclofen (5–20 μM) inhibited transmitter release and produced paired pulse facilitation. Subsequent application of 8-Br-cGMP (500 μM) increased the amplitude of the first EPSC and restored paired pulse depression. Scale bar: Control, 500 pA; Baclofen and Baclofen + 8-Br-cGMP, 250 pA.

(A2) Summary of the effects of 8-Br-cGMP on the amplitude of the first EPSC in the presence of baclofen (n = 6).

(B) Average effect of 8-Br-cGMP on paired pulse ratio ($EPSC_2$/$EPSC_1$) in baclofen (n = 5). Points represent the average of three consecutive responses.

not alter the PPR? We considered the possibility that P_r was already so high under normal conditions that a CNGC-mediated enhancement in P_r could not be resolved as a further decrease in PPR. To test this hypothesis, we examined the actions of cyclic nucleotides on the PPR under conditions of reduced P_r. We lowered P_r by applying the $GABA_B$ receptor agonist baclofen (5–20 μM). To blunt the effects of 8-Br-cGMP on fiber excitability, these experiments were performed in aCSF containing an elevated concentration of divalent ions (4 mM Ca^{2+}, 4 mM Mg^{2+}). In the presence of baclofen, 8-Br-cGMP (500 μM) increased the amplitude of the first EPSC by ~75% (Figure 9A) and changed the PPR from facilitation (2.11 ± 0.15) to depression (0.91 ± 0.06; Figure 9B, $p < 0.01$). 8-Br-cGMP had similar effects when release probability was lowered by applying the Ca^{2+} channel blocker Cd^{2+} (50–75 μM) or performing the experiment in aCSF with low extracellular Ca^{2+} (0.5–1 mM; data not shown, n = 5).

We also examined the actions of low concentrations of cyclic nucleotides when release probability was reduced. In normal aCSF containing either baclofen (5 μM) or Cd^{2+} (10 μM), application of 100–150 μM 8-Br-cGMP increased the amplitude of ON-evoked EPSCs (~100%) and dramatically changed the PPR (from 4.5 ± 1.3 to 1.3 ± 0.1, n = 3). Taken together, these data demonstrate that activating presynaptic CNGCs can enhance nerve-evoked transmission under conditions in which P_r is reduced.

Discussion

In this study, we identify a novel role for olfactory CNGCs in neurotransmission. cAMP and cGMP modulate transmission between olfactory nerve fibers and their targets in the olfactory bulb but have no effect on synaptic transmission in mice lacking CNGCs, indicating that the effects of cyclic nucleotides are mediated entirely by CNGC activation. Strong activation of CNGCs depresses evoked transmission by altering olfactory nerve fiber excitability while both weak and strong CNGC activation increase spontaneous transmitter release. These effects are specific to ON-mediated transmission, since cyclic nucleotides modulate ON- but not mitral cell input to olfactory bulb glomeruli. The actions of cyclic nucleotides on synaptic transmission are accompanied by a marked increase in basal Ca^{2+} in ORN synaptic terminals. Finally, we show that activation of presynaptic CNGCs can counteract manipulations that reduce the probability of release at olfactory nerve terminals.

At central synapses, cyclic nucleotides typically facilitate synaptic transmission (Arancio et al., 1995; Sakaba and Neher, 2001; Weisskopf et al., 1994). These effects are generally attributed to direct actions on the exocytotic apparatus or protein kinase-dependent phosphorylation of targets that regulate transmitter release. We find that high concentrations of both cAMP and cGMP dramatically inhibit nerve-evoked transmission while in-

creasing spontaneous glutamate release from olfactory nerve terminals in the olfactory bulb. These actions are unlikely to require kinase activity since a combination of kinase inhibitors, including staurosporine, H-89, and H-7, did not block the effects of cyclic nucleotides on ON-mediated transmission.

Several observations indicate that cyclic nucleotides modulate transmitter release from the olfactory nerve via activation of the olfactory subtype of CNGC. First, forskolin and 8-Br-cGMP both strongly modulated ON-mediated synaptic transmission. This result implicates the olfactory subtype of CNGCs in the actions of cyclic nucleotides because olfactory CNGCs, unlike their counterparts in the retina, are nearly equally well gated by cAMP and cGMP (Zagotta and Siegelbaum, 1996). Second, cyclic nucleotides modulated ON- but not mitral cell-evoked transmission in olfactory bulb glomeruli. This synapse specificity is consistent with immunohistochemical data suggesting that CNGCs are restricted to olfactory nerve fibers in the bulb (Matsuzaki et al., 1999). Third, and most importantly, cyclic nucleotides have no effect on evoked or spontaneous transmitter release from olfactory nerve fibers in mice lacking olfactory CNGCs.

Cyclic nucleotides produced a large increase in presynaptic Ca^{2+} in ORN terminals. This result fits with the high calcium permeability of olfactory CNGCs (Frings et al., 1995). We cannot exclude the possibility, however, that cyclic nucleotides triggered release of Ca^{2+} from internal stores, though we are unaware of any studies that support this possibility.

Increasing presynaptic Ca^{2+} typically enhances transmitter release. Indeed, cyclic nucleotides increased the frequency of miniature EPSCs in PG cells. What could account for the simultaneous increase in presynaptic Ca^{2+} and reduction in evoked transmission in response to strong CNGC activation? It is conceivable that the depression of evoked transmission could be a consequence of vesicle depletion due to the increase in spontaneous transmitter release. However, we saw no obvious correlation between the relative inhibition of evoked responses and the increase in sEPSC frequency produced by cyclic nucleotides in the same cells. This suggests that changes in vesicle availability do not underlie the depression of evoked transmission.

An alternative explanation for the inhibition of evoked release is that strong CNGC-mediated membrane depolarization of olfactory nerve terminals alters ON excitability. Consistent with this idea, high concentrations of cyclic nucleotides abolished the ON fiber volley. This effect was mimicked by depolarizing nerve fibers with an elevated K^+ aCSF. The changes in ON excitability produced by cyclic nucleotides were not due to Ca^{2+} influx because 8-Br-cGMP inhibited the fiber volley in the absence of extracellular Ca^{2+}. This observation rules out the possibility that hyperpolarization due to presynaptic Ca^{2+}-activated K^+ conductances (Bielefeldt and Jackson, 1993) could underlie the reduction of the fiber volley. We believe the most parsimonious explanation is that CNGC-mediated currents can depolarize presynaptic terminals to the point where voltage-dependent Na^+ channels inactivate, thereby blocking action potential propagation into ON terminals. Thus, strong activation of presynaptic CNGCs can decrease the number of functional ON fibers contributing to evoked transmission.

Several other types of ionotropic receptors, including nicotonic acetylcholine receptors (MacDermott et al., 1999) and glycine receptors (Turecek and Trussell, 2001) have been shown to enhance transmitter release from central nerve terminals by facilitating calcium influx. In contrast, activation of depolarizing presynaptic $GABA_A$ receptors can inhibit transmitter release through the classical mechanism of primary afferent depolarization (PAD) (Eccles et al., 1962; Nicoll and Alger, 1979) in the spinal cord. PAD leads to presynaptic inhibition by inactivating Na^+ channels and blocking action potential propagation into nerve terminals. Recently, it has been suggested that strong activation of ionotropic kainate receptors at mossy fiber terminals in the hippocampus can inhibit transmitter release via mossy fiber depolarization (Schmitz et al., 2000). Our results indicate that strong activation of CNGCs similarly inhibits ON-mediated glutamate release through a PAD-like mechanism.

Presynaptic kainate receptors produce a concentration-dependent bidirectional modulation of transmission at hippocampal mossy fiber synapses (Schmitz et al., 2001) and parallel fiber synapses in the cerebellum (Delaney and Jahr, 2002). At these synapses, high concentrations of kainate receptor agonists inhibit transmission, while low concentrations enhance transmission (Delaney and Jahr, 2002; Schmitz et al., 2001). Although it is significantly more difficult to titrate the concentration of intracellular cyclic nucleotides, we find that moderate concentrations of 8-Br-cGMP (which are more likely to be physiologically relevant) enhance spontaneous transmitter release without inhibiting evoked transmission. Indeed, simply blocking endogenous phosphodiesterase activity with IBMX increased spontaneous transmitter release, suggesting that regulation of endogenous cyclic nucleotide levels in ON terminals can influence activation of CNGCs. Presumably moderate activation of presynaptic CNGCs enables Ca^{2+} influx to facilitate transmitter release but does not produce enough depolarization to block action potential propagation into ON terminals.

ON-evoked EPSCs show marked paired pulse depression, suggesting that release probability is high under normal conditions. Although activation of presynaptic CNGCs increased spontaneous transmitter release, under normal conditions, we observed no change in the paired pulse ratio of evoked transmission. However, we show that activation of presynaptic CNGCs can indeed increase release probability since cyclic nucleotides alter the paired pulse ratio when release probability is low. Several studies indicate that presynaptic metabotropic receptors can mediate strong inhibition of olfactory nerve transmission (Aroniadou-Anderjaska et al., 2000; Ennis et al., 2001). Our data suggest that the regulation of presynaptic cyclic nucleotide levels can provide a mechanism by which olfactory nerve input to the bulb may be enhanced.

Might presynaptic CNGCs play additional roles in the olfactory bulb? It has been proposed that odorant receptors on olfactory nerve terminals contribute to the precise targeting of olfactory receptor axons to particular glomerular targets in the bulb (Mombaerts et al., 1996; Wang et al., 1998). This raises the possibility that axon

targeting in the bulb relies on activation of odorant receptors and the subsequent generation of cAMP (Yoshida et al., 2002). Nerve terminal CNGCs represent a potential downstream target for cAMP generated by odorant receptor activation in the bulb. However, it has been suggested that axons from ORNs expressing the same odorant receptor converge normally onto specific glomeruli in CNGC mutant mice (Lin et al., 2000). In contrast, other studies indicate that the axons of some ORNs do not target the appropriate glomeruli in CNGC mutant mice (Baker et al., 1999; Zhao and Reed, 2001; Zheng et al., 2000). These deficits have generally been attributed to a loss of odor-evoked activation of CNGCs in the dendritic cilia of ORNs. An alternative to this activity-dependent model is that CNGCs in the axons of ORNs contribute to precise axonal pathfinding either by modulating ON synaptic transmission or by increasing nerve terminal calcium. The dramatic effects of CNGC activation on ON-mediated synaptic transmission support the intriguing possibility that CNGCs in ON nerve endings contribute to axon pathfinding.

Experimental Procedures

Slice Preparation and Electrophysiology

Olfactory bulb slices were prepared from 2- to 4-week-old rats (Sprague-Dawley) and mice (c57bl6) and maintained in artificial cerebrospinal fluid (aCSF) containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgS0_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 22 mM glucose, and 2.5 mM $CaCl_2$ equilibrated with 95% O_2/5% CO_2. All voltage-clamp experiments (Vm = −80mV) were performed in the presence of picrotoxin (100 μM). For most experiments examining the actions of forskolin, the phosphodiesterase inhibitor IBMX (50–100 μM) was included in the aCSF. Cells were visualized using infrared-DIC optics (BX-51WI, Olympus, Melville, NY). Whole-cell patch-clamp electrodes (4–7 MΩ) were filled with a solution containing 135 mM D-gluconic acid, 135 mM CsOH, 5 mM NaCl, 10 mM HEPES, 12 mM phosphocreatine, 0.2–0.5 mM EGTA, 3 mM Mg-ATP, and 0.2 mM Na-GTP (pH ~7.35, 300 mOsm). Pipettes for field recordings (2–3 MΩ) were filled with aCSF. Series resistance for whole-cell recordings was always ≤10 MΩ and compensated electronically by ≥90%. Synaptic currents and field potentials were recorded with an Axopatch 200B amplifier (Axon instruments, Foster City, CA), filtered at 2 kHz, and collected and digitized at 5–20 kHz (ITC-18; Instrutech, Mineola, NY). Data acquisition and analysis were performed with Axograph (Axon instruments) and IGOR Pro software (Wavemetrics, Lake Oswego, OR). EPSCs and field potentials were evoked (0.05–0.066 Hz) via a bipolar electrode placed in the olfactory nerve and/or mitral cell layer. Most experiments were performed at room temperature; experiments performed at 30°C–32°C were not qualitatively different. The amplitudes of synaptic responses were measured over a 0.5–2 ms window centered around the peak. Fiber volley amplitude was measured as the maximum negative inflection point after digital subtraction of the stimulus artifact in tetrodotoxin (TTX, 0.5–1 μM). Representative traces are the average of 5–20 consecutive episodes. All data are shown as mean ± SEM. The student's t test was used to determine statistical significance.

Fluorescent Ca^{2+} Dye Loading and Imaging

Oregon Green or Calcium Green-1 dextran (2.5%–5%; 10 kDa MW [Molecular Probes, Eugene, OR]) was dissolved in 2%–5% Triton-X100 and carefully injected with a flexible plastic syringe tip into both nostrils of young (P12–P14) rats. Imaging experiments were performed 3–5 days after injecting the Ca^{2+} dye. Image acquisition (488 nm excitation) and analysis were performed with a cooled-CCD camera system (T.I.L.L. Photonics, Gräfelfing, Germany).

Olfactory CNGC Mutant Mice

CNGC mutant mice were generated by crossing female heterozygous mutant mice (Brunet et al., 1996) with wild-type males. To increase the viability of CNGC mutant offspring, newborn pups were transferred to foster mothers (CD1 strain). All experiments were performed blind with respect to genotype. Genotyping was performed post hoc on preserved tissue by PCR for the *CNGC*, *neo*, and *sry* genes (indicating the presence of the wild-type CNGC gene, the targeted CNGC mutant gene, and Y chromosome, respectively).

Acknowledgments

We are indebted to John Ngai and Lisa Brunet for providing *CNGC*$^{-/+}$ mice, helpful advice, and reagents. We thank Jane Sullivan for help with mouse genotyping and Jianhua Cang for comments on the manuscript. G.J.M. received support from an NIGMS training grant, a Merck Fellow award, and an NRSA predoctoral fellowship (NIDCD). J.S.I. is supported by a Burroughs Wellcome Fund Career Award, McKnight Scholar Award, and the NIDCD (R01DC04682).

Received: August 23, 2002
Revised: December 20, 2002

References

Arancio, O., Kandel, E.R., and Hawkins, R.D. (1995). Activity-dependent long-term enhancement of transmitter release by presynaptic 3′,5′-cyclic GMP in cultured hippocampal neurons. Nature *376*, 74–80.

Aroniadou-Anderjaska, V., Zhou, F.M., Priest, C.A., Ennis, M., and Shipley, M.T. (2000). Tonic and synaptically evoked presynaptic inhibition of sensory input to the rat olfactory bulb via GABA(B) heteroreceptors. J. Neurophysiol. *84*, 1194–1203.

Baker, H., Cummings, D.M., Munger, S.D., Margolis, J.W., Franzen, L., Reed, R.R., and Margolis, F.L. (1999). Targeted deletion of a cyclic nucleotide-gated channel subunit (OCNC1): biochemical and morphological consequences in adult mice. J. Neurosci. *19*, 9313–9321.

Beaumont, V., and Zucker, R.S. (2000). Enhancement of synaptic transmission by cyclic AMP modulation of presynaptic Ih channels. Nat. Neurosci. *3*, 133–141.

Bielefeldt, K., and Jackson, M.B. (1993). A calcium-activated potassium channel causes frequency-dependent action-potential failures in a mammalian nerve terminal. J. Neurophysiol. *70*, 284–298.

Bradley, J., Zhang, Y., Bakin, R., Lester, H.A., Ronnett, G.V., and Zinn, K. (1997). Functional expression of the heteromeric "olfactory" cyclic nucleotide- gated channel in the hippocampus: a potential effector of synaptic plasticity in brain neurons. J. Neurosci. *17*, 1993–2005.

Brunet, L.J., Gold, G.H., and Ngai, J. (1996). General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron *17*, 681–693.

Delaney, A.J., and Jahr, C.E. (2002). Kainate receptors differentially regulate release at two parallel fiber synapses. Neuron *36*, 475–482.

Eccles, J.C., Magni, F., and Willis, W.D. (1962). Depolarization of central terminals of group 1a afferent fibers from muscle. J. Physiol. *160*, 532–540.

Ennis, M., Zhou, F.M., Ciombor, K.J., Aroniadou-Anderjaska, V., Hayar, A., Borrelli, E., Zimmer, L.A., Margolis, F., and Shipley, M.T. (2001). Dopamine D2 receptor-mediated presynaptic inhibition of olfactory nerve terminals. J. Neurophysiol. *86*, 2986–2997.

Frings, S., Seifert, R., Godde, M., and Kaupp, U.B. (1995). Profoundly different calcium permeation and blockage determine the specific function of distinct cyclic nucleotide-gated channels. Neuron *15*, 169–179.

Katz, B. (1969). The Release of Neural Transmitter Substances (Liverpool, UK: Liverpool University Press).

Kramer, R.H., and Tibbs, G.R. (1996). Antagonists of cyclic nucleotide-gated channels and molecular mapping of their site of action. J. Neurosci. *16*, 1285–1293.

Kuzmiski, J.B., and MacVicar, B.A. (2001). Cyclic nucleotide-gated channels contribute to the cholinergic plateau potential in hippocampal CA1 pyramidal neurons. J. Neurosci. *21*, 8707–8714.

Lin, D.M., Wang, F., Lowe, G., Gold, G.H., Axel, R., Ngai, J., and Brunet, L. (2000). Formation of precise connections in the olfactory bulb occurs in the absence of odorant-evoked neuronal activity. Neuron *26*, 69–80.

MacDermott, A.B., Role, L.W., and Siegelbaum, S.A. (1999). Presynaptic ionotropic receptors and the control of transmitter release. Annu. Rev. Neurosci. *22*, 443–485.

Matsuzaki, O., Bakin, R.E., Cai, X., Menco, B.P., and Ronnett, G.V. (1999). Localization of the olfactory cyclic nucleotide-gated channel subunit 1 in normal, embryonic and regenerating olfactory epithelium. Neuroscience *94*, 131–140.

Mellor, J., Nicoll, R.A., and Schmitz, D. (2002). Mediation of hippocampal mossy fiber long-term potentiation by presynaptic Ih channels. Science *295*, 143–147.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Nakamura, T., and Gold, G.H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. Nature *325*, 442–444.

Nicoll, R.A., and Alger, B.E. (1979). Presynaptic inhibition: transmitter and ionic mechanisms. Int. Rev. Neurobiol. *21*, 217–258.

Pape, H.C. (1996). Queer current and pacemaker: the hyperpolarization-activated cation current in neurons. Annu. Rev. Physiol. *58*, 299–327.

Rieke, F., and Schwartz, E.A. (1994). A cGMP-gated current can control exocytosis at cone synapses. Neuron *13*, 863–873.

Sakaba, T., and Neher, E. (2001). Preferential potentiation of fast-releasing synaptic vesicles by cAMP at the calyx of Held. Proc. Natl. Acad. Sci. USA *98*, 331–336.

Schmitz, D., Frerking, M., and Nicoll, R.A. (2000). Synaptic activation of presynaptic kainate receptors on hippocampal mossy fiber synapses. Neuron *27*, 327–338.

Schmitz, D., Mellor, J., and Nicoll, R.A. (2001). Presynaptic kainate receptor mediation of frequency facilitation at hippocampal mossy fiber synapses. Science *291*, 1972–1976.

Shepherd, G.M., and Greer, C.A. (1998). Olfactory bulb. In The Synaptic Organization of the Brain, G.M. Shepherd, ed. (Oxford: Oxford University Press), pp. 159–203.

Southan, A.P., Morris, N.P., Stephens, G.J., and Robertson, B. (2000). Hyperpolarization-activated currents in presynaptic terminals of mouse cerebellar basket cells. J. Physiol. *526*, 91–97.

Turecek, R., and Trussell, L.O. (2001). Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. Nature *411*, 587–590.

Wachowiak, M., and Cohen, L.B. (2001). Representation of odorants by receptor neuron input to the mouse olfactory bulb. Neuron *32*, 723–735.

Wang, F., Nemes, A., Mendelsohn, M., and Axel, R. (1998). Odorant receptors govern the formation of a precise topographic map. Cell *93*, 47–60.

Weisskopf, M.G., Castillo, P.E., Zalutsky, R.A., and Nicoll, R.A. (1994). Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. Science *265*, 1878–1882.

Yau, K.W., and Baylor, D.A. (1989). Cyclic GMP-activated conductance of retinal photoreceptor cells. Annu. Rev. Neurosci. *12*, 289–327.

Yoshida, T., Ito, A., Matsuda, N., and Mishina, M. (2002). Regulation by protein kinase A switching of axonal pathfinding of zebrafish olfactory sensory neurons through the olfactory placode-olfactory bulb boundary. J. Neurosci. *22*, 4964–4972.

Zagotta, W.N., and Siegelbaum, S.A. (1996). Structure and function of cyclic nucleotide-gated channels. Annu. Rev. Neurosci. *19*, 235–263.

Zhao, H., and Reed, R.R. (2001). X inactivation of the *OCNC1* channel gene reveals a role for activity-dependent competition in the olfactory system. Cell *104*, 651–660.

Zheng, C., Feinstein, P., Bozza, T., Rodriguez, I., and Mombaerts, P. (2000). Peripheral olfactory projections are differentially affected in mice deficient in a cyclic nucleotide-gated channel subunit. Neuron *26*, 81–91.

Zucker, R.S., and Regehr, W.G. (2002). Short-term synaptic plasticity. Annu. Rev. Physiol. *64*, 355–405.