Regulation of Main Olfactory Bulb Mitral Cell Excitability by Metabotropic Glutamate Receptor mGluR1

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Heinbockel, Thomas, Philip Heyward, François Conquet, and Matthew Ennis. Regulation of main olfactory bulb mitral cell excitability of metabotropic glutamate receptor mGluR1. J Neurophysiol 92: 3085–3096, 2004. First published June 22, 2004; 10.1152/jn.00349.2004. In the rodent main olfactory bulb (MOB), mitral cells (MCs) express high levels of the group I metabotropic glutamate receptor (mGluR) subtype, mGluR1. The significance of this receptor in modulating MC excitability is unknown. We investigated the physiological role of mGluR1 in regulating MC activity in rat and mouse MOB slices. The selective group I agonist (RS)-3,5-dihydroxyphenylglycine (DHPG), but not group II or III agonists, induced potent, dose-dependent, and reversible depolarization and increased firing of MCs. Effects persisted in the presence of blockers of fast synaptic transmission, indicating that they are due to direct activation of mGluRs on MCs. Voltage-clamp recordings showed that DHPG elicited a voltage-dependent inward current consisting of multiple components sensitive to potassium and calcium channel blockade and intracellular calcium chelation. MC excitatory responses to DHPG were absent in mGluR1 knockout mice but persisted in mGluR5 knockout mice. Broad-spectrum LY341495, MCPG, as well as preferential mGluR1 LY367385 antagonists blocked the excitatory effects of DHPG and also potently modulated MC spontaneous and olfactory nerve-evoked excitability. mGluR antagonists altered spontaneous membrane potential bistability, increasing the duration of the up and down states. mGluR antagonists also substantially attenuated MC responses to sensory input, decreasing the probability and increasing the latency of olfactory nerve-evoked spikes. These findings suggest that endogenous glutamate tonically modulates MC excitability and responsiveness to olfactory nerve input, and hence the operation of the MOB circuitry, via activation of mGluR1.

INTRODUCTION

Sensory transmission from olfactory nerve terminals to the principal projection neurons of the main olfactory bulb (MOB), mitral and tufted cells (MCs/TCs), is mediated by glutamate acting at AMPA and N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor subtypes (Aroniadou-Anderjaska et al. 1997; Bardoni et al. 1996; Chen and Shepherd 1997; Ennis et al. 1996, 2001; Keller et al. 1998). MCs/TCs are also glutamatergic. Glutamate released from MCs/TCs, acting at both ionotropic receptor subtypes, mediates dendrodendritic transmission at synapses with juxtaglomerular and granule cell interneurons (Aroniadou-Anderjaska et al. 1999a; Bardoni et al. 1996; Isaacson and Strowbridge 1998; Schoppa et al. 1998). Recent electrophysiological studies suggest that ionotropic glutamate receptors mediate recurrent excitatory interactions among apical and lateral dendrites of MCs/TCs (Aroniadou-Anderjaska et al. 1999b; Carlson et al. 2000; Isaacson 1999; Salin et al. 2001; Schoppa and Westbrook 2001).

Neuroanatomical studies demonstrate that MCs/TCs and other neuronal subtypes in the MOB express high levels of metabotropic glutamate receptors (mGluRs), suggesting that they play an important role(s) in olfactory processing. The eight mGluRs identified to date are subdivided into three groups based on sequence homology, signal transduction mechanisms, and pharmacology (Conn and Pin 1997): group I mGluRs (mGluR1, mGluR3), group II mGluRs (mGluR2, mGluR5), and group III mGluRs (mGluR4, mGluR6-8). MCs/TCs express high levels of mGluR1 (Martin et al. 1992; Masu et al. 1995; Sahara et al. 2001; Shigemoto et al. 1992; van den Pol 1995). Electron microscopy studies demonstrated that mGluR1 is present on the somata and apical and lateral dendrites of MCs/TCs (van den Pol 1995). This expression pattern suggests that mGluR1 could mediate MC/TC responses to glutamatergic inputs from olfactory nerve terminals and/or could function as auto- or heteroreceptors for glutamate released from apical or lateral dendrites of MCs/TCs. MCs/TCs also express mRNA for mGluR7 and mGluR8 (Kinzie et al. 1995; Saugstad et al. 1997), although immunocytochemical studies indicate that these receptors are present on their axon terminals in the granule cell layer and in perifovea cortex (Kinoshita et al. 1998; Wada et al. 1998).

There is limited information about the physiological actions and function of group I mGluRs on either MCs or TCs. Studies in dissociated cultured rat and frog MOB neuronal preparations reported that activation of group I mGluRs increased Ca2+ release from internal stores in MCs and bulb interneurons (Carlson et al. 1997; Geiling and Schild 1996) or depolarized MCs and increased the frequency of miniature excitatory postsynaptic currents (Schoppa and Westbrook 1997). The function(s) of group I mGluRs on MCs in the MOB network has not been investigated. The goal of the present study therefore was to investigate the actions of group I mGluRs on MC excitability and responsiveness to sensory input from olfactory nerve terminals. To address these issues, we studied the functional consequences of activation or blockade of mGluRs on MCs using whole cell patch-clamp recording in MOB slices from rats, wild-type mice, and mice with targeted mGluR1 knockout genes.

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gene deletions of group I mGluRs. Parts of this study have been published in abstract form (Heinbockel and Ennis 2002; Heinbockel et al. 2001a,b).

METHODOLOGY

Animals and genotyping

Animal experimentation and usage was in accordance with guidelines of the University of Maryland and Institutional Animal Care and Use Committee and the National Institutes of Health. Experimental animals were commercially available male and female rats (Sprague-Dawley; Zivic Laboratories, Zelienople, PA) and mice (C57Bl/6J, Jackson Laboratory, Bar Harbor, ME) and mGluR1 and mGluR5 mutant mice from our colony. Breeder stock of the mGluR1 and mGluR5 mutant mice (C57Bl/6J background) were provided by F. Conquet; the generation of mGluR1 and mGluR5 receptor knockout mice has been reported previously (Chiamulera et al. 2001; Conquet et al. 1994). mGluR5 mice were provided as a homozygous mutant line. mGluR1 mice, generated as a lacZ knock-in, were maintained by heterozygous mating and were genotyped by polymerase chain reaction (PCR) of DNA from tail tip digests. Homozygous mGluR1 mutant mice were also identified phenotypically by their characteristic progressive mobility deficit at several weeks of age (Conquet et al. 1994). For PCR genotyping, tail tips were digested at 56°C in 20 μl proteinase K (1 μg/μl) in 48 mM Tris-HCl pH 8.0, 11 mM NaCl, 0.5 mM NaEDTA, and 0.5% sodium dodecylsulfate until tips were completely dissolved (1–2 h). Sterile water (780 μl) was added to each digest, and the samples were heated at 95°C for 20 min to inactivate the protease K. Each PCR sample (25 μl) contained 1 μl DNA digest in 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM each dTTP, dCTP, dATP, and dGTP, and 0.625 U Taq polymerase. The oligonucleotide primers were 20 pmol/25 μl. The thermocycler program was initiated by a 1-min denaturation step at 94°C followed by 39 amplification cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The reaction products were separated by electrophoresis on 1.6% agarose gels, and the amplicons were visualized with ethidium bromide under UV illumination. The oligonucleotides for the neomycin resistance gene were 5’ GTGAAAT- GAAGTCGAGGACCA and 5’ ATACATTTTCTCGGCAAGGCA and generated a 170-bp amplicon. The oligonucleotides for lacZ were 5’ CATTCTACCAAGCTAAGTACTC and 5’ GATAACGTC- CGTACTCTCAAGCG and generated a 296-bp amplicon.

β-galactosidase immunohistochemistry

Conventional immunohistochemical techniques were used to map the distribution of neurons expressing β-galactosidase (β-gal) in tissue sections harvested from mGluR1 transgenic animals (see preceeding text). Mice were deeply anesthetized (pentobarbital sodium, 50 mg/kg ip) and transcardially perfused with 5–10 ml 0.9% saline followed by 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer (7.4 pH). Brains were removed and postfixed for 1–2 h at 4°C and then immersed in 20% sucrose phosphate buffer overnight. Forty-micrometer-thick sections were cut on a freezing microtome and placed in 0.1 M phosphate-buffered saline (PBS). Sections from wild-type (+/+) and homozygous (−/−) mice were processed simultaneously in the same solutions to reduce variability in immunohistochemical processing. Immunohistochemistry was performed in free-floating sections, as follows. 1) immersion in 1% hydrogen peroxide for 10 min, followed by 4 × 5-min rinses in PBS; 2) 30-min incubation in PBS containing 0.1% bovine serum albumin and 0.05% Triton X-100 (PBS+); 3) overnight (10–16 h) incubation in anti-β-gal polyclonal primary antibody (1:5,000; rabbit: Covance, Madison, WI); 4) 10-min incubation in a 1:500 dilution of biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS+, followed by 3 × 5-min rinses in PBS+; 5) 1 h incubation in avidin-biotin-peroxidase complex solution (ABC-elite, 1:1,500; Vector) in PBS+, followed by 3 × 5-min rinses in PBS+; and 6) 10-min incubation in 0.02% 3,3'-diaminobenzidine (DAB) in 0.05 M phosphate buffer and 0.03% hydrogen peroxide, followed by 3 × 5-min rinses in PBS. Sections were mounted on coated slides, air-dried for 16 h, dehydrated in alcohol and xylenes, and coverslipped with DPX.

Sections were examined under brightfield optics using a Leica DMRXA photomicroscope (Leica, Deerfield, IL). Digital microscopy images were captured using a Phase I digital camera (PhaseOne, Northport, NY), sized, and balanced for brightness and contrast using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA). Photomicrographs were printed on a Fujif Pictography 3000 printer (Fujif PhotoFilm, Tokyo).

Slice preparation and electrophysiology

Juvenile (21- to 31-day old) rats or mice were decapitated, the MOB dissected out and immersed in artificial cerebrospinal fluid (ACSF, see following text) at 4°C as previously described (Heyward et al. 2001). Horizontal slices (400-μm-thick) of the MOB were cut parallel to its long axis using a vibratome (Vibratome Series 1000, Ted Pella, Redding, CA). After a period of recovery (30 min) at 30°C, the slices were incubated in a holding bath at room temperature (22°C) until used. For recording, a single brain slice was placed in a perfusion-bath recording chamber mounted on a microscope stage and maintained at 30 ± 0.5°C. Slices were submerged in ACSF (see following text for details on composition) flowing at 2.5–3 ml/min.

Visually guided recordings from neurons in the MC layer were made with near-infra red differential interference contrast (NIR DIC) optics, water-immersion objectives, and a BX50WI microscope (Olympus Optical, Tokyo) (Stuart et al. 1993). NIR transillumination was at 900 nm (filter transmission, 850–950 nm) concentric with the objective and optimized for DIC. A 0.25-in CCD camera (CCD 100, Dage, Stamford, CT), fitted with a 3-to-1 zooming coupler (Optem, Fairport, NY) was used. Contrast was enhanced in real-time using an image processor (Model 794, Hughes Aircraft Company, Carlsbad, CA) and the image was displayed on a monochrome monitor (Dage HR120, Dage-MTI, Michigan City, IN).

Recordings were made using conventional whole cell patch-clamp methods. Recording pipettes were pulled on a Flaming-Brown P-97 puller (Sutter Instrument, Novato, CA) from standard-wall filamented 1.5-mm-diam borosilicate glass; tip diameter was 2–3 μm, tip resistance was 5–8 MΩ. Seal resistance was routinely >1GΩ. Liquid junction potential was 9–10 mV; reported voltage measurements were not corrected for this potential. Data were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Analog signals were recorded, low-pass Bessel filtered at 2 kHz, and digitized on videotape (AR Vetter, Rebersburg, PA) and computer disk (Axoscope/Clampex, Axon Instruments). Data were also collected through a Digidata-1200A Interface (Axon Instruments) and digitized at 10 kHz. Holding currents were generated under manual control by the recording amplifier.

Membrane depolarizations were calculated from the down-state membrane potential associated with mitral cell (MC) bistability. In some cases during agonist application, the down-state stability was eliminated (see Fig. 7A). In this case, the membrane potential was calculated from the potential immediately after an action potential. Current versus voltage (I-V) plots were obtained from steady-state currents (see Fig. 6B, inset) over potentials from −120 to +20 mV using a voltage step (10-mV step intervals, 600 ms/step, Vhold = −60 mV) protocol in the presence of synaptic blockers and TTX. Membrane resistance was calculated from the amount of steady-state current required to hyperpolarize the resting potential of the cells by 10 mV, from −60 to −70 mV. Distributions of membrane potential (see Fig. 7D) were constructed by all-points analysis of digitized
records (pClamp, Axon Instruments), with voltage data points (excluding action potential peaks) in digitized records (sampled at 2 kHz) binned by amplitude. For each time point of the digitized recording, the membrane potential was determined, counted, and subsequently plotted in binned format. As previously described (Heyward et al. 2001), this analysis reveals the range of membrane potentials encompassing MC bistability as well as the duration of time the cell spends at each potential. Curve fitting was performed using pClamp analysis software (Axon Instruments). Mean spike rates were calculated over a 30 s period prior to, during, and after drug application. In current-clamp recordings, membrane input resistance was calculated from voltage changes in response to hyperpolarizing current pulses (1 s, −40 to −80 pA, 0.05 Hz). In voltage-clamp recordings, membrane resistance was calculated from current changes in response to voltage pulses (1 s, −10 to −20 mV, 0.05 Hz). Numerical data are expressed as the means ± SE. Unless otherwise described, tests for statistical significance (P < 0.05) were performed using Student’s t-test.

Biocytin (0.05% to 0.1%, Molecular Probes) was added to the pipette solution in some experiments to allow histological confirmation of recorded cells. The presence of biocytin had no evident effect on MC electrophysiology. After recording, slices were fixed overnight in phosphate-buffered 4% paraformaldehyde at 4°C. Slices were incubated with Cy3-conjugated Streptavidin (Jackson ImmunoResearch Laboratories) and processed for later visualization and cell identification with laser-scanning confocal microscopy (FluoView confocal microscope, Olympus Instruments, Long Beach, CA) as described previously (Puche and Shipley 2001).

Olfactory nerve stimulation

The olfactory nerve layer was focally stimulated with a bipolar electrode constructed from a pair of twisted stainless-steel wires (70 μm), insulated except for bluntly cut tips. The tips of the wires were placed tangentially along the peripheral surface of the olfactory nerve layer, slightly rostral to the estimated location of the recorded MC. Stimuli were isolated monophasic square wave pulses (10−200 μA in amplitude, 0.1 ms in duration) delivered by a Grass S8800 stimulator (Astro-Med, West Warwick, RI) and an isolated constant current source (Grass PSIU6, Astro-Med).

Drugs and solutions

The standard ACSF consisted of (in mM) 120 NaCl, 3 KCl, 1.3 CaCl2, 1.3 MgSO4, 10 glucose, 25 NaHCO3, and 5 N,N,N,N-tetraacetic acid (BAPTA)-AM (20 μM). In a subset of experiments, slices were incubated in bis-(o-aminophenoxy)-N,N,N,N′-tetraacetic acid (BAPTA)-AM (20 μM) to block potassium channels. In other experiments, slices were incubated in (−)-carboxycyclopropyl)-9H-xanthine-9-propanoic acid (LY341495), (S)(−)-α-amino-4-carboxy-2-methylbenzenecarboxylic acid (MS41495), nickel chloride (Ni2+), TEA, and tetrodotoxin (TTX). All drugs were supplied by Tocris (Ellisville, MO), except for 4-AP, barium chloride, sodium chloride, cesium chloride, gabazine, nickel chloride, TEA chloride, and TTX (Sigma-Aldrich, St. Louis, MO). A salt-agar bridge was used as the reference electrode during experiments in which MCGP was bath applied to avoid changes in junction potential at the silver/silver chloride reference electrode otherwise seen with this drug.

Results

MCs were identified visually with NIR-DIC microscopy by their large cell bodies located in the distinct narrow band of the MC layer and by intrinsic properties such as membrane potential bistability (Heyward et al. 2001; see Fig. 7A) and input resistance (rat, 207±10.4 MΩ, n = 42; mouse, 177±14.4 MΩ, n = 86). Location and bistability distinguish MCs from TCS in the adjacent external plexiform layer and from the small, high resistance (typically >1GΩ) granule cells (GCs) present in the MC layer (Heyward et al. 2001; Schoppa et al. 1998). In some experiments, MC identification was verified by subsequent histological examination (n = 6). Data are reported for recordings from 97 rat and 174 mouse MCs.

Group I/II mGluR agonists depolarize and increase MC firing rate

We first investigated the actions of the selective group I/II mGluR agonist, ACPD, on MC membrane potential and spontaneous spike generation in current-clamp recording mode. As shown in Fig. 1A, bath application of ACPD (50 μM) strongly depolarized all MCs tested. The depolarizing effect of ACPD on MCs was substantially larger in rats than in wild-type mice (peak membrane depolarization (ΔmV) in rats = 14.1±1.4 mV, n = 23; Δ mV in mice = 6.1±0.7 mV, n = 12; P = 2.97E-4). ACPD-evoked depolarization was accompanied by an increased action potential firing rate. With prolonged application (>2 min) of ACPD, the initial excitation was invariably followed by spike broadening, decreased spike amplitude, and gradual reduction in firing of MCs (i.e., depolarization block). All of these effects were reversible during washout and were repeatable without apparent desensitization (Fig. 1B).

To determine if these effects were mediated by direct activation of mGluRs on MCs, ACPD was applied in the presence of blockers of ionotropic glutamate and GABA_A receptors (Figs. 1B and 2): CNQX (10 μM), APV (50 μM), and gabazine (5 μM). Under these conditions, the depolarizing action of ACPD persisted (Δ mV in rat MCs = 14.3±2 mV, n = 6) and was virtually identical to that observed in control ACSF (14.1±1.4 mV, n = 23; P = 0.94). Taken together, the results suggest that depolarization and increased firing elicited by ACPD are mediated directly by activation of group I/II mGluRs on MCs.

Bath application of MCGP (100–500 μM), a nonselective group I/II mGluR antagonist, blocked the effects of ACPD (50 μM) in rat MCs (Figs. 1C and 2); Δ mV in the presence of ACPD and ACPD + MCGP was 14.1±1.4 mV (n = 23) and 2.9±0.6 mV (n = 13), respectively. Application of LY341495 (10–100 μM), a blocker of all mGluR subtypes at micromolar concentrations (Kingston et al. 1998), prevented or reversed both the ACPD-evoked membrane depolarization and increased firing (Δ mV in presence of LY341495 + ACPD = −1.2±0.1 mV, P = 0.14, n = 8). After washout of LY341495,
FIG. 1. The group I/II metabotropic glutamate receptor (mGluR) agonist (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD) and the group I mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) depolarize and increase the firing of mitral cells (MCs). A: bath application of 50 μM ACPD resulted in membrane potential depolarization and increased action potential firing in this rat MC. The initial excitation was followed by gradual spike broadening, decreased spike amplitude and reduction in firing indicative of depolarization block. B: a similar response to ACPD (50 μM) was obtained in the presence of blockers of fast synaptic transmission [6-cyano-7-nitroquinolinolene-2,3-dione (CNQX), 2-amino-5-phosphonovaleric acid (APV), and 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide (gabazine)] in another rat MC. There was no apparent desensitization with repeated application of ACPD. C: the group I/II mGluR antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG, 100 μM) blocks the response of another MC to ACPD. After washout (>5 min), re-application of ACPD depolarized and increased spontaneous discharge of the same cell. D–G: MCs were activated by group I, but not group II or III, mGluR agonists. Bath application of the group III mGluR agonist L(±)-2-aminobutyric acid (AP4; 100 μM, D) or the group II mGluR agonist (2S,3S,4S)-CCG/(2S,1R,2S)-2-(carboxycyclopropyl)glycine (L-CCG-I; 20 μM, E) did not alter the membrane potential or firing rate of this mouse MC in current-clamp recordings. F: the same cell was activated by the selective group I agonist DHPG. G: the selective mGluR1 antagonist (S)-α-amino-α-[(1S,2S)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY367385, 100 μM) blocked the actions of the group I mGluR agonist DHPG (50 μM) in another mouse MC. After washout (>15 min), re-application of DHPG robustly depolarized and increased MC spontaneous discharge. All experiments (except in A) were performed in the presence of blockers of fast synaptic transmission: CNQX (10 μM), APV (50 μM), and gabazine (5 μM).

FIG. 2. Bar graph summarizing the effects of mGluR agonists and antagonists on MC membrane potential. Responses are expressed as the change in membrane potential elicited by application of mGluR agonists, alone or in combination with mGluR antagonists. All data shown, except that for ACPD in the first bar, were collected in the presence of synaptic blockers (CNQX, 10 μM; APV, 50 μM; gabazine, 5 μM). *, statistically significant difference from control (P < 0.05).
re-application of ACPD depolarized and increased the firing rate of MCs.

**Selective group I mGluR agonists activate MCs**

To investigate the specific class of mGluRs mediating the effect of ACPD, we next explored the actions of preferential agonists for the three mGluR groups. In current-clamp recordings from mouse MCs, neither the group II agonist L-CCG-I (20 μM) nor the group III agonist AP4 (100 μM) depolarized MCs or increased their firing rate (Fig. 1D and E; \(P = 0.24\) and \(P = 0.27\), respectively, \(n = 5\)). Similarly, neither L-CCG-I (\(n = 7\), \(P = 0.21\)) nor AP4 (\(n = 9\), \(P = 0.32\)) elicited significant shifts of the holding current in voltage-clamp recordings (\(V_{\text{hold}} = -60\) mV). By contrast, the preferential group I agonist DHPG (50 μM) depolarized mouse and rat MCs and increased their firing rate (Figs. 1F and 2). DHPG (50 μM) elicited a peak membrane depolarization of 14.7 ± 1.9 mV in mice (\(P = 1.37E-6\), \(n = 16\)). In voltage clamp (\(V_{\text{hold}} = -60\) mV), DHPG (50 μM) evoked a robust inward current of \(-110.0 ± 10.9\) pA (\(P = 4.45E-10\), \(n = 25\)) in the presence of synaptic blockers [CNQX (10 μM), APV (50 μM), and gabazine (5 μM)].

Group I mGluRs include the mGluR1 and mGluR5 subtypes. MCs express high levels of mGluR1, but mGluR5 appears to be absent or expressed at very low levels in MCs. This suggests that the effects of DHPG are mediated via activation of mGluR1s on MCs. To test this hypothesis, we first investigated the effects of the preferential mGluR5 agonists as selective mGluR1 agonists are not available. The mGluR5 agonist CHPG (50 μM) was without excitatory effect on MCs (\(P = 0.18\), \(n = 8\)), suggesting that the effects of ACPD and DHPG are mediated through the mGluR1 receptor subtype.

We next assessed the specificity of DHPG with a preferential mGluR1 antagonist, LY367385 that has negligible actions on group II and III mGluRs and antagonizes mGluR5 only at concentrations in excess of 100 μM (Clark et al. 1997; Salt et al. 1999). In the presence of LY367385 (100 μM), DHPG did not change the membrane potential of MCs (Figs. 1G, left and 2); \(\Delta\) mV in DHPG = 14.7 ± 1.9 mV (\(n = 16\)) versus DHPG + LY367385 = 0.4 ± 0.01 mV (\(n = 7\)). After washout, re-application of DHPG depolarized and increased MC firing rate (Fig. 1G, right).

**DHPG-evoked excitation of MCs is present in mGluR5 knockout mice but absent in mGluR1 knockout mice**

The preceding pharmacological results suggest that the effect of DHPG on MCs is mediated by activation of mGluR1. However, because the specificity of mGluR agonists and antagonists is limited and varies with concentration, we took advantage of mice with targeted deletion of the mGluR1 receptor gene to further investigate the function of this receptor subtype on MCs (Conquet et al. 1994). In this transgenic strain, the mGluR1 gene sequence has been replaced with that for β-galactosidase (β-gal) by homologous recombination; therefore in “knockout” (KO) mice β-gal is present only in cells that would normally express mGluR1. We first examined the location of β-gal-positive (β-gal+) neurons in mGluR1 KO and wild-type littermate (WT) mice using immunohistochemistry (see METHODS). As shown in Fig. 3, the distribution of β-gal+ neurons in the MOB and other brain structures is consistent with previous reports on the distribution of neurons expressing mGlu1 receptors in mGluR1 knockout and wild-type mice as revealed by β-gal immunohistochemistry (see METHODS for details). A: in sections of mGluR1 mutant (−/−) mice, β-gal staining is prominent in the cerebellum (Cb), hippocampus (Hc), thalamus (Th), lateral septum (LS), superior colliculus (SC), inferior olive (I0), main olfactory bulb (MOB), and throughout the cortex (Cx). B: higher magnification illustrates the staining pattern in the MOB. β-gal staining is present in neurons in the MC (MCL) and glomerular layers (GL) and also in scattered cells in the external plexiform layer (EPL). No staining was present in the internal plexiform (IPL) or granule cell layers (GCL). C: β-gal staining was absent in sections harvested from mGluR1 wild-type (+/+) mice.
mGluR1 KO mice (Fig. 4B), the same concentration of DHPG had no discernible effects on MCs (ΔmV = 0.02 ± 0.03 mV, P = 0.92, n = 9). Likewise, in slices from mGluR1 KO mice (Fig. 4C), the group I/II mGluR agonist ACPD did not depolarize MCs (ΔmV = −0.5 ± 0.5 mV, P = 0.38, n = 8); in some of these slices, ACPD produced a small reduction of MC firing rate, although this trend did not reach statistical significance (P = 0.15). MC input resistance and resting membrane potential were similar in wild-type [177 ± 14.4 MΩ (n = 86), −52.2 ± 0.8 mV (n = 28)] and mGluR1 KO mice [152.4 ± 42.9 MΩ (n = 12), −54.2 ± 2.4 mV (n = 8)]. In slices from mGluR5 KO mice, DHPG activated MCs in a similar manner to that seen in WT mice (Fig. 4D; ΔmV = 14.7 ± 1.3 mV, P = 2.69E-8, n = 8). As seen in WT mice, the response of MCs in mGluR5 KO mice to DHPG was much stronger than the response to ACPD (ΔmV = 6.0 ± 1.2 mV, P = 3.54E-4, n = 8). Taken together, the preceding results indicate that the excitatory effect of DHPG is mediated entirely by direct activation of mGluR1 on MCs.

DHPG elicits an inward current in MCs

We next investigated the properties of the DHPG-evoked current in MCs in voltage-clamp recording mode (Vhold = −60 mV) in the presence of synaptic blockers and TTX (1 μM) to prevent indirect effects of DHPG. The amplitude of the DHPG-evoked current in synaptic blockers (−110.0 ± 10.9 pA, P = 4.45E-10, n = 25) was similar to that in blockers and TTX (−101.2 ± 32.9 pA, n = 6), indicating that fast sodium channels are not substantially involved in the inward current. DHPG evoked an inward shift of the holding current in a dose-dependent manner (Fig. 5A). The dose-response relationship revealed an EC50 of 27.4 μM (Fig. 5B). The preferential mGluR5 agonist CHPG (50–100 μM) had no effect on the holding current, consistent with its lack of effect in current-clamp recordings.

The I-V relationship of the DHPG-evoked current is shown in Fig. 6. DHPG (50 μM) induced an inward shift in holding current at all membrane potentials examined (Fig. 6A). The DHPG current obtained by subtracting the control (normal ACSF) and DHPG I-V curves (difference current) exhibited voltage dependency with currents of larger amplitude at more depolarized membrane potentials (Fig. 6B). For example, the amplitude of the DHPG current at −30 mV (−129.7 ± 13.3 pA) was significantly larger (P = 0.0001, n = 9) than that at −90 mV (−61.4 ± 8.3 pA); the peak current occurred at −20 mV (−148.3 ± 10.9 pA). There was no significant change in membrane input impedance associated with the DHPG-induced inward current (121.2 ± 22.4 vs. 123.2 ± 25.2 MΩ, P = 0.53, n = 11). This suggests that either the DHPG-induced current is mediated without change in input impedance or that several ionic conductances are activated in response to DHPG, and the resulting current reflects increased activation of inward currents and inhibition of outward currents.

mGluR1-induced depolarizations and inward currents in CNS neurons, including MCs in culture, have been ascribed to the inhibition of K+ currents (Anwyl 1999; Charpak et al. 1990; Guérit et al. 1994; Schoppa et al. 1997). To investigate the potential involvement of K+ ions, we studied the effects of K+ channel blockers, 120 mM Cs+ and 20 mM TEA (included in the pipette solution, see METHODS), on the DHPG-
evoked current (Fig. 6, C and D). This reduced the inward current at relatively depolarized membrane potentials, i.e., positive to −40 mV, suggesting that at least part of the current is mediated by closure of K⁺ channels. The inward current increased at more hyperpolarized potentials and persisted in the presence of the K⁺ blockers, suggesting that DHPG current consists of multiple components. Because activation of mGluR1 is also known to induce currents that are triggered by a rise in intracellular Ca²⁺ levels, we next investigated the effects of the K⁺ blockers combined with bath-applied BAPTA-AM (20 μM) to chelate intracellular Ca²⁺ (Tsien 1980). Under these conditions (Fig. 6, E and F), the DHPG current was eliminated except in a narrow voltage range between −60 and −40 mV. This residual current was completely blocked by further addition of the voltage-dependent Ca²⁺ channel blockers Cd²⁺ (100 μM) and Ni²⁺ (100 μM; Fig. 6, G and H). Taken together, these results indicate that the DHPG current consists of multiple components.
Tonic modulation of MC membrane excitability by mGluR activation

Previous studies indicate that MCs exhibit membrane bistability, a property that significantly influences their excitability and sensory responses (Heyward et al. 2001). Although bistability is generated by MC intrinsic membrane properties (Heyward et al. 2001), it may be modulated by extrinsic input. Therefore we investigated the effects of activation and inactivation of mGluRs on bistability. As reported previously (Heyward et al. 2001) and shown in Fig. 7A, MCs exhibit intrinsic membrane potential bistability, spontaneously alternating between two discrete membrane potentials differing by ~10 mV: a more depolarized “up state,” perithreshold for spike generation, and a more hyperpolarized “down state” devoid of spiking. Bistability is present in both rat and mice MCs, but the up state is more pronounced and prolonged in rat than mouse (Heinbockel and Heyward, unpublished observations). In the presence of synaptic blockers, ACPD (50 μM) or DHPG (50 μM) increased MC firing and modulated MC bistability. During ACPD-evoked depolarizations, down-state stability was eliminated, such that MCs spiked repetitively, returning immediately to the up state after a spike (Fig. 7A, middle).

Application of mGluR antagonists also dramatically altered bistability in rat MCs. MCPG (100 to 500 μM) significantly prolonged the duration of the up and down states (Fig. 7, B and C; down state, $P = 8.33E-5$, $n = 36$; up state, $P = 1.51E-7$, $n = 36$). These effects were accompanied by a significant reduction in spontaneous firing rate ($4.3 \pm 0.6$ vs. $3.2 \pm 0.51$ Hz, $n = 32$; $P = 3.53E-6$). MCPG also reduced spontaneous spiking in mouse MCs, although this was not quantified. All-points analysis of the membrane potential (Heyward et al. 2001) revealed a shift toward more time spent in the down state and a hyperpolarizing shift of the up-state membrane potential (Fig. 7D). Similar effects on membrane bistability were observed with the broad-spectrum mGluR antagonist LY341495 (100 μM, $n = 4$) and the mGluR1-selective antagonist LY367385 (100 μM, $n = 6$). All of these effects occurred in the presence of fast synaptic blockers (i.e., CNQX, APV, gabazine), which had no effect on bistability ($n = 33$) as previously reported (Heyward et al. 2001). Application of synaptic blockers did, however, eliminate MC responses to olfactory nerve stimulation ($n = 9$), indicating that iotopic glutamate receptor-mediated synaptic inputs to these cells were completely blocked. These results suggest that bistability and MC excitability are tonically modulated, at least in part, by endogenously released glutamate acting at mGluR1.

Blockade of mGluRs reduces MC responses to olfactory nerve input

Because mGluR antagonists potently modulated bistability, a property that regulates the responsiveness of MCs to olfactory nerve input (Heyward et al. 2001), we investigated the effect of mGluR antagonists on rat MCs to single-pulse (0.1 Hz) stimulation of the olfactory nerve. The intensity of olfactory nerve input (Heyward et al. 2001), we investigated the effect of mGluR antagonists on rat MCs to single-pulse (0.1 Hz) stimulation of the olfactory nerve. The intensity of olfactory nerve input was adjusted to elicit a short-latency spike (~60–80% of the stimulus trials). The selective mGluR1 antagonist LY367385 (10–100 μM) reversibly reduced the response probability to olfactory nerve stimulation, i.e., the occurrence of a short-latency spike (Fig. 8, A and B) from.
73.2 ± 4.9 to 50.4 ± 7.5% (n = 10, P = 0.0011). LY367385 also significantly increased the latency of olfactory nerve-evoked short-latency spikes (Fig. 8, A and C) from 10.6 ± 1.1 to 13.0 ± 1.3 ms (n = 10, P = 1.41E-4). These effects on olfactory nerve-evoked responses were not accompanied by change in overall membrane potential. Similar effects were observed in wild-type mouse MCs where the mGluR antagonists LY341495 or LY367385 (100 μM) reduced the probability of olfactory nerve-evoked responses from 89.2 ± 4.4 to 41.5 ± 8.7% (n = 9, P = 5.07E-4). Thus selective inactivation of mGluR1 reduces sensory-evoked spiking in MCs.

To investigate if the preceding effects were due to presynaptic modulation of olfactory nerve terminals versus postsynaptic modulation of MC excitability, we recorded olfactory nerve-evoked excitatory postsynaptic currents (EPSCs) before and during bath application of LY367385. The amplitude, latency, and time course of olfactory nerve-evoked EPSCs were not altered by LY367385 (Fig. 8D, n = 6; amplitude: 106 ± 29.7 vs. 108.5 ± 26.5 pA, P > 0.05; latency: 2.1 ± 0.5 vs. 1.8 ± 0.4 ms, P > 0.05; 10–90% rise time: 1.6 ± 0.4 vs. 1.8 ± 0.4 ms, P > 0.05; half-width: 4.4 ± 2.1 vs. 5.3 ± 1.9 ms, P > 0.05). These findings indicate that LY367385 does not presynaptically alter the excitability of olfactory nerve terminals.

**DISCUSSION**

The present results demonstrate an important role of mGluR1 in modulating the excitability of MCs. Broad-spectrum (i.e., ACPD) and selective group I (i.e., DHPG) mGluR agonists potently and dose dependently depolarized and increased the firing rate of MCs. These effects were due to direct activation of mGluR1 on MCs as they persisted undiminished in the presence of synaptic blockers and/or TTX and were absent in transgenic animals with targeted deletion of the mGluR1 gene. Blockade of group I receptors or mGluR1 significantly reduced MC spontaneous firing and olfactory nerve-evoked discharge of MCs, suggesting that endogenously released glutamate acting at mGluR1 tonically modulates the basal and sensory-evoked discharge of MCs.

**Activation of mGluR1 directly depolarizes MCs**

Application of either the mixed group I/II agonist ACPD or the preferential group I agonist DHPG resulted in a uniform and reversible membrane potential depolarization and increased rate of action potential generation in both rat and mouse MCs. DHPG was typically more potent in depolarizing MCs than ACPD at similar concentrations, consistent with the higher potency of DHPG at group I receptors (Conn and Pin 1997; Schoepp et al. 1999). Overall, mGluR agonists had more robust effects in rats than in mice, possibly linked to different levels of receptor expression or differences in intracellular messenger machinery. The excitatory effects of ACPD or DHPG were not mediated by activation of ionotropic glutamate receptors.
receptors as a result of increased glutamate release from MCs or other MOB neurons nor due to decreased activation of GABA_A receptors on MC (i.e., disinhibition). This was ascertained by the lack of significant effects of synaptic blockers (CNQX, AP5, gabazine) on ACPD- or DHPG-evoked depolarizations. In voltage clamp, the inward current elicited by ACPD or DHPG persisted undiminished in synaptic blockers and/or TTX, suggesting that such currents are not mediated by changes in action-potential transmitter release from neuronal elements presynaptic to MCs.

ACPD and DHPG can activate both group I receptor subtypes, mGluR1 and mGluR5. Several results indicate that the actions of these agonists on MCs are mediated exclusively via activation of mGluR1: 1) the excitatory effects of ACPD and DHPG were completely absent in mice with targeted deletion of the mGlu1 but not the mGlu5 receptors; 2) the effects of DHPG were blocked by the highly selective mGluR1 antagonist LY367385 (IC_50 = 8.8 µM) (Clark et al. 1997; Marino et al. 2001; Rivadulla et al. 2002) at concentrations that do not affect responses mediated by ionotropic glutamate receptors or other mGluR subtypes (Clark et al. 1997; Salt et al. 1999); and 3) the preferential mGluR5 agonist CHPG was without effect. Additionally, neither group II nor group III receptors contribute discernibly to ACPD- or DHPG-evoked depolarizations or inward currents in MCs as preferential agonists for these mGluR classes, CCG and AP4, respectively, were without effect. We cannot exclude the possibility, however, that group II or III receptors modulate other currents, e.g., high-threshold Ca^{2+} currents (Trombley and Westbrook 1992) that would have been inactive at the holding potentials used in these experiments (−50 to −60 mV).

Activation of mGluR1 induces an inward current in MCs

Both ACPD and DHPG elicited an inward current in voltage-clamp recordings of rat and mouse MCs. The EC_50 of the DHPG-evoked current, 27.4 µM, is comparable to that reported elsewhere (Schoepp et al. 1999). This inward current, similar to DHPG currents in neurons in several brain areas (see Anwyl 1999 for review) has multiple components. One component of the inward current was mediated by a K^+ conductance as it was abolished by TEA and Cs^{2+}. This result agrees in part with previous observations by Schoppa and Westbrook (1997) that intracellular Cs^{2+} reduces inward currents evoked by DHPG in cultured MCs. A second component requires a rise in intracellular Ca^{2+} as it was abolished by BPAT-AM. The identity of this BPAT-sensitive current remains to be determined but may involve Ca^{2+}-dependent nonselective cation conductances (Congar et al. 1997; Crepel et al. 1994; Guerinneau et al. 1995; Raggenbass et al. 1997) or electroneutral Na^+/Ca^{2+} exchangers (Keele et al. 1997; Lee and Boden 1997; Staub et al. 1992). A third component, present at membrane potentials from −60 to −40 mV in the presence of BPAT, TEA and Cs^{2+}, appears to involve voltage-gated Ca^{2+} channels as it was eliminated by Cd^{2+} and Ni^{2+}. Additional experiments are necessary to delineate the I-V characteristics and other properties of the individual conductances elicited in MCs by DHPG.

Endogenous glutamate tonically modulates MC excitability via activation of mGluR1

An important finding of the present study is that blockade of mGluRs reduces MC spontaneous and sensory-evoked excitability via modulation of membrane bistability, an intrinsic property of MCs. mGluR antagonists (LY367385, LY341495, MCPG), in the presence of fast synaptic blockers, lengthened the periodicity of bistability by significantly prolonging the duration of both the up and down states. Because the prolongation of the subthreshold down state was proportionately greater than that of the up state, mGluR1 inactivation causes MCs to spend more time at this hyperpolarized level in which they do not spontaneously spike (Heyward et al. 2001). Additionally, mGluR inactivation produced a hyperpolarizing shift of the range of normally perithreshold up-state membrane potentials, further biasing MCs away from spike threshold. Together, these actions reduce MC excitability, consistent with the observed reduction in spontaneous firing rate. These actions of mGluR antagonists contrast with the lack of effect of last synaptic blockers on MC bistability (Heyward et al. 2001).

Bistability is an important determinant of MC responses to olfactory nerve input. In the perithreshold up state, MCs readily launch short-latency spikes in response to olfactory nerve input (Hayar et al. 2001; Heyward et al. 2001). By contrast, in the hyperpolarized down state, olfactory nerve input often fails to elicit spikes in MCs or spikes are evoked at long latency. The increase in latency corresponds to the time to depolarize from the down state to ramp or up-state potentials close to spike threshold (Heyward et al. 2001). Thus events that prolong the down state and/or produce a hyperpolarizing shift of the up-state membrane potentials should render MCs less likely to launch spikes and/or increase the latency of spikes in response to olfactory nerve input. Both of these effects were observed during inactivation of mGluR1: olfactory nerve-evoked spiking in MCs was significantly reduced and the latency of evoked spikes increased. As mGluR1 antagonism did not modify olfactory nerve-evoked EPSCs in MCs, the effects on olfactory nerve-evoked spiking appear to be mediated by modulation of bistability. These results, consistent with in vitro findings in rat sensorimotor cortex (Bandrowski et al. 2003), indicate that ambient glutamate levels tonically modulate MC excitability via activation of mGluR1.

Functional considerations

Localization of mGluR1 to MC apical dendrites suggests a role of these receptors at glutamatergic synapses from olfactory nerve terminals (van den Pol 1995) and/or glutamate-mediated recurrent excitation (i.e., spillover) among MC apical dendrites (Aroniadou-Anderjaska et al. 1999b; Carlson et al. 2000; Friedman and Strowbridge 2000; Isaacsen 1999; Salin et al. 2001; Schoppa and Westbrook 2001). Another possible source of ambient glutamate is glial cells, which have intimate relationships with MC dendrites in the glomerular layer (Chao et al. 1997; Kasowski et al. 1999). Tonic activation of mGluR1 by baseline glutamate levels may serve to facilitate or amplify sensory responses of MCs. mGluR1-mediated excitation is typically slow in time course and preferentially engaged by high-frequency activity (Anwyl 1999). Thus mGluR1 responses in MCs would tend to be preferentially activated over
successive sniff cycles during odor inhalation. MC responses to odors are characterized by specific temporal firing patterns, including oscillations and synchronous activity (Friedrich and Lauren 2001; Kauer 1998; Kay and Laurent 1999: Luo and Katz 2001; Spors and Grinvald 2002). In this regard, it is noteworthy that mGluR antagonism (i.e., with MCPG) was found to significantly reduce olfactory nerve-evoked rhythmic oscillations in MCs (Schoppa and Westbrook 2001). It is reasonable to speculate therefore that activation of mGluR1s on MC/TC apical dendrites plays an important role in odor-evoked oscillations and coding of olfactory stimuli.

mGluR1s are also present on portions of MC lateral dendrites presynaptic to GC dendrites (van den Pol 1995). This suggests that mGluR1 may function, in part, to increase the degree or duration of depolarization of the lateral dendrite after glutamate release from neighboring MCs/TCs. Spillover-mediated activation of mGluRs among MCs strongly activated by a particular odorant may presynaptically enhance glutamate release onto GCs, increasing lateral inhibition and thus contrast between strongly and weakly activated MCs (Kinzie et al. 1998; van den Pol 1995).

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