Dopamine D2 Receptor–Mediated Presynaptic Inhibition of Olfactory Nerve Terminals

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Ennis, Matthew, Fu-Ming Zhou, Kelly J. Ciombor, Vassiliki Aroniadou-Anderjaska, Abdallah Hayar, Emiliana Borrelli, Lee A. Zimmer, Frank Margolis, and Michael T. Shipley. Dopamine D2 receptor–mediated presynaptic inhibition of olfactory nerve terminals. *J Neurophysiol* 86: 2986–2997, 2001. Olfactory receptor neurons of the nasal epithelium project via the olfactory nerve (ON) to the glomeruli of the main olfactory bulb, where they form glutamatergic synapses with the apical dendrites of mitral and tufted cells, the output cells of the olfactory bulb, and with juxtaglomerular interneurons. The glomerular layer contains one of the largest population of dopamine (DA) neurons in the brain, and DA in the olfactory bulb is found exclusively in juxtaglomerular neurons. D2 receptors, the predominant DA receptor subtype in the olfactory bulb, are found in the ON and glomerular layers, and are present on ON terminals. In the present study, field potential and single-unit recordings, as well as whole cell patch-clamp techniques, were used to investigate the role of DA and D2 receptors in glomerular synaptic processing in rat and mouse olfactory bulb slices. DA and D2 receptor agonists reduced ON-evoked synaptic responses in mitral/tufted and juxtaglomerular cells. Spontaneous and ON-evoked spiking of mitral cells was also reduced by DA and D2 agonists, and enhanced by D2 antagonists. DA did not produce measurable postsynaptic changes in juxtaglomerular cells, nor did it alter their responses to mitral/tufted cell inputs. DA also reduced *1*) paired-pulse depression of ON-evoked synaptic responses in mitral/tufted and juxtaglomerular cells and *2*) the amplitude and frequency of spontaneous, but not miniature, excitatory postsynaptic currents in juxtaglomerular cells. Taken together, these findings are consistent with the hypothesis that activation of D2 receptors presynaptically inhibits ON terminals. DA and D2 agonists had no effect in D2 receptor knockout mice, suggesting that D2 receptors are the only type of DA receptors that affect signal transmission from the ON to the rodent olfactory bulb.

INTRODUCTION

Odor signals are transduced by olfactory receptor neurons in the nasal epithelium and relayed to the glomeruli of the main olfactory bulb (MOB) via the olfactory nerve (ON). In the glomeruli, ON terminals form glutamatergic, axodendritic synapses with mitral and tufted (M/T) cells (Aroniadou-Anderjaska et al. 1997; Berkowicz et al. 1994; Ennis et al. 1996), the output cells of the MOB, and with juxtaglomerular (JG) interneurons (Bardoni et al. 1996; Keller et al. 1998; Kosaka et al. 1997; Pinching and Powell 1971). While the glomeruli are the first stage of synaptic processing in the olfactory system, little is known about intraglomerular synaptic mechanisms that process sensory input.

Previous studies demonstrate that most JG cells contain GABA (Ribak et al. 1977) and/or dopamine (DA) (Davis and Macrides 1983; Halasz et al. 1981; McLean and Shipley 1988). The DA JG neurons are abundant; the rat MOB contains more DA neurons (100,000–150,000) (McLean and Shipley 1988) than the entire substantia nigra and ventral tegmental area midbrain DA system (~30,000) (Björklund and Lindvall 1984). Many JG cells colocalize GABA and DA (Gall et al. 1987; Kosaka et al. 1985). In the rat, 69% of immunoreactive neurons in the glomerular layer (GL) colocalize DA and GABA, while only 4% contained DA alone (Gall et al. 1987; Kosaka et al. 1985).

The roles of GABAergic and dopaminergic JG neurons in glomerular synaptic processing are not understood, although recent studies suggest that JG interneurons may function, in part, to presynaptically regulate sensory input to the glomeruli. GABA$_B$ receptors are present on ON terminals in the glomeruli (Bonino et al. 1999; Margeta-Mitrovic et al. 1999), where they inhibit glutamate release from the ON (Aroniadou-Anderjaska et al. 2000). Anatomical evidence also indicates that DA D2 receptors are present on ON terminals. Olfactory receptor neurons express DA D2 receptors, and in the MOB, D2 receptors are localized exclusively in the ON and glomerular layers (Coronas et al. 1997; Koster et al. 1999; Nickell et al. 1991). Bulbectomy, a manipulation that causes retrograde degeneration of olfactory receptor neurons, eliminates D2 receptor mRNA in the olfactory epithelium (Koster et al. 1999). These findings suggest that D2 receptors are expressed by olfactory receptor neurons and are translocated to ON terminals in MOB.

Previous studies have shown that D2 receptors in the MOB are functional, since DA reduces Ca^{2+} influx in ON terminals (Wachowiak and Cohen 1999) and reduces ON-evoked synaptic responses of M/T cells in rats (Hsia et al. 1999). However, D2 receptor expression has also been reported in JG cells (Mansour et al. 1990) and D1-like (i.e., D1 and D5 subtypes) ligand binding is present at very low levels in the subglomerular layers of the MOB (Coronas et al. 1997; Nickell et al. 1991). Thus it is still unclear whether DA inhibits transmission from the ON to the MOB via pre- and/or postsynaptic actions, and whether such inhibition is mediated solely by the D2 receptor subtype. Additionally, the influence of D2 receptor activation on spike output from mitral cells, and the role of these receptors in regulating JG neuronal activity are unknown. To address these issues, we investigated the actions of DA on spontaneous and ON-evoked activity in M/T and JG cells in rats, and in wildtype mice and in mice with targeted deletion of the D2 receptor gene.

METHODS

Animals

The following experimental procedures were conducted so as to minimize animal suffering and the number of animals used, and were approved by the animal welfare committee of the University of Maryland. Juvenile (12–28 day old) male rodents (Sprague-Dawley rats from Zivic Miller, C57BL/6 mice from Jackson Labs, and DA D2 knockout mice from our colony) were used. The generation of DA D2 receptor knockout (D2 knockout) mice has been reported previously (Baik et al. 1995). The D2 knockout line had been backcrossed to C57BL/6J, and all mice were at least 95% C57BL/6J. Mice were genotyped by polymerase chain reaction (PCR) of DNA in tail tip digests. Tail tips were digested at 56°C in 20 μL of 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 (approximately 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 proteinase K. The PCR sample (25 μL) contained 1 μL of DNA digest in 1 times PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dTTP, dCTP, dATP, dGTP, and 0.625 U of *Taq* polymerase. The oligonucleotide primers were 5 pmol/25 μL for neomycin resistance gene (Neo) or 20 pmol/25 μL for the D2 receptor. The thermocycler program was initiated by a 1-min denaturation step at 95°C followed by 40 amplification cycles: 95°C for 1 min, 60°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 visualized with ethidium bromide under ultraviolet (UV) illumination. The D2 receptor oligonucleotides were 5′ CAGATA-GACGACCCAGGGCATAAC and 5′ CAATGGATCCACTGAAC-CTGTCCTG and generated a 284 bp amplicon. The oligonucleotides for Neo were 5′ GCTATTCGGCTATGACTGGG and 5′ GAAGGC-GATAGAAGGCGATG and generated a 725 bp amplicon. Electrophysiological experiments in hemi- and homozygous mice were performed blindly (without knowledge of the genotype of the slice).

Slice preparation

Rat and mouse MOB slices were prepared as previously described (Aroniadou-Anderjaska et al. 1997, 1999; Ciombor et al. 1999). Horizontal slices (400 μm thick) were transferred to an interface or submerged recording chamber maintained at 25–30°C and were continuously perfused at a rate of 1–2 ml/min with oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 3.5 KCl,

2.5 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, and 10 D-glucose (pH 7.4). Experiments were initiated 1–2 h after the slices were placed in the chamber. Focal stimulation pulses were applied to the slice with a bipolar electrode (paired 50-μm-diam stainless steel wires, insulated except for bluntly cut tips). Isolated, constant-current monophasic square-wave stimuli (10–400 μA in amplitude, 0.1 ms in duration) were delivered by a Grass S8800 stimulator.

Extracellular recordings

Extracellular recordings from single mitral cells were obtained with glass micropipettes (2–3 μm tip diam, 10–20 MΩ) filled with a 2% solution of pontamine sky blue in 0.5 M sodium acetate. Electrode signals were amplified, discriminated, and displayed using conventional electrophysiological techniques as previously described (Ciombor et al. 1999). Spontaneous and ON-evoked spiking activity of mitral cells was acquired and analyzed using modified CED hardware and software as previously described (Ciombor et al. 1999). Field excitatory postsynaptic potentials (fEPSPs) were recorded in the GL with glass micropipettes (2–4 μm tip diam, 0.5–2 MΩ) filled with 2 M NaCl. fEPSPs were filtered (3 kHz low-pass), and digitized on-line at 10 kHz with Axon Instruments hardware and software (pClamp8). Measurement of fEPSP amplitudes was done with pClamp8 software. A moving average (5 points) of the data was generated using Origin 6 (Microcal Software, Northampton, MA). Group data, expressed as means $\pm$ SE, were statistically analyzed with paired *t*-tests.

Whole cell recordings

JG neurons were visualized with an Olympus BX50WI upright microscope equipped with a $\times$60 water immersion lens and near-infrared-DIC optics. They were identified based on their periglomerular location (Shipley and Ennis 1996), the size of their soma, and their high-input resistance ($\sim$2 GΩ). All recordings were made at 25–28°C. Patch electrodes were prepared from Garner KG-33 glass tubing using a Narishige PP-830 puller. Series resistance (range 8–15 MΩ) was not compensated but was carefully monitored for constancy. Data were discarded when series resistance varied by >20% or was larger than 15 MΩ. The intracellular solution contained (in mM) 135 KCl (or 135 CsCl and 5 mM QX222), 10 HEPES, 2 Mg_2-ATP, 0.2 Na_2-GTP, and 0.5 EGTA; pH and osmolarity were adjusted to 7.3 and 280 mOsm, respectively. Electrical signals were recorded using an Axopatch-200B amplifier (Axon Instruments), filtered at 5 kHz, and stored on videotape. Evoked events were digitized on-line and stored on hard disk. Off-line, signals were filtered at 2 kHz and digitized at 20 kHz for capturing individual spontaneous events, and at 0.5–1 kHz for capturing long stretches of recordings. The liquid junction potential after achieving whole cell was about 4 mV and was subtracted from the membrane potentials presented below. The decay of synaptic currents was fitted to the following function: $I(t) = A_f \exp(-t/\tau_f) + A_s \exp(-t/\tau_s) + C$, in which $I(t)$ was the amplitude of EPSCs at time t, A_f and A_s were the amplitudes of the fast and slow components, respectively, τ_f and τ_s were the decay time constants of the fast and slow components, respectively, and C was the residual current at the end of the fitting interval. As will be presented below, stimulation of the lateral olfactory tract (LOT) or mitral cell layer (MCL) produced prolonged synaptic responses in JG cells. The synaptic charge transfer of these responses was estimated by subtracting the holding current and then numerically integrating the remaining current beginning at the time that the stimulus was applied (Schoppa et al. 1998). All values are means $\pm$ SE.

Chemicals

The following agents were diluted in oxygenated ACSF and applied by bath perfusion: quinelorane (40–500 μM), quinpirole (50–100

μM), eticlopride (10–50 μM), sulpiride (100 μM), DA hydrochloride (40–300 μM), D(−)-2-amino-5-phosphonopentanoic acid (APV, 50–100 μM), bicuculline methiodide (10 μM) (all from Sigma), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris Neuramin).

RESULTS

Dopamine reduces the ON-evoked fEPSP in the glomerular layer

Previous studies demonstrate that ON shocks elicit a two-component fEPSP in the GL that reflects glutamatergic synaptic currents generated in the apical dendrites of M/T cells (Aroniadou-Anderjaska et al. 1997, 1999). The first experiment investigated the effects of DA, and D2 receptor agonists and antagonists, on the ON-evoked fEPSP in the rat. Bath application of DA (40 μM) reduced the peak amplitude of the ON-evoked fEPSP from 1.2 ± 0.1 (SE) mV to 0.8 ± 0.1 mV (Fig. 1*A*, $n = 5$, $P = 0.005$), a 30.1% reduction. Subsequent addition of the D2 antagonist sulpiride (100 μM) completely reversed the effect of DA and caused an increase in the fEPSP amplitude to 106% of control (Fig. 1*A*; $n = 4$). The inhibitory action of DA on the fEPSP was mimicked by the selective D2 receptor agonist, quinpirole. Quinpirole (100 μM) reduced the peak amplitude of the fEPSP from 0.9 ± 0.1 to 0.6 ± 0.1 mV ($n = 10$, $P < 0.0001$), a decrease of 32.7% (Fig. 1*C*). Subsequent addition of the D2 antagonist sulpiride (100 μM) reversed the effects of quinpirole to within 99% of control levels ($n = 10$; Fig. 1*C*). The effects of DA and quinpirole were reversible after wash out.

DA reduces paired-pulse depression of the ON-evoked fEPSP in the glomerular layer

Next, we examined whether D2 receptor activation affects M/T cell synaptic responses to paired-pulse stimulation of the rat ON. Two successive, identical stimuli delivered at various interstimulus intervals (ISIs) may produce depression (paired-pulse depression) or facilitation of the response to the second (test) pulse. Whether paired-pulse depression or facilitation is produced depends largely on the probability of transmitter release in response to the first (conditioning) pulse (Debanne et al. 1996; Manabe et al. 1993; Thompson et al. 1993). If DA acts presynaptically to reduce the probability of glutamate release from ON terminals, then the ratio of conditioning versus test response amplitude should be altered (i.e., the degree of paired-pulse depression or facilitation). By contrast, if DA acts postsynaptically, both conditioning and test responses should be reduced to the same degree, and therefore their amplitude ratio will remain unchanged.

As shown in Fig. 1*B*, paired-pulse stimulation of the ON (100-ms ISI) produced a pronounced depression of the second (test) fEPSP; the test fEPSP was 52.3 ± 7.0% of the conditioning shock fEPSP ($n = 4$). DA (40 μM) disproportionately reduced the conditioning versus test fEPSPs. In the presence of DA, the amplitude of the test fEPSP was 65 ± 10.4% of the conditioning fEPSP, a significant reduction of the degree of paired-pulse depression (Fig. 1*B*; $n = 4$, $P = 0.01$). In the presence of sulpiride, DA did not affect the degree of paired-pulse depression; the test fEPSP was 52.0 ± 4.6% of the
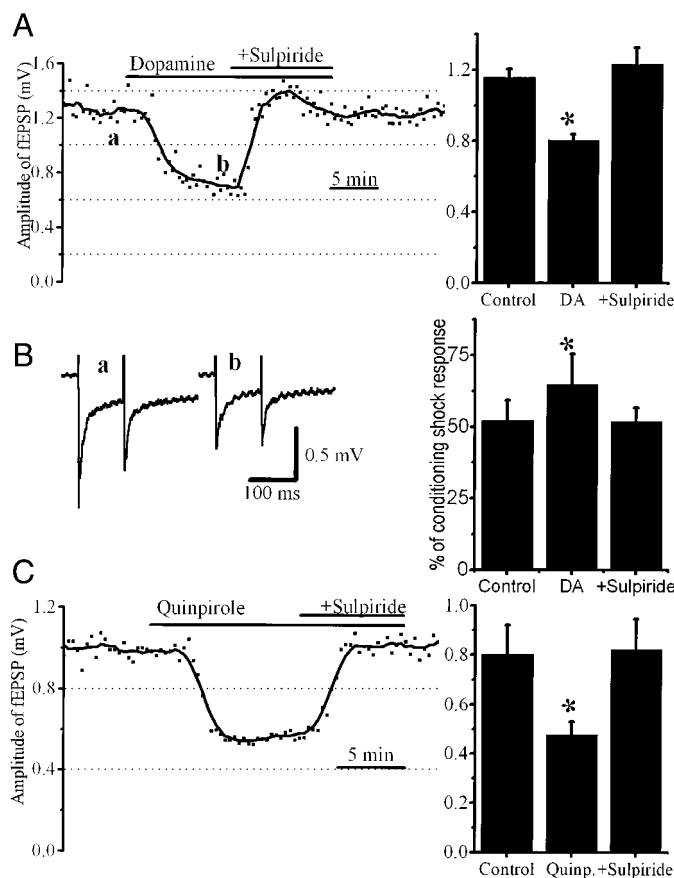

FIG. 1. Dopamine (DA) suppresses the olfactory nerve (ON)–evoked field excitatory postsynaptic potential (fEPSP) recorded in the rat glomerular layer (GL). *A*: line graph from a typical experiment showing the suppression of the GL fEPSP by bath application of DA (40 μM). Values represent the peak amplitude ± SE of the fEPSP. DA-induced suppression (30.7%) was fully reversed by the D2 antagonist sulpiride (100 μM). Group data at the right from similar experiments show that DA significantly suppresses ($n = 5$, * $P = 0.005$) the ON-evoked fEPSP, an effect fully reversed by sulpiride ($n = 4$). *B*: records show responses to paired-pulse stimulation of the ON (100-ms interstimulus intervals) before (Control) and during application of DA (DA); control and DA records correspond to time points a and b indicated in *A*. In control artificial cerebrospinal fluid (ACSF), paired ON shocks produced pronounced paired-pulse depression of the test fEPSP. DA preferentially suppressed the conditioning shock fEPSP and decreased paired-pulse depression. Traces are averages of 5 sweeps. Bar graph of group data showing the change in paired-pulse responses to ON stimulation; data are expressed as the amplitude of the test fEPSP as a percentage of the conditioning fEPSP. Note that DA significantly reduced paired-pulse depression ($n = 4$, * $P = 0.01$). *C*: line graph showing the suppression of the GL fEPSP by bath application of quinpirole (100 μM). Sulpiride (100 μM) fully reversed the quinpirole-induced suppression. Group data for similar experiments are shown in the bar graphs to the *right* ($n = 10$, * $P < 0.0001$).

conditioning response ($n = 4$), similar to paired-pulse depression in control media. These results suggest that DA reduced the probability of glutamate release from ON terminals, but they do not rule out concomitant postsynaptic effects.

DA reduces spontaneous and ON-evoked spiking of mitral cells

To determine whether D2 receptor activation has an impact on the output from M/T cells, we examined the effects of DA
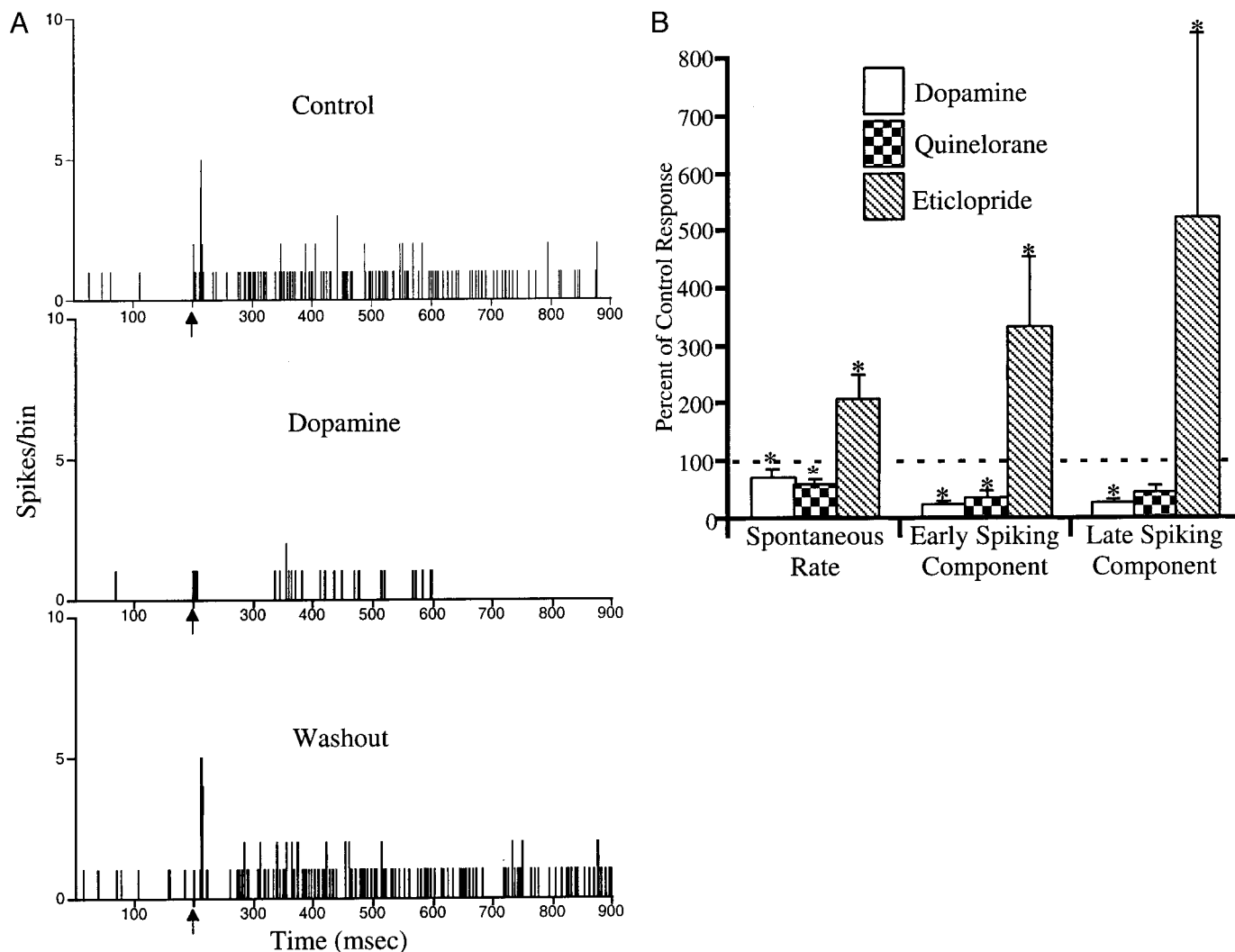
FIG. 2. DA inhibits spontaneous and ON-evoked discharge of rat mitral cells. *A*: peristimulus time-histograms (PSTHs) showing the response of a mitral cell to ON stimulation (at arrows) before (*top PSTH*), during (*middle PSTH*), and after (*bottom PSTH*) bath application of DA (100 μM). DA significantly decreased both the early and late spiking responses of this mitral cell. ON-evoked spiking recovered within 15–25 min after DA wash out. All PSTHs were generated for 25 consecutive ON shocks delivered at 0.2 Hz. *B*: bar graph summarizing the effects of DA, quinelorane, and eticlopride on the spontaneous and ON-evoked spiking of mitral cells. All values are expressed as percent of control values ± SE. Dotted line represents control (i.e., 100%). Statistical tests were performed on raw pre- and postagent data; * $P < 0.05$ in all cases (see text for details). Note that DA and the D2 receptor agonists comparably reduced spontaneous and ON-evoked spiking, while these measures were increased by the D2 receptor antagonist eticlopride.

on ON-evoked mitral cell spiking in the rat. Most mitral cells exhibit a characteristic biphasic excitatory response to single ON shocks consisting of an early, brief excitation, followed by a short period of inactivity, and then a later, prolonged spiking component lasting up to 700 ms (Aroniadou-Anderjaska et al. 1997; Ennis et al. 1996). As shown in Fig. 2, *A* and *B,* DA application (100–300 μM) reduced the early spiking component in all mitral cells tested by 43.0–100%; mean reduction, 73.1 ± 4.4% ($n = 20$, $P < 0.0001$). DA also decreased the late spiking component in all cells by 27.0–100%; mean reduction, 70.3 ± 6.0% ($n = 19$, $P = 0.005$). In addition, DA (100–300 μM) decreased the baseline spontaneous discharge rate of 15/18 mitral cells. The mean discharge rate decreased from 2.8 ± 0.6 to 1.7 ± 0.5 spikes/s, a 26.9 ± 12.9% reduction ($n = 18$, $P = 0.02$; Fig. 2, *A* and *B*). The actions of DA were fully

reversible. Similar inhibitory effects were produced by the D2 receptor agonist, quinelorane (100–300 μM), which decreased both the spontaneous and ON-evoked activity of mitral cells (Fig. 2*B*). Spontaneous discharge rate was reduced by 37.6 ± 8.2% ($n = 12$, $P = 0.005$). The early spiking component was reduced by 61.6 ± 12.0% ($n = 13$, $P = 0.0006$). The late spiking component was also depressed in six of nine cells, although this trend did not reach statistical significance (mean reduction, 53.5 ± 12.9%, $P = 0.06$). The four remaining cells did not exhibit a late spiking component.

As summarized in Fig. 2*B,* the D2 receptor antagonist eticlopride (10 μM) increased the mean spontaneous discharge rate of mitral cells from 1.5 ± 0.5 to 2.4 ± 0.8 spikes/s ($n = 8$, $P = 0.02$), a 109 ± 43.4% increase. Eticlopride also increased the magnitude of the early (234.5 ± 122.2%, $n = 9$,
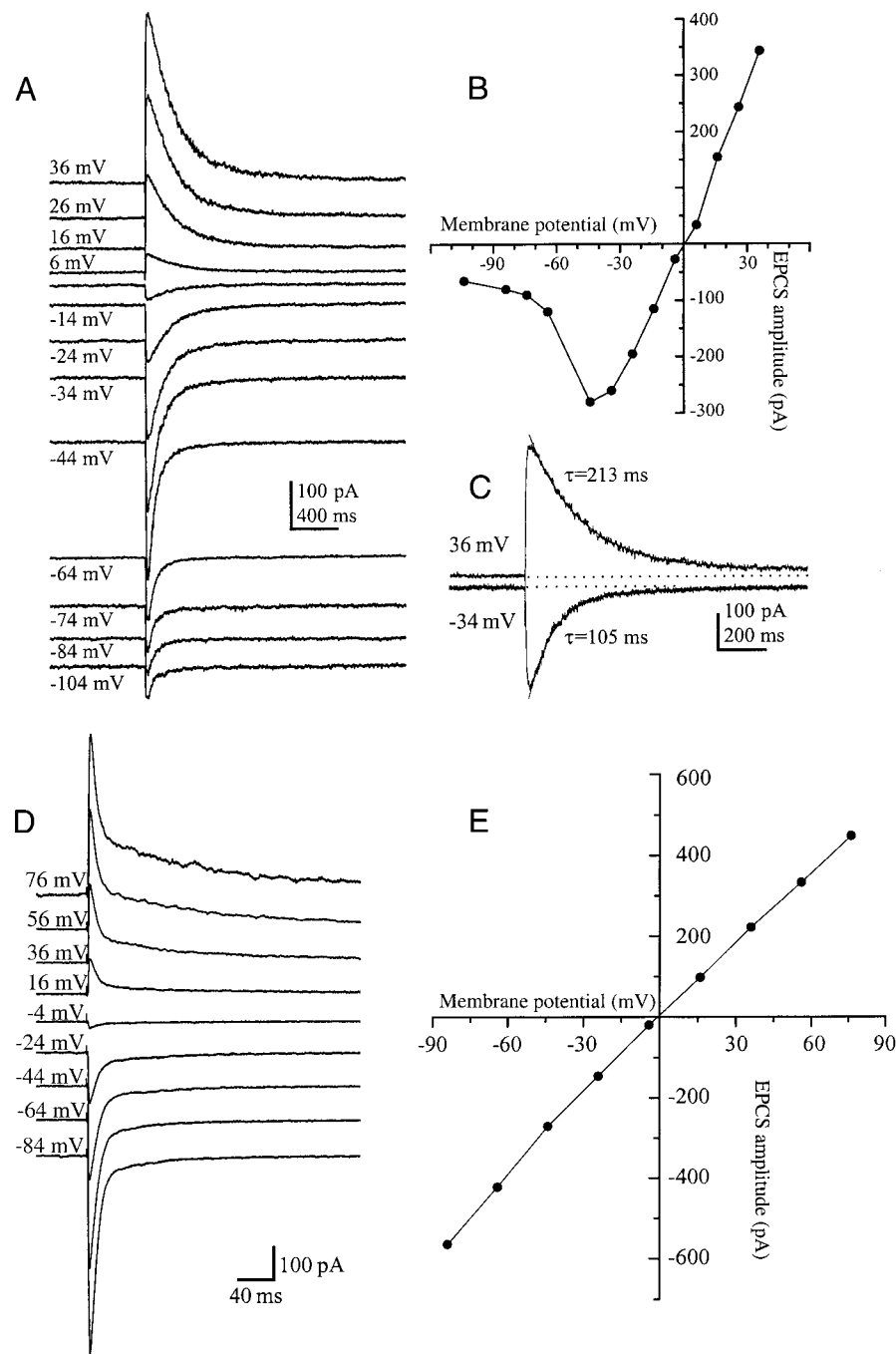
FIG. 3.   Properties of ON-evoked, *N*-methyl-D-as-partate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor—dependent excitatory postsynaptic currents (EPSCs) in mouse juxtaglomerular (JG) cells. *A–C*: ON-evoked, NMDA receptor–mediated EPSCs in a JG cell at different holding potentials; the pipette solution contained 135 mM CsCl, and the bath contained 10 μM bicuculline and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The ON-evoked EPSC reversed around 0 mV. Note that the current-voltage (*I-V*) curve (*B*) exhibits a region of negative slope conductivity at holding potentials of -40 to -80 mV, characteristic of NMDA receptor–mediated currents in the presence of extracellular Mg^{2+} (1.3 mM). *C*: kinetics of ON-evoked NMDA receptor–mediated EPSCs at 36 and -34 mV. The decay of these currents was slower at positive holding potentials than at negative ones. *D* and *E*: linear *I-V* relation of ON-evoked, AMPA receptor–dependent EPSCs; the pipette solution contained 135 mM CsCl, and the bath contained 10 μM bicuculline and 50 μM D($-$)-2-amino-5-phosphonopentanoic acid (APV). The ON-evoked non-NMDA EPSCs exhibited a linear *I-V* relationship and a reversal potential near 0 mV. Traces are averages of 2–4 sweeps.

$P = 0.02$) and late (423.8 $\pm$ 320.1%, $n = 6$, $P = 0.02$) spiking components evoked by ON stimulation. These results suggest that D2 receptors may be tonically activated by endogenous DA.

Characteristics of ON-evoked EPSCs in JG cells

JG cells receive glutamatergic synaptic input from the ON. Thus if DA presynaptically inhibits ON terminals, then it should also reduce ON-evoked, glutamate-mediated excitatory postsynaptic currents (EPSCs) in JG cells. To test this hypothesis, whole cell patch-clamp recordings were obtained from mouse JG cells. JG cell synaptic responses to ON inputs have

not been investigated in mice. Therefore we first characterized the *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor–mediated components of ON-evoked EPSCs in these cells (Fig. 3). In the presence of 10–20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μM bicuculline, 1.3 mM Mg^{2+}, and with a -64-mV holding potential, ON shocks induced inward synaptic currents with a 10–90% rise time (T_{10-90}) of 6.8 $\pm$ 0.5 ms ($n = 7$). The decay of the EPSC could be fitted with a double exponential function with τ_1 of 49 $\pm$ 3.0 ms (80% of the total amplitude) and τ_2 of 151 $\pm$ 6.8 ms ($n = 7$). In 3 JG cells, ON-evoked NMDA receptor–dependent EPSCs were recorded

over a range of holding potentials (from approximately -100 mV to approximately $+50$ mV, Fig. 3, *A* and *B*). EPSCs were relatively small at -60 mV or more negative holding potentials and were larger at potentials positive to -40 mV. The currents reversed polarity around 0 mV (Fig. 3, *A* and *B*). The characteristics of the current-voltage (*I-V*) curves for the NMDA receptor–mediated currents attest to the excellent voltage control of JG cells. The decay of these currents was faster at negative holding potentials than at positive ones (Fig. 3*C*). The *I-V* relationship was linear after perfusion with a Mg^{2+}-free bathing solution ($n = 2$, data not shown). In the presence of CNQX, application of 50 μM APV blocked ON-evoked EPSCs in all JG cells tested ($n = 4$, data not shown), confirming that they were mediated by NMDA receptors. These biophysical and pharmacological properties are characteristic for NMDA receptor–mediated currents (Konnerth et al. 1990; Lester et al. 1990; Nowak et al. 1984). Thus JG cells receive typical NMDA receptor–mediated synaptic inputs from ON terminals.

Next we characterized ON-evoked non-NMDA receptor–mediated EPSCs in the presence of 1.3 mM Mg^{2+}, 50 μM APV, and 10 μM bicuculline. At a holding potential of -64 mV, ON shocks evoked relatively fast inward synaptic currents in 25 JG cells (Fig. 3, *D* and *E*). The peak amplitude was 450 ± 47 pA ($n = 25$). T_{10-90} rise time was 2.3 ± 0.2 ms ($n = 25$). The decay time course was variable with the 90% decay time ranging from 2 to 40 ms. The *I-V* relationship for ON-evoked, non-NMDA receptor EPSCs in JG cells in the presence of 1.3 mM Mg^{2+} was linear with a reversal potential around 0 mV in all JG cells tested ($n = 4$, Fig. 3, *D* and *E*), typical for

non-NMDA receptor–mediated currents. ON-evoked EPSCs in the presence of APV were abolished on addition of 20 μM CNQX (data not shown).

DA suppresses ON-evoked EPSCs in JG cells

At a holding potential of -66 mV, bath application of 40 μM DA reversibly reduced the amplitude of the ON-evoked EPSCs in all 26 mouse JG cells. Most cells (20 of 26) were tested in the presence of 40–100 μM APV, and the rest were tested without APV. As the results were not different, the two data sets were pooled. EPSC amplitude in the presence of 40 μM DA was $38 \pm 1.9\%$ of control level ($P < 0.01$, $n = 26$, Fig. 4*A*). Despite the pronounced effect on ON-evoked EPSCs, we did not observe any DA-induced changes in holding current or input resistance in JG cells. In nine JG cells recorded with a KCl-based intracellular solution, the holding current and input resistance were -5.0 ± 1.5 pA and 2.1 ± 0.3 GΩ in the control condition, and -5.3 ± 1.6 pA and 2.0 ± 0.3 GΩ in the presence of 20–100 μM DA ($P > 0.05$). These results suggest that DA did not directly (i.e., postsynaptically) act on any of the JG cells sampled in this study.

Paired-pulse ON stimulation at ISIs of 50–400 ms produced pronounced depression (i.e., paired-pulse depression) of the second (test shock) evoked EPSC in JG cells. DA strongly and disproportionately depressed the conditioning versus the test EPSC, thereby decreasing the degree of paired-pulse depression. In control media, paired-pulse ON stimulation (100-ms ISI) reduced the amplitude of the test EPSC to $23 \pm 0.3\%$ of the conditioning EPSC. In the presence of 40 μM DA, the test
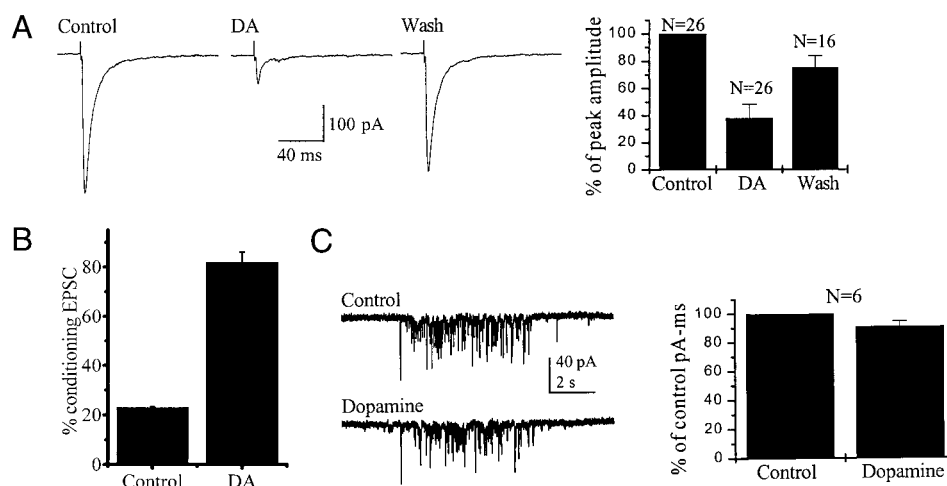

FIG. 4. DA reduces ON-evoked EPSCs in mouse JG cells. *A*: records from an individual experiment (*left*) and group data (*right*) showing the effects of DA on EPSCs evoked in JG cells. Single ON shocks elicit short-latency EPSCs in this JG cell (KCl-based intracellular solution). Application of 40 μM DA depressed the ON-evoked EPSC by ~75%, an effect that largely recovered within 10 min after wash out. No change in holding current or input resistance was observed. Traces are averages of 4 sweeps. Bar graph of group data for 26 JG cells shows that 40 μM DA significantly suppressed evoked EPSCs to $38 \pm 1.9\%$ of control level ($P < 0.01$). In 16 cells, significant recovery was achieved after wash out; in 10 cells no wash was performed due to loss of the cells. *B*: bar graph summarizing the effects of DA on paired-pulse depression of ON-evoked EPSCs in JG cells. Before application of DA, paired-pulse stimulation of the ON (100-ms interstimulus interval) produced pronounced paired-pulse depression; the amplitude of the test shock EPSC was $23 \pm 0.3\%$ of the conditioning shock EPSC. Application of 40 μM DA significantly reduced paired-pulse depression of the test EPSC to $82 \pm 4\%$ of the conditioning EPSC ($n = 17$, $P < 0.01$). *C*: DA has no effect on mitral cell–evoked EPSCs in JG cells. Stimulation in the mitral cell layer or lateral olfactory tract (see text for details) evoked bursts of EPSCs in JG cells. In presence of 40 μM DA, the charge transfer of the synaptic response was ~90% of the control response. The bar graph at *right* summarizes group data for 6 cells. DA did not significantly decrease mitral cell–evoked JG EPSCs ($P = 0.056$); the small decline is likely due to a gradual deterioration of the recorded JG cell or synaptic connections.

EPSC was 82 ± 4% of the conditioning shock EPSC, a significant reduction in the degree of paired-pulse depression (Fig. 4B, $P < 0.01$, $n = 17$). The reduction of paired-pulse depression suggests that DA decreases the probability of glutamate release from ON terminals.

JG cells also receive excitatory glutamatergic input from the apical dendrites of M/T cells (Pinching and Powell 1971; Shipley and Ennis 1996). If indeed DA has no direct effects on M/T and JG cells, then JG cell EPSCs evoked by antidromic activation of M/T cells should not be affected by DA. In six mouse JG cells, lateral olfactory tract (1 cell) or mitral cell layer (5 cells) stimulation evoked bursts of EPSCs (Fig. 4C). Due to the bursting nature of the responses, we measured the total charge transfer induced by antidromic stimuli by calculating the integral of the response (see METHODS). The charge transfer in the presence of DA (40–100 μM) was only slightly smaller than those under control conditions (90 ± 2% of control). This slight reduction was attributable to gradual rundown because strong stimuli (~2 times the strength of ON shocks used) evoked antidromic responses that showed a tendency to slowly decline over time in the absence of DA. Under the same conditions, DA reduced ON-evoked EPSCs in JG cells to 30% of control values (see above). These results, taken with the finding that DA did not produce a measurable effect on holding current or input resistance in JG cells, indicate either that D2 receptors are not present in JG cells or that the receptors are too few in number or located at sites too remote (e.g., autoreceptors at DA release sites) to appreciably influence JG cell responses to M/T cell synaptic inputs.

DA depresses spontaneous, but not miniature, EPSCs in JG cells

To further investigate a presynaptic locus of DA action, we compared the effects of DA on the frequency and amplitude of action potential–dependent spontaneous EPSCs versus action potential–independent miniature EPSCs in mouse JG cells. In four JG cells tested in the presence of 50 μM APV, DA reduced spontaneous EPSC (sEPSC) frequency by 44 ± 7.5% and amplitude by 42 ± 6.5% ($P < 0.001$; Fig. 5). The reduction in sEPSC frequency is consistent with a presynaptic locus of DA action. The reduction of sEPSC amplitudes could be attributed to a postsynaptic DA effect on JG cells and/or a reduction in the frequency of multiple, overlapping sEPSCs via presynaptic inhibition. To address this issue, we next investigated the effect of DA on the frequency and amplitude of spontaneous miniature EPSCs (mEPSCs). mEPSCs are believed to represent spontaneous, quantal release of transmitter from presynaptic sites in an action potential/Ca^{2+}–independent manner. An effect of DA on the amplitude of mEPSCs would suggest a postsynaptic action of DA. In the presence of 0.5 μM TTX and 50 μM APV, mEPSCs were recorded in seven JG cells at −64 mV (Fig. 6). mEPSCs had a rapid time course with a T_{10-90} of 0.32 ± 0.01 ms ($n = 7$). A double exponential function with a fast and slow term was needed to fit the decay of the averaged mEPSC. The fast component had a decay time constant (τ_f) of 1.2 ± 0.05 ms, and the small slow component had a decay time constant (τ_s) of 5.0 ± 0.04 ms ($n = 7$). These kinetics are similar to those of non-NMDA receptor–dependent mEPSCs described elsewhere (Zhou and Hablitz 1997), and, consistent with this, mEPSCs were completely blocked by bath application of 10 μM CNQX ($n = 3$, data not shown). DA
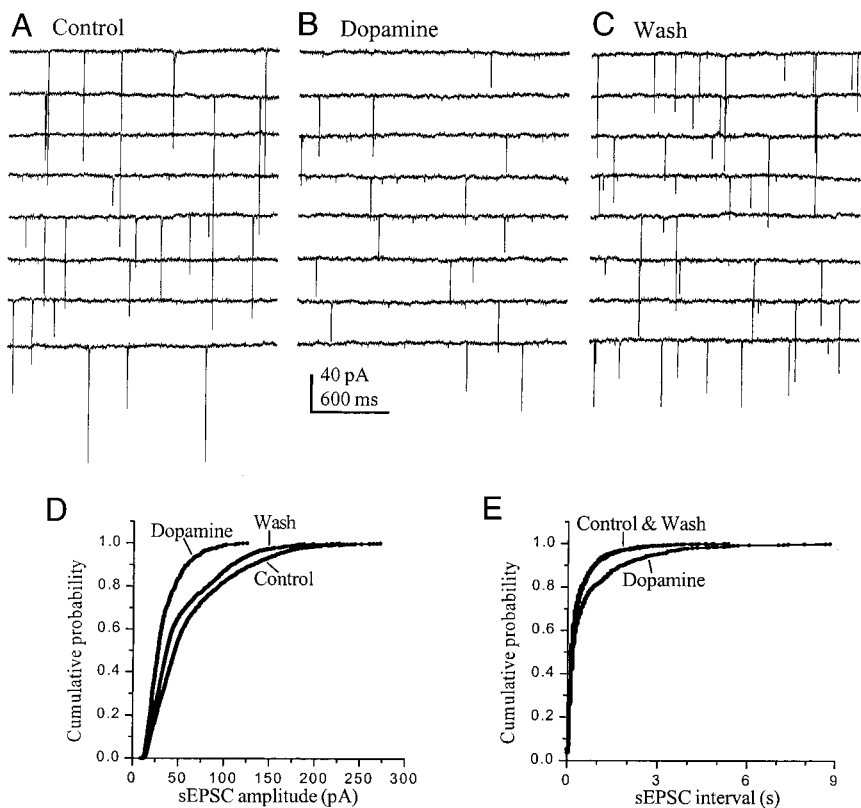

FIG. 5. DA depresses spontaneous EPSCs (sEPSCs) in mouse JG cells. A–C: individual traces from a single cell showing examples of sEPSCs before (A), during (B), and after (C) application of 40 μM DA; the pipette solution contained 135 mM CsCl, and the bath contained 50 μM APV. Note that DA reduces the frequency of sEPSCs. D: histogram showing the cumulative probability of the amplitude of sEPSCs before and during application of 40 μM DA, and after DA wash out. DA significantly reduced the mean amplitude of sEPCSs from 62.5 ± 1.0 pA (control, $n = 1,841$) to 33.0 ± 0.6 pA (DA, $n = 994$, $P < 0.001$). sEPSC amplitudes returned to near control values (52.0 ± 0.9 pA, $n = 1,555$) after DA wash out. E: histogram showing the cumulative probability of the frequency of sEPSCs before and during application of 40 μM DA, and after DA wash out. DA significantly increased the mean interval between sEPCSs from 0.34 ± 0.1 s (control, $n = 1,841$) to 0.63 ± 0.3 s (DA, $n = 994$, $P < 0.001$). The interval between sEPSCs returned to near control values (0.35 ± 0.54 s, $n = 1,555$) after DA wash out.
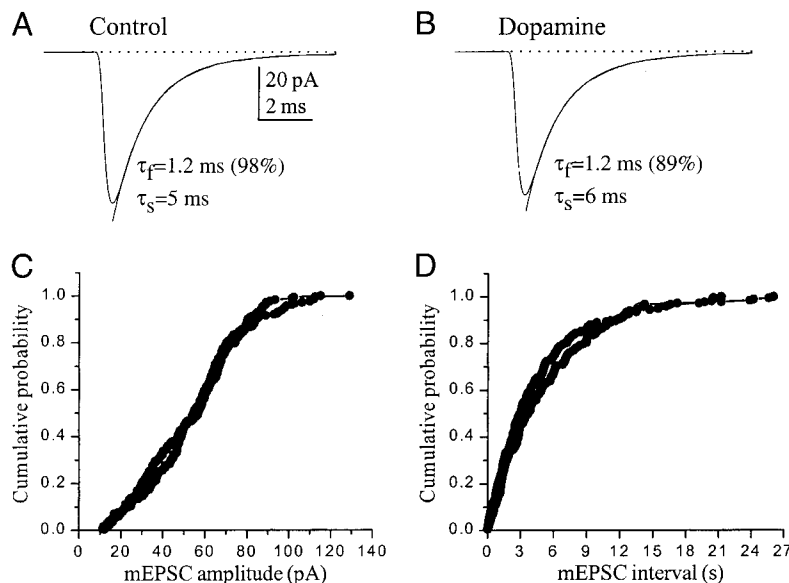
FIG. 6. DA does not affect miniature EPSCs (mEPSCs) in mouse JG cells. *A* and *B*: waveform of averaged mEPSCs before and during application of 40 μM DA; the pipette solution contained 135 mM CsCl, and the bath contained 0.5 μM TTX and 50 μM APV. DA did not alter the kinetics of mEPSCs. *C*: cumulative probability histogram shows that 40 μM DA did not affect the distribution of mEPSC amplitudes. The mean amplitude of mEPSCs before and during application of DA were 54.0 $\pm$ 1.6 pA (n = 217) and 54.0 $\pm$ 1.6 pA (n = 183, P = 0.47), respectively. *D*: cumulative probability histogram shows that 40 μM DA did not affect the distribution of mEPSC intervals. The mean EPSC intervals before and during application of DA were 4.5 $\pm$ 0.3 s (n = 217) and 5.2 $\pm$ 0.4 s (n = 183, P = 0.27), respectively.

had no effect on the frequency, amplitude, or kinetics of mEPSCs (n = 5, P > 0.1; Fig. 6, *A–D*). Because JG cells receive glutamatergic synaptic inputs from M/T cells as well as ON terminals, the sEPSCs and mEPSCs could arise from either source. However, as the previous experiment showed that M/T cell–evoked EPSCs in JG cells were not affected by DA, these findings taken together support the hypothesis that DA acts to presynaptically inhibit glutamate release from ON terminals.

DA has no effect on ON-evoked responses in D2 receptor knockout mice

D2-like actions can be produced by DA receptor subtypes D3 and D4 (Niznik and Van Tol 1992; Sibley and Monsma 1992). Previous studies have shown that ON terminals express D2 receptors, but it is not known whether D3 or D4 receptors are also present and thus contribute to the presynaptic effects shown in the preceding experiments. To address this question and to provide an alternative to pharmacological antagonism of the D2 receptors, we compared the effects of DA and D2 receptor agonists in D2 knockout mice versus wildtype littermates. Quinelorane (100 μM) differentially influenced the ON-evoked fEPSP in the GL recorded simultaneously in MOB slices from wildtype and D2 knockout mice. As shown in Fig. 7*A,* quinelorane suppressed the ON-evoked fEPSP in slices (n = 4) harvested from wildtype (37.5 $\pm$ 4.8% reduction; P < 0.02), but not D2 knockout mice (2.5 + 2.5% reduction).

Next, we investigated the effect of DA on ON → JG cell EPSCs in wildtype and D2 knockout mice. DA reduced the amplitude of ON → JG cell EPSCs in all 11 JG cells from heterozygous D2 knockout mice in a manner identical to that observed in wildtype mice (39.6 $\pm$ 2.3% of control level, P < 0.01; Fig. 7*B*). DA also reduced the degree of paired-pulse depression in these cells, increasing the amplitude of the test EPSC from 31 $\pm$ 2% of the conditioning EPSC in control media to 73 $\pm$ 3% in the presence of DA (n = 11, P < 0.01). By contrast, DA (20–100 μM) did not alter the amplitude or waveform of ON → JG cell EPSCs in slices from homozygous D2 knockout mice (n = 6, Fig. 7*B*). The EPSC amplitude in the

presence of DA was 97 $\pm$ 1.4% of control amplitude (Fig. 7*B, P* > 0.05). DA also had no effect on paired-pulse depression evoked by paired ON shocks in slices from homozygous D2 knockout mice; the amplitude of the test EPSC was 30 $\pm$ 1% of the conditioning EPSC amplitude in control media, and 31 $\pm$ 1% in the presence of 40 μM DA (n = 6, P > 0.05).

DISCUSSION

The major finding of this study is that DA, a neurotransmitter contained in JG neurons, substantially decreases sensory input to the MOB by a presynaptic action on ON terminals. DA and D2 agonists markedly attenuated the responses of both M/T and JG cell excitatory responses to monosynaptic ON input. This inhibitory effect of DA is mediated exclusively by D2 receptors as DA was without effect in MOB slices harvested from mice with targeted deletion of the D2 receptor gene.

Dopamine decreases M/T and JG cell responses to ON input

DA significantly reduced the fEPSP recorded in the GL evoked by ON stimulation. This fEPSP reflects, for the most part, glutamatergic synaptic currents generated in the apical dendrites of M/T cells in response to ON input (Aroniadou-Anderjaska et al. 1997, 1999). The reduction of the ON-evoked GL fEPSP observed here with DA and quinpirole is comparable to the effects of quinpirole reported by Hsia et al. (1999). Consistent with the reduction of the ON-evoked fEPSP, DA significantly decreased both spontaneous and ON-evoked action potentials in mitral cells. The inhibitory effects of DA were mimicked by specific D2 receptor agonists, and prevented or reduced by D2 receptor antagonists. A reduction of ON-evoked spiking in mitral cells by DA and D2 receptor agonists was recently reported in the turtle MOB (Berkowicz and Trombley 2000). Finally, DA also reduced spontaneous and ON-evoked EPSCs in JG cells. Thus DA or D2 agonists reduced spontaneous and ON-evoked synaptic responses in all MOB neurons known to be directly targeted by ON synaptic inputs.
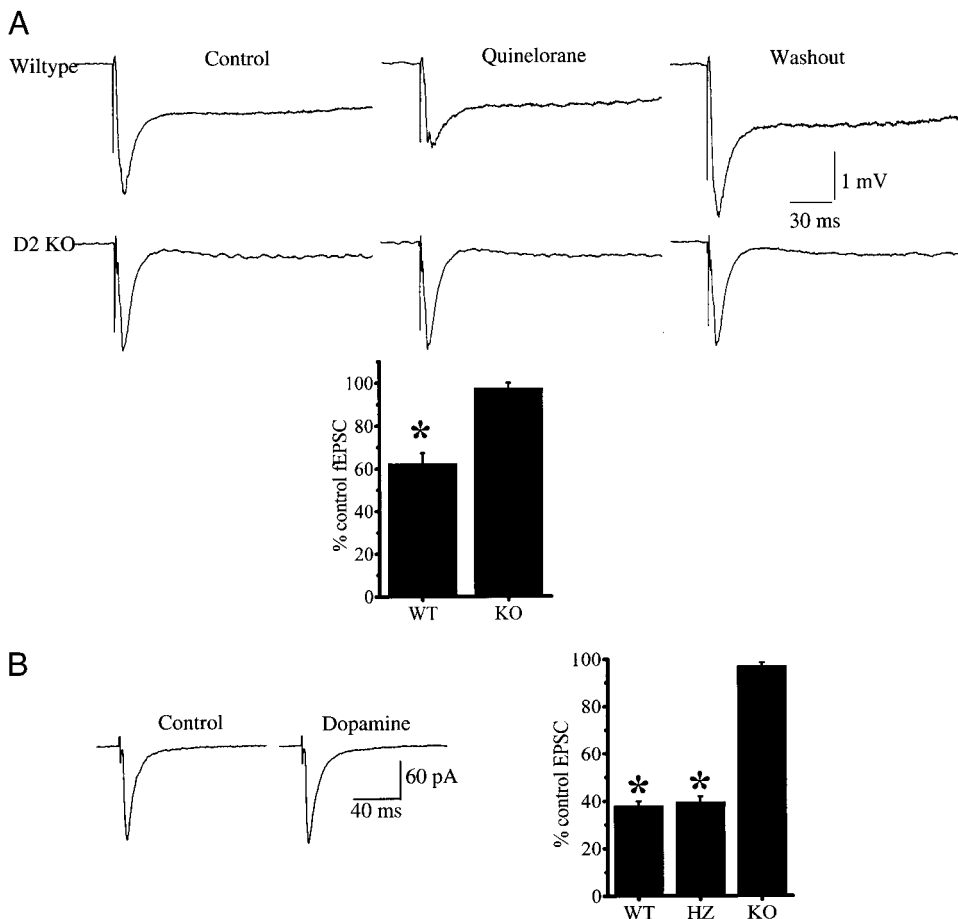
A

B

FIG. 7. Dopamine has no effect in D2 knockout mice. *A*: ON-evoked fEPSPs in the glomerular layer were simultaneously recorded in slices from wildtype and homozygous D2 knockout mice. Application of the D2 receptor agonist quinelorane (100 μM) reversibly suppresses the fEPSP in wildtype, but not D2 knockout, mice. Traces are averages of 10 sweeps. Bar graph shows group data of DA's effect on ON-evoked fEPSP in wildtype (WT) and homozygous D2 knockout (KO) mice ($n = 4$, * $P < 0.02$). *B*: individual examples of ON-evoked EPSCs in JG cells from homozygous D2 KO mice before (Control) and during application of 40 μM DA. Tracers are averages of 4 sweeps. Bar graph at *right* shows group data of DA's effect on ON-evoked EPSCs wildtype (WT), heterozygous D2 KO (HZ), and homozygous D2 KO (KO) mice. Note that DA comparably decreases the ON-evoked EPSC in WT ($n = 26$) and HZ ($n = 11$) mice (* $P < 0.01$) but has no effect in the homozygous KO mice ($n = 6$, $P > 0.05$).

It should be noted that bath-applied DA differs from synaptically released DA with regard to concentration, duration, and/or sites of action. Thus some of the actions of bath-applied DA observed in the present study may differ from the effects of DA released in response to odorant stimuli.

DA effects are mediated by D2 receptors

Anatomical studies demonstrate that the D2 receptor is the predominant DA receptor subtype expressed in the MOB (Coronas et al. 1997; Koster et al. 1999; Nickell et al. 1991). However, D1-like (i.e., D1 and D5 subtypes) ligand binding is present at very low levels in the subglomerular layers of the MOB (Coronas et al. 1997; Koster et al. 1999; Nickell et al. 1991). While the DA receptor agonists and antagonists used in the present study support the interpretation that DA's actions are mediated by D2 receptors, D2 pharmacological reagents are not very precise tools. To circumvent this problem, we took advantage of a mouse line with a targeted deletion of D2 receptors (D2 knockout mice). In these D2 knockout mice, DA was completely ineffective in modulating the ON-evoked fEPSP and ON-evoked EPSCs in JG cells. This strongly supports the idea that D2 receptors, alone, function to presynaptically reduce glutamate release from ON terminals.

Presynaptic locus of DA action

Several lines of evidence from the present study indicate that DA presynaptically inhibits glutamate release from ON termi-

nals. First, DA significantly altered the degree of paired-pulse depression of both M/T and JG cell synaptic responses to ON stimulation. In a paired-pulse paradigm, a postsynaptic action of DA should change the amplitudes of the conditioning and test responses proportionally (i.e., with no significant change in the degree of paired-pulse depression). The present experiments showed that DA significantly and disproportionately suppressed the amplitude of the conditioning versus test fEPSP recorded in the GL, thereby attenuating the degree of paired-pulse depression. Further, DA also attenuated the degree of paired-pulse depression of the ON-evoked EPSC in JG cells. Thus DA appears to reduce the probability of glutamate release from ON terminals onto all MOB neurons known to receive ON synaptic inputs.

Second, DA had no discernible influence on the membrane properties of JG cells. Because JG neurons have much smaller cell bodies and dendritic trees than mitral cells (Pinching and Powell 1971), they provide good space-clamp conditions that should optimize the ability to discern postsynaptic actions of DA. DA consistently suppressed both spontaneous and ON-evoked EPSCs in JG cells; this reduction was not accompanied by any detectable change in the holding current or input resistance in JG cells. This indicates that the predominant locus for the reduction of ON → JG cell EPSCs is pre- rather than postsynaptic.

Third, DA did not significantly alter M/T → JG cell synaptic responses. JG cells receive glutamatergic synaptic inputs from the intraglomerular apical dendrites of M/T cells (Pinching and

Powell 1971; Shipley and Ennis 1996). A direct postsynaptic inhibitory action of DA either on JG cells or on the apical dendrites of M/T cells would be expected to reduce the responses of JG cells to M/T cell inputs. However this was not observed.

Fourth, DA reduced sEPSCs, but not mEPSCs in JG cells. DA reduced both the frequency and amplitude of AMPA receptor–dependent sEPSCs. The decreased frequency of sEPSCs in the presence of DA is consistent with a presynaptic locus of action; the decreased sEPSC amplitude could be due to a postsynaptic locus of action or, alternatively, to a decreased incidence of temporally overlapping sEPSCs in JG cells. In agreement with this latter possibility, DA had no effect on the amplitude, frequency, and kinetics of mEPSCs recorded in JG cells in the presence of TTX. Since mEPSCs are believed to represent random release of single (i.e., quantal) neurotransmitter packets, the failure of DA to alter their characteristics provides further evidence that DA does not have postsynaptic actions on JG cells. Taken together, the most parsimonious explanation of these findings is that DA reduces glutamate release from ON terminals; a presynaptic mechanism. While the present results cannot rule out some small inhibitory postsynaptic action of DA, the evidence indicates that the majority, if not all, of the reduction of ON-evoked responses by DA is mediated by presynaptic D2 receptors on ON terminals. This is consistent with anatomical data showing that olfactory receptor neurons, whose axons form the ON, contain mRNA for D2 receptors, and that D2 receptor binding sites are restricted to the ON and GL (Coronas et al. 1997; Koster et al. 1999; Nickell et al. 1991).

There are several mechanisms by which DA could presynaptically inhibit glutamate release from ON terminals: D2 receptors are known to reduce transmitter release by suppressing Ca^{2+} currents (Formenti et al. 1998; Koga and Momiyama 2000; Lledo et al. 1992; Wachowiak and Cohen 1999; Williams et al. 1990). Alternatively, D2 receptors could *1*) increase potassium conductances, which would decrease the excitability of ON terminals (Lacey et al. 1988), or *2*) directly modulate the release machinery, thereby reducing transmitter release (Man-Son-Hing et al. 1989; Wu and Saggau 1997).

Recent studies in the turtle showed that DA and D2 receptor agonists decrease Ca^{2+} influx in ON terminals without affecting presynaptic ON action potentials (Wachowiak and Cohen 1999). DA and D2 receptor agonists were also reported to presynaptically inhibit glutamate release in the ventral tegmental area of the rat via a Ca^{2+}-dependent mechanism (Koga and Momiyama 2000). In the present experiments, DA reduced both the frequency and amplitude of sEPSCs but had no effect, however, on mEPSCs recorded in the presence of TTX. On the basis of these findings, we suggest that DA reduces voltage-activated Ca^{2+} currents in ON terminals. Additional experiments are needed to identify the presynaptic mechanism involved in D2-mediated inhibition of ON terminals in the rodent MOB.

Presynaptic regulation of ON terminals

Olfactory receptor neurons expressing the same odorant receptors (an olfactory receptor neuron cohort) converge on the same MOB glomeruli (for review, see Mombaerts 1999; Mori

et al. 1999). Odor molecules differentially activate multiple cohorts of olfactory receptor neurons, with the result that different odors evoke specific patterns of glomerular activity (Belluscio and Katz 2001; Friedrich and Korsching 1997; Guthrie et al. 1993; Johnson and Leon 1996; Johnson et al. 1998; Laurent 1996; Shepherd 1994). Less is known about the representation of odor concentration. If increasing concentrations of an odorant molecule cause increased neural activity among olfactory receptor neurons of the same cohort, then the range of concentrations that can be encoded might be limited by concentrations that produce maximal transmitter release by ON terminals. Presynaptic inhibition of ON terminals is a potential mechanism for increasing the range of concentrations that can be processed by MOB neurons: as activity increases in ON terminals, DA JG cells would be more strongly excited and thus exert negative feedback onto ON terminals, effectively increasing the dynamic range of information transfer from olfactory receptor neurons to MOB neurons.

In most mammals, investigative sniffing typically entails repetitive sniffs at 100- to 200-ms intervals (Komisaruk 1970; Welker 1964). Presynaptic inhibition of ON terminals may also play a role in determining spatial and temporal components of glomerular activation during repetitive sniffing by adjusting the level of glomerular excitation as a function of sniff frequency.

We recently reported that GABA acting via $GABA_B$ receptors on ON terminals causes presynaptic inhibition (Aroniadou-Anderjaska et al. 2000) that is similar to that reported here for DA. Why are ON terminals presynaptically regulated by both DA and GABA? One possibility is that the functions conjectured above for DA (e.g., scaling concentration range, modulating ON input across sniff cycles) may be further enhanced by dual transmitter regulation. Another possibility may be related to the differential regulation of DA and GABA in JG neurons by ON activity. Manipulations that reduce ON synaptic activation of JG cells downregulates tyrosine hydroxylase and DA in JG cells (Baker 1990; Baker et al. 1983, 1984; Brunjes et al. 1985) but do not reduce GABA in the same JG neurons (Baker 1990). Thus if certain odor molecules are infrequently encountered, this might lead to downregulation of DA in JG cells in the glomeruli targeted by the olfactory receptor neurons activated by that odor molecule. Exposure to that odor would evoke less presynaptic inhibition by DA. Conversely, if the animal is tonically exposed to a background odor, the glomeruli activated by the odor might release more DA and thus produce greater presynaptic inhibition, which would decrease responses to the maintained background odor. As GABA is not regulated in an activity-dependent manner, this transmitter might function to presynaptically regulate ON terminals independently of the animal's odor exposure history.

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