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Cholinergic Modulation of Dopaminergic Neurons in the Mouse Olfactory Bulb

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Abstract

Considerable evidence exists for an extrinsic cholinergic influence in the maturation and function of the main olfactory bulb. In this study, we addressed the muscarinic modulation of dopaminergic neurons in this structure. We used different patch-clamp techniques to characterize the diverse roles of muscarinic agonists on identified dopaminergic neurons in a transgenic animal model expressing a reporter protein (green fluorescent protein) under the tyrosine hydroxylase promoter. Bath application of acetylcholine (1 mM) in slices and in enzymatically dissociated cells reduced the spontaneous firing of dopaminergic neurons recorded in cell-attached mode. In whole-cell configuration no effect of the agonist was observed, unless using the perforated patch technique, thus suggesting the involvement of a diffusible second messenger. The effect was mediated by metabotropic receptors as it was blocked by atropine and mimicked by the m2 agonist oxotremorine (10 μM). The reduction of periglomerular cell firing by muscarinic activation results from a membrane-potential hyperpolarization caused by activation of a potassium conductance. This modulation of dopaminergic interneurons may be important in the processing of sensory information and may be relevant to understand the mechanisms underlying the olfactory dysfunctions occurring in neurodegenerative diseases affecting the dopaminergic and/or cholinergic systems.

Key words: dopaminergic neurons, muscarine, olfactory bulb, patch-clamp

Introduction

In vertebrates, the olfactory bulb (OB) is the first relay for olfactory processing, receiving information from the olfactory epithelium and conveying it to higher brain structures via its projection neurons, the mitral and the tufted cells. These neurons interact with 2 classes of local inhibitory interneurons: (1) periglomerular (PG) cells that make synapses onto the primary dendrites of mitral/tufted cells, and (2) granule cells that release GABA onto mitral cell secondary dendrites (for a review, see Kratskin and Belluzzi [2003]).

The OB is under a massive extrinsic cholinergic innervation, which strongly influences its maturation and function (Halász and Shepherd [1983]; for a review see Kratskin and Belluzzi [2003]); the main source of cholinergic afferents to the OB is the nucleus of the horizontal limb of the diagonal band (NHDB) (Carson 1984; Zaborszky et al. 1986). Extrinsic cholinergic inputs in the OB were once thought to terminate predominantly onto granule spines, leading to the conclusion that the main role of acetylcholine (ACh) is to modulate granule cell inhibition of mitral cells (Halász and Shepherd 1983; Macrides and Davis 1983). Subsequent observations, however, have reported significant cholinergic innervation also in the glomerular layer (Nickell and Shipley 1988b; Ravel et al. 1990; Le Jeune and Jourdan 1993; Kasa et al. 1995; Crespo et al. 1999), where cholinergic terminals innervate preferentially—albeit not exclusively—a subpopulation of tyrosine hydroxylase (TH)–positive (dopaminergic) PG cells (Le Jeune and Jourdan 1994). Importantly, many of these contacts are morphologically of the symmetric type, which is generally associated with inhibitory synaptic actions.

Although functional and behavioral studies have stressed the importance of cholinergic inputs in olfactory memory (Ravel et al. 1994; Levy et al. 1995) and in odor processing (Nickell and Shipley 1988a; Elaagouby and Gervais 1992; Linster and Hasselmo 1997), the sites and mechanisms of cholinergic action have not been clearly defined. Electrophysiological experiments have yielded conflicting results. For example, electrical stimulation of the NHDB in vivo was reported either to depress (Nickell and Shipley 1988a) or to increase (Kunze et al. 1991) mitral cell firing through cholinergic modulation of GABAergic inhibition, and direct

application of ACh in vivo gave results at odds on mitral cell activity (Ravel et al. 1990; Elaagouby and Gervais 1992). Possible explanations of these conflicting results may reside on the one side in the different targets of cholinergic terminals and on the other side in the dual organization of the OB cholinergic system, due to the segregation of muscarinic and nicotinic receptors: The glomerular layer is considered preferentially receptive to nicotinic agonists (Elaagouby et al. 1991; Castillo et al. 1999), in contrast to the external plexiform layer, internal plexiform layer, and granule cell layer, which seem to be more susceptible to modulation by muscarinic receptors (Castillo et al. 1999).

In order to clarify some of these discrepancies, it seemed important to analyze the cholinergic responses in single and univocally identified neuronal subpopulations. In the present study, we have investigated the cholinergic responses of dopaminergic neurons. We have focused our attention on these cells because they are preferential targets of cholinergic terminals in the entry circuits of the OB, the glomerular layer (Le Jeune and Jourdan 1994), and because of their key role in the signal processing.

For this purpose, we used different patch-clamp techniques to identify and characterize the diverse roles of cholinergic agonists on dopaminergic cells in a transgenic animal model expressing a reporter protein (enhanced green fluorescent protein [eGFP]) under the TH promoter so that dopaminergic cells could be viewed in living preparations and recorded under direct visual control.

Materials and methods

Animals and surgical procedures

Experimental procedures were carried out so as to minimize animal suffering and the number of mice used. The procedures employed were in accordance with the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes and were approved by the Campus Veterinarian of the Ferrara University. A total of 20 mice of ages between 2 and 12 months have been used. All experiments were performed using the transgenic mice TH-GFP/21-31 line carrying the eGFP gene under the control of the TH promoter (Sawamoto et al. 2001; Matsushita et al. 2002). The transgene construct contained the 9.0-kb, 5′-flanking region of the rat TH gene, the second intron of the rabbit β-globin gene, cDNA-encoding green fluorescent protein (GFP), and polyadenylation signals of the rabbit β-globin and simian virus 40 early genes. Transgenic mice were identified by PCR on the genomic DNA extracted from tail biopsies. A 475-bp fragment of DNA was amplified by PCR using the primer, to detect tail DNA bearing the GFP sequence. Transgenic lines were maintained as heterozygous by breeding with to C57BL/6J inbred mice.

Slice preparation

Adult mice were deeply anaesthetized (intraperitoneal injection of 60 mg/kg of sodium pentobarbital) and decapitated. The brain was exposed and chilled with oxygenated artificial cerebrospinal fluid (ACSF). And the OBs were dissected. Thin slices (100–150 μm) were obtained by cutting the OB in the coronal plane, placed in the recording chamber (1 cm^3 volume), and mounted on an Olympus BX50WI microscope. The slices were constantly superfused with physiological saline using a gravity flow system (2 ml/min).

Cell dissociation

Adult mice were used to isolate OB neurons. Two solutions were used for the preparation: a dissecting solution and Tyrode solution. The dissecting medium (DM) contained (in millimoles) 82 Na_2SO_4, 30 K_2SO_4, 10 HEPES, 5 $MgCl_2$, 10 glucose, and 0.001% phenol red indicator; pH was adjusted to 7.4 with NaOH; and the solution was continuously bubbled with 100% O_2. Tyrode solution contained (in millimoles) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 20 glucose; the pH was adjusted to 7.4 with NaOH; and the solution was continuously bubbled with 100% O_2. Dissociation of the OB by enzymatic digestion and mechanical trituration was performed following the procedure described by Gustincich et al. (1997), with minor changes. After dissecting and slicing the bulbs, small pieces of the preparation were transferred to a solution containing DM and 0.3% protease type XXIII (Sigma, St Louis, MO) for 30–45 min at 37 °C. After enzymatic digestion, the bulbs were transferred to solution containing DM, 0.1% bovine serum albumin (Sigma) and 0.1% trypsin inhibitor (Sigma) to stop protease activity (10 min, 37 °C). Bulbs were finally suspended in Tyrode solution and triturated using fire-polished Pasteur pipettes of varying gages. The cell suspension was centrifuged at 500 × g (5 min), and the pellet was resuspended in Tyrode solution. The dissociated OB neurons were plated on a glass coverslip previously coated with concanavalin A (1 mg/ml) to allow sedimentation of cells. The cells were allowed to set on the glass for at least 1 h before commencement of recordings. Isolated dopaminergic cells were identified under epifluorescence microscope.

Electrophysiological methods

Membrane currents were recorded and acquired with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and a 12-bit A/D–D/A converter (Digidata 1440A; Axon Instruments); off-line analysis was performed using version 10 of pClamp (Axon Instruments).

Pipettes had a resistance of 4–5 MΩ when filled with standard intracellular (IC) solution; the seal resistance was always greater than 3 GΩ.

Solutions

The extracellular (EC) solutions used had the following composition (in millimoles): standard ACSF: 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 15 glucose; high K EC solution: 115 NaCl, 12 KCl, 26 $NaHCO_3$,

1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 15 glucose. Saline was continuously bubbled with 95% O_2/5% CO_2; the osmolarity was adjusted at 305 mOsm with glucose. In order to avoid possible synaptic contaminations from the GABAergic and glutamatergic terminals impinging onto dopaminergic cells, in slice preparation the EC solution was always supplemented with kynurenate (1 mM) and bicuculline (50 μM).

The standard pipette-filling IC solution had the following composition (in millimoles): 120 KCl, 10 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 2 Na-ATP, and 10 glucose.

Amphotericin B was included in the recording electrode-filling solution as perforating agent (200 μg/ml plus 300 μg pluronic F-127). In order to make sure of the integrity of the perforated patch, EGTA was omitted from IC solution and the concentration of $CaCl_2$ was raised to 3 mM. Data were collected after the series resistance fell to <50 MΩ.

In all IC solutions, the osmolarity was adjusted to 295 mOsm with glucose and the pH to 7.2 with KOH.

All drugs and fine chemicals were purchased from Sigma. Drugs were locally applied with a rapid solution changer (RSC-160, Biologic, Claix, France).

Statistics

Data were analyzed using Origin 7.5 software.

Results

When recorded in the cell-attached configuration, both in enzymatically dissociated preparations (n = 32) and in thin slices (n = 74), dopaminergic neurons were spontaneously active, as previously reported (Pignatelli et al. 2005). In cell-attached mode, the application of ACh (1 mM) produced an evident inhibitory effect on the spontaneous firing, leading to a marked frequency reduction and occasionally to a complete block of the action currents (Figure 1A). This effect, however, was lost after rupture of the patch and the

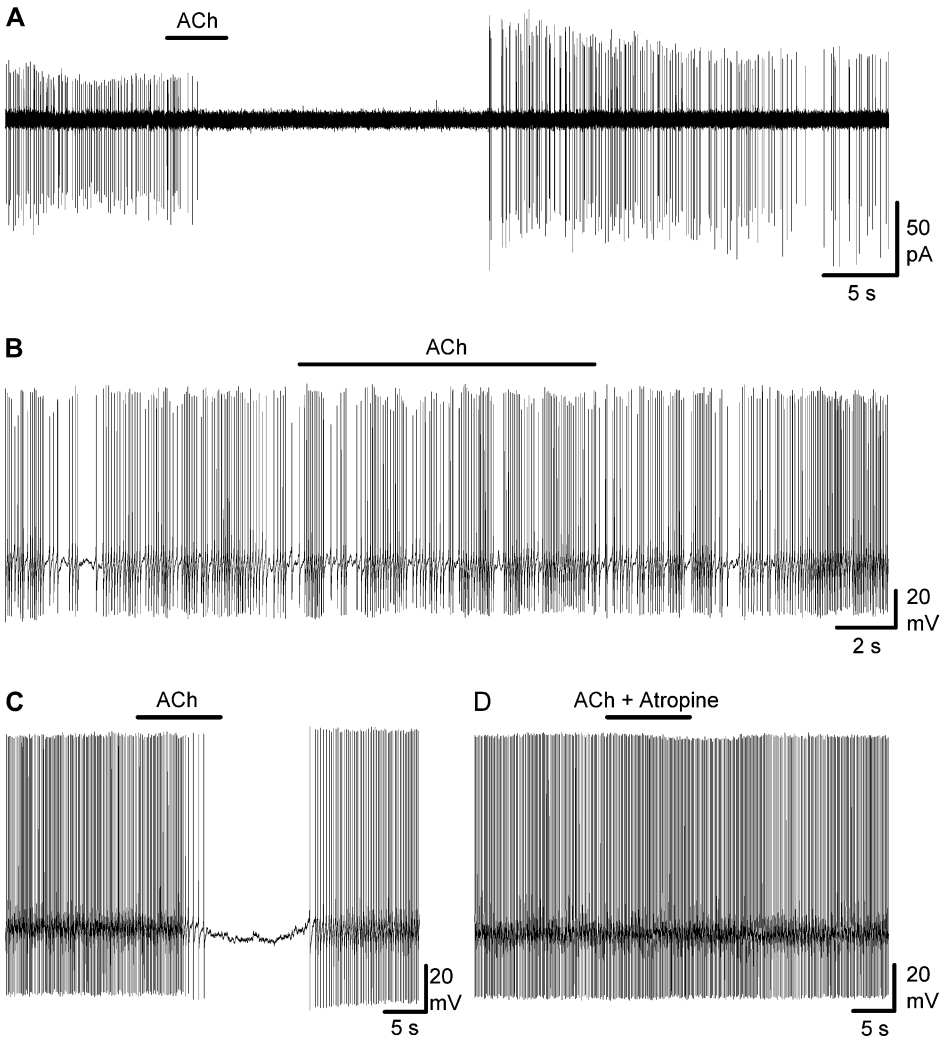

Figure 1 Effect of ACh on spontaneous firing. **(A)** Effect of ACh (1 mM) on action currents recorded in cell-attached mode. **(B)** In the same cell, an even longer application of ACh is ineffective few minutes after the passage to the whole-cell configuration, suggesting the involvement of a diffusible factor. **(C,D)** Whole-cell recording in perforated patch: responses to ACh alone (left) and the presence of a muscarinic blocker (atropine 10 μM, right). Recordings made in slices.

consequent passage to the whole-cell configuration (Figure 1B, same cell), suggesting the involvement of some diffusible factor which was washed out after rupture of the patch. We therefore made all our whole-cell recordings in amphotericin perforated patches (see Materials and methods). Using this protocol, under current-clamp conditions, bath application of ACh evoked a 2 to 3 mV hyperpolarization, occasionally leading to the block of spontaneous firing (Figure 1C); the effect was fully reversible upon washout.

The cholinergic effect in dopaminergic cells was mediated exclusively by metabotropic receptors as atropine (10 μM) prevented any inhibition of ACh on spontaneous firing (Figure 1D).

The effect mediated by ACh in the glomerular layer was restricted to dopaminergic cells. Nondopaminergic periglomerular cells were not spontaneously active; and therefore, the effect had to be evaluated from ACh-induced variations in membrane potential and current. In 13 nondopaminergic cells examined in thin slices, we could never observe any hyperpolarization in current-clamp mode—or any outward current in voltage-clamp mode—set off by ACh (data not shown); routinely, these experiments were repeated at 3 different potentials (−30, −70, and −90 mV, see Figure 4 below), and at none of these potentials, we observed any effect.

Bath application of muscarine (50 μM), in slices (n = 6) and in enzymatically dissociated cells (n = 7), reproduced the effect of ACh reducing reversibly spike discharge of dopaminergic cells (data not shown).

Immunohistochemical analysis has shown that the main subtype of muscarinic receptor present in the glomerular layer is m2 (Fonseca et al. 1991; Crespo et al. 2000), so we further investigated the effect of the m2 agonist oxotremorine (Gillard et al. 1987) in thin slices (n = 28) and in dissociated cells (n = 9). Oxotremorine (10 μM) reproduced the action of ACh and of muscarine on firing frequency (Figure 2A) and on membrane potential (Figure 3).

The reduction of firing frequency and the hyperpolarization produced by the activation of muscarinic receptors was paralleled by 2 additional effects: an increase of the velocity of repolarization (Figure 2C) and an increase of the amplitude of the afterhyperpolarization (Figure 2D). Because all these effects could be accounted for by an increase in a potassium conductance, we compared the reversal potential of the hyperpolarization induced by the activation of muscarinic receptors with the potassium equilibrium potential in dissociated cells (n = 13). We first measured the amplitude of the currents evoked in response to local application of the muscarinic agonists at different membrane potentials under voltage-clamp conditions using standard solutions (Figure 4A). The reversal potential so calculated was −92.5 mV (Figure 4B), in reasonable agreement with the nernstian potassium equilibrium potential (E_K = −97.8 mV). We then repeated the set of experiments after modification of the EC concentration of K ions (see Materials and methods) so that E_K was −60 mV; under these conditions, the new reversal potential calculated was −57.8 mV (Figure 4C,D).

In spite of the multiple evidence of the activation of a potassium conductance, we were unable to evidence any reduction of the membrane resistance following the activation of metabotropic cholinergic receptors (data not shown). The input resistance, evaluated by measuring the voltage responses to the injection of hyperpolarizing current steps (30 pA amplitude, 80 ms duration, 1 p.p.s.), did not show any significant variation, whereas the hyperpolarization was always present, although limited to few millivolts.

The standard EC solution used for these experiments included the tryptophan metabolite kynurenate (see Materials and methods) to avoid synaptic interferences from other cells. However, kynurenate, a classical antagonist of glutamate at ionotropic receptors, has been reported to affect the α7 nicotinic receptors (Hilmas et al. 2001). The α7 subunit has been found in the periglomerular layer (Le Jeune et al. 1995), and the activation of receptors of this type in the OB leads to a facilitation of glutamatergic neurotransmission (Girod et al. 2000). In order to exclude any nicotinic-mediated contribution to the effects described above, in a series of experiments in dissociated cells ACh has been applied in the presence of atropine (10 μM) and without kynurenate in the external saline. In these conditions, ACh had no effects on frequency discharge or on membrane potential, confirming the purely muscarinic action on dopaminergic cells. Finally, in dissociated cells (n = 8), the nicotinic agonist DMPP (50 μM) has been focally applied to TH-GFP+ cells, and in no case, we have been able to observe any response.

Discussion

In this paper, we have analyzed the responsiveness to cholinergic agonists of one of the cell types present in the glomerular layer, the periglomerular cells. We show that only a subset of them, the dopaminergic, respond to cholinergic stimulation and that their response is mediated only by metabotropic receptors. Nondopaminergic monopolar periglomerular cells do not show any response, either ionotropic or metabotropic, to cholinergic agonists. We show also that the activation of cholinergic receptors induces an inhibitory response, leading to a marked reduction of the spontaneous firing, a hallmark of dopaminergic neurons. This inhibitory response consists in a small hyperpolarization (3–4 mV), which is paralleled by an outward current in voltage-clamp conditions. A few millivolts hyperpolarization might appear ineffectual, but we have observed experimentally—and shown in numerical models of these cells (Pignatelli et al. 2005)—that the delicate and precise equilibrium between pacemaker currents can be effectively perturbed by a hyperpolarization of this magnitude.

A number of converging observations suggest that the reduction of periglomerular cell firing by muscarinic receptor activation may result from a membrane-potential

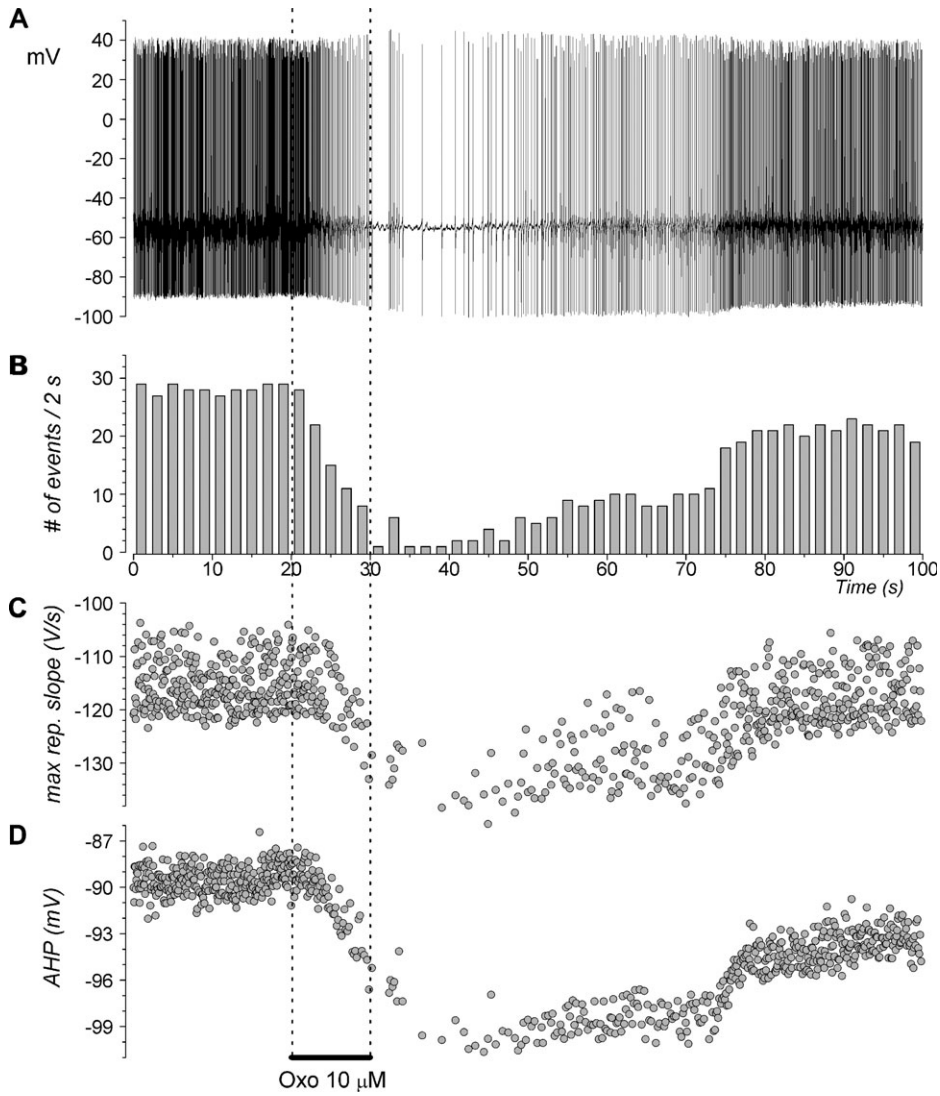

Figure 2 Effects of m2 muscarinic agonist. **(A)** Whole-cell recording in perforated patch; response to the application of oxotremorine 10 μM; **(B)** Frequency count of the tracings shown in A, at the same time base; bin width of 2 s; **(C)** Measure of the max repolarizing rate, in volts per second, with an increase of about 20% following application of the muscarinic agonist; explanation in the text; **(D)** Measure of the afterhyperpolarization amplitude. Recordings made in slices.

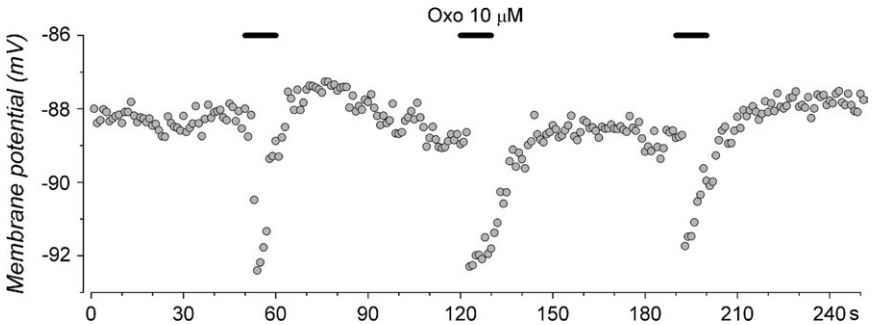

Figure 3 Effect of multiple application of the m2 muscarinic agonist on membrane potential. Each point is the resting potential averaged over 100 ms measured at 1-s interval. Recordings made in slices.

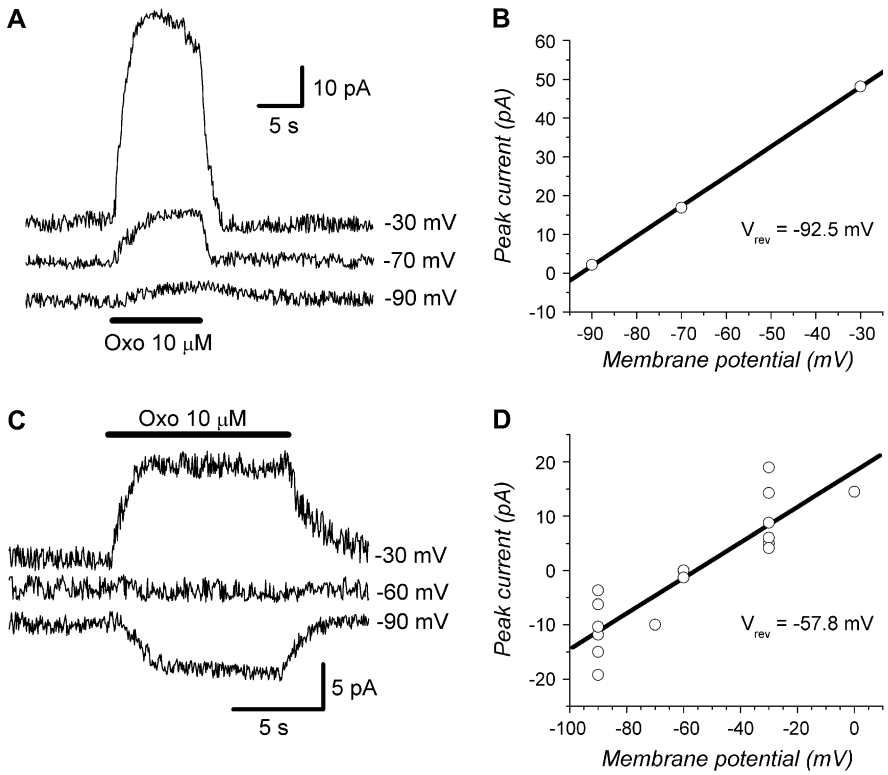

Figure 4 Reversal potential of the conductance opened by the muscarinic agonist. **(A)** Responses to local application of oxotremorine in standard solutions (E_K = −97.8 mV) at the indicated voltages. **(B)** Amplitudes of the currents shown in A as a function of membrane potential; the reversal potential, calculated from the regression line, is −92.5 mV; **(C)** Responses to local application of oxotremorine in high potassium EC saline (E_K = −60 mV) at the indicated voltages; **(D)** Evoked currents versus membrane potential; the regression line shows a reversal potential at −57.8 mV. Recordings made in enzymatically dissociated cells.

hyperpolarization caused by activation of a potassium conductance. The strongest evidence is that the muscarinic-activated current has a reversal potential nearly coincident with the potassium equilibrium potential, but the role of a K conductance is further substantiated by additional observations. First is the increase of the action potentials hyperpolarizing rate in a system lacking transient potassium currents: the presence of this type of conductance would explain the increase of the repolarizing rate by the removal of inactivation, but the K conductances present in these cells are only of the delayed rectifier type (Pignatelli et al. 2005). Second is the increase of the afterpotential hyperpolarization. In this context, it is interesting to observe that a muscarinic control on dopaminergic cell firing has been described also in other brain structures (Egan and North 1986; Mccormick and Pape 1988; for review, see Brown et al. 1997).

We have not made a systematic study of cholinergic responses in the other neuronal types present in the glomerular layer, namely, external tufted cells and short-axon cells, for which there are indications of an excitatory response mediated by nicotinic receptors (Nickell and Shipley 1988b; Castillo et al. 1999). We can confirm and extend to a larger number of observations the report of Castillo et al. (1999) that monopolar periglomerular cells (dopaminergic or not) do not respond to nicotinic stimulation.

Functional aspects

The muscarinic modulation exerted by centrifugal fibers on dopaminergic neurons adds further complexity to the already intricate mechanism of odor processing in the OB. Within this process, the role of dopaminergic neurons has never been truly elucidated, and therefore, any speculation about the functional implications of muscarinic modulation of these cells would be perhaps premature. Nevertheless, some consideration can be attempted.

The cholinergic input to the glomerular layer predictably influences olfactory processing at the first level of synaptic integration. However, up to date, the focus has been limited to the nicotinic action of cholinergic input. Nickell and Shipley (1988b) identified the cholinoceptive targets in the glomerular layer as short-axon cells, and Elaagouby et al. (1991) proposed that this cholinergic action would be mediated via nicotinic receptors. The dominance of nicotinic action the glomerular layer seemed to be confirmed by the demonstration that activation of nicotinic receptors excites bipolar periglomerular cells (Castillo et al. 1999).

The possibility of a muscarinic modulation of the bulbar circuits in the glomerular layer has never been considered until Crespo et al. (2000), using immunohistochemical techniques, showed that a large subset of "juxtaglomerular" cells

expressed m2-type receptor in their somatodendritic domain. Our results confirm and extend this observation. Several papers report that muscarinic agonists applied to the OB lead to an impairment of odor discrimination (Ravel et al. 1994; Ghatpande et al. 2006; Mandairon et al. 2006). However, muscarinic receptors are present at many levels in the OB, so it is impossible to individuate the specific contribution of dopaminergic modulation to this effect.

It is well established that the periglomerular cells are a rather heterogeneous population under both anatomical (Kosaka et al. 1997) and functional (Puopolo and Belluzzi 1998) aspects. An important characteristic of theirs is that only 1 subset, GABAergic/dopaminergic, is innervated by the olfactory axons (Toida et al. 2000), whereas the other subsets (calbindin and calretinin positive) are not (Kosaka et al. 1997). In the olfactory glomeruli, dopamine can inhibit glutamate release from olfactory receptor neuron nerve terminals via a presynaptic D2 receptor–mediated mechanism (Hsia et al. 1999; Berkowicz and Trombley 2000; Ennis et al. 2001; Davila et al. 2003). Immunohistochemical evidence indicates that dopaminergic cells express only muscarinic receptors (Crespo et al. 2000), and the data presented here indicate that their activation leads to an inhibition of the spontaneous activity of these cells. Putting all these data together, one might expect that the cholinergic input onto dopaminergic cells would potentiate the release of neurotransmitter from olfactory nerve terminals. However, further studies are needed in order to investigate a possible convergence of muscarinic actions in the bulbar transmission, at different levels and possibly through different muscarinic receptor subtypes.

Another order of considerations is related to the peculiar property of dopaminergic neurons, which are generated and added to the bulbar circuitry also in adulthood. Interestingly, activation of the cholinergic system (nicotinic and muscarinic) promotes survival of newborn neurons in the adult dentate gyrus and OB under both normal and stressed conditions (Kaneko et al. 2006), and it is interesting to observe the presence of m2 receptors on dopaminergic neurons.

This muscarinic effect on dopaminergic interneurons may be important in modulating OB output to central structures required for driven behaviors and may be relevant to understanding mechanisms underlying the perturbations of cholinergic inputs to cortex that occur in Alzheimer's disease.

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