Inward rectifier potassium (K\textsubscript{ir}) current in dopaminergic periglomerular neurons of the mouse olfactory bulb

Mirta Borin\textsuperscript{1}, Alex Fogli Iseppe\textsuperscript{1}, Angela Pignatelli and Ottorino Belluzzi* 

Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

INTRODUCTION

The background potassium conductance mediated by inward rectifying potassium channels impacts on many physiological processes, from the excitability profile of nerve and muscle cells to hormone release. A voltage-dependent block of the channel pore by polyamines and intracellular magnesium is thought to be responsible for the inward rectification of these channels (Lopatin et al., 1995); for a review see Hibino et al. (2010) which, opening at potentials close to $E_{K}$, tend to maintain the membrane in a hyperpolarized state.

Dopaminergic (DA) periglomerular (PG) neurons are critically placed at the entry of the bulbar circuitry, directly in contact with both the terminals of olfactory sensory neurons and the apical dendrites of projection neurons; they are autorhythmic and are the target of numerous terminals releasing a variety of neurotransmitters. Despite the centrality of their position, suggesting a critical role in the sensory processing, their properties—and consequently their function—remain elusive. The current mediated by inward rectifier potassium ($I_{K\text{ir}}$) channels in DA-PG cells was recorded by adopting the perforated-patch configuration in thin slices; $I_{K\text{ir}}$ could be distinguished from the hyperpolarization-activated current ($I_{h}$) by showing full activation in <10 ms, no inactivation, suppression by $Ba^{2+}$ in a typical voltage-dependent manner ($IC_{50}$ 208 $\mu$M) and reversal potential nearly coincident with $E_{K}$. $Ba^{2+}$ (2 mM) induces a large depolarization of DA-PG cells, paralleled by an increase of the input resistance, leading to a block of the spontaneous activity, but the $K_{ir}$ current is not an essential component of the pacemaker machinery. The $K_{ir}$ current is negatively modulated by intracellular cAMP, as shown by a decrease of its amplitude induced by forskolin or 8Br-cAMP. We have also tested the neuromodulatory effects of the activation of several metabotropic receptors known to be present on these cells, showing that the current can be modulated by a multiplicity of pathways, whose activation in some case increases the amplitude of the current, as can be observed with agonists of D2, muscarinic, and GABA\textsubscript{A} receptors, whereas in other cases it has the opposite effect, as it can be observed with agonists of α1 noradrenergic, 5-HT and histamine receptors. These characteristics of the $K_{ir}$ currents provide the basis for an unexpected plasticity of DA-PG cell function, making them potentially capable to reconfigure the bulbar network to allow a better flexibility.

Keywords: olfactory bulb, dopaminergic neurons, periglomerular cell, $K_{ir}$ channels, patch-clamp techniques

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MATERIALS AND METHODS

ETHIC STATEMENT
A total of 123 mice have been used. The experimental process was designed so as to minimize animal number and suffering of the animals used. The protocols adopted were designed according to European Council Directives (609/1986 and 63/2010) and Italian laws (DL 116/92) on the protection of animals used for scientific purposes. The experimental procedures were approved by the Ethical Committee for Animal Experiments of the Ferrara University (CEASA), by the Directorate-General for Animal Health of the Ministry of Health, and supervised by the Campus Veterinarian of the University of Ferrara.

ANIMALS AND SURGICAL PROCEDURES
For these experiments we used a transgenic mice strain (TH-GFP/21–31), carrying the eGFP transgene under the control of the TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002). The TH-GFP strain was maintained as heterozygous by breeding with C57BL/6j inbred mice.

RECORDING CONDITIONS
The temperature of the 1-mL recording chamber was controlled using Peltier devices (RS Components, Milan, Italy) and measured with a high-precision, type K thermocouple (RS Components).

For current and voltage recordings an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) was used, and the signals were digitized and acquired with a Digidata 1440A (Molecular Devices) 16 bit A/D–D/A converter; correction for the junction potential was calculated using the related function of the acquisition software (pClamp 10, Molecular Devices).

Patch pipettes were built from borosilicate glass capillaries (1.5 o.d., 0.87 i.d., with filament; Hilgenberg, Malsfeld, Germany) with a Zeitz-DMZ puller (Martinsried, Germany), and showed a resistance of 4–5 MΩ when filled with standard intracellular solution (see below); the seal formation was assisted by a MCPU-3 air pressure controller (MPI, Lorenz Meßgerätebau, Katlenburg-Lindau, Germany); the seal resistance obtained was always greater than 3 GΩ.

SOLUTIONS
The solutions used had the following composition (mM):

- **EC0**, standard ACSF extracellular (EC) solution: 125 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 15 glucose.
- **EC1**, high K EC solution: 95 NaCl, 32.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 15 glucose.
- **EC2**, K-TEA EC solution: 100 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 20 TEA, and 10 glucose.
- **EC3**, high K-TEA EC solution: 70 NaCl, 32.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 20 TEA, and glucose.

All EC solutions were continuously bubbled with 95% O2 and 5% CO2, and the osmolarity was corrected to 305 mOsm with glucose.

**Standard pipette-filling intracellular (IC) solution:**
120 KCl, 10 NaCl, 2 MgCl2, 0.5 CaCl2, 5 EGTA, 10 HEPES, 2 Na-ATP, 10 glucose; in this solution, the free IC calcium concentration was calculated to be 16 nM (http://www.stanford.edu/~cpatton/downloads.htm).

For perforated patches, 200 μg/ml amphotericin B was added to the IC solution (plus 300 μg/ml pluronic F-127). EGTA was omitted and CaCl2 concentration was increased to 3 mM in the electrode filling solution to control of the integrity of the perforated patch, as in case of unexpected rupture, the massive entry of calcium from the pipette would cause a rapid cell death. Data were collected after the series resistance dropped below 50 MΩ.

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

Except where indicated, when recording from slices, the EC solutions included two mixtures of blockers:

- **BL1**, for ligand-gated channels (1 mM kynurenic acid and 10 μM bicuculline).
- **BL2**, for voltage-dependent channels (TTX 0.6 μM, Cd2+ 100 μM and ivabradine 10 μM).

ANALYSIS OF CURRENT RECORDINGS

I_Kir amplitude was measured as instantaneous current at the beginning (I_{inst}) and at the end of test voltage pulses as steady-state current (I_{ss}).

The temperature-dependence of activation and deactivation rate constants were calculated as:

\[ Q_{10} = \left( \frac{\text{rate}(T_2)}{\text{rate}(T_1)} \right)^{\frac{10}{T_2-T_1}} \]

where \( Q_{10} \) is the fold-change as a consequence of increasing the temperature by 10°C, calculated between the two temperatures \( T_1 \) and \( T_2 \).

DATA ANALYSIS
To evoke the \( K_r \) current, a series of hyperpolarizing voltage steps in −10 mV increments were imposed from the holding potential of −40 to −130 mV at 10 s intervals. Unless otherwise indicated, the current amplitudes were measured at the end of the hyperpolarizing step (steady-state current).

When box charts are used to represent data ensembles, the central square represents the mean, the central line the median, the range of the boxes represent the S.E, and the whiskers define the 10–90% range of data samples.

Offline analysis was performed using version 10.3 of pClamp (Molecular Devices) and version 8.1 of OriginPro (OriginLab Corporation, Northampton, MA).

Unless otherwise indicated, data are presented as means ± s.e.m.; for the statistical analysis we used the software Prism 5 (GraphPad, La Jolla, CA). The statistical significance was assessed with Two-Way analysis of variance (ANOVA), or Student’s t-test for paired samples as indicated; in Two-Way ANOVA multiple comparisons post-tests were performed using the Bonferroni method.
$P$ value of $< 0.05$ was considered significant; in figures, 1 to 4 asterisks represent differences significant at the 0.05, 0.01, 0.001, 0.0001 level, respectively.

RESULTS

The data are based on recordings from 285 TH+ PG neurons from the glomerular layer. Most OB DA cells are small, PG interneurons (about 5–8 μm in diameter), but there is also a certain number of external tufted (ET) cells (about 10–15 μm in diameter) (Baker et al., 1993; Kosaka and Kosaka, 2009, 2011). In this study, we restricted the analysis to PG cells; these were selected on the basis of their location around the glomerular border, dendritic arborization extending within the glomerular neuropil, membrane capacitance (8.0 ± 0.2 pF; $n = 297$) and input resistance (979.4 ± 33.4 MΩ; $n = 276$). In addition to the evident differences in dimension (Kosaka and Kosaka, 2008), membrane capacity and input resistance (Pignatelli et al., 2005), DA-PG cells show a regular firing pattern, whereas DA-ET cells show burst pattern activity (Hayar et al., 2004). Finally, short-axon cells, which have membrane capacitance and input resistance very similar to PG cells, can be usually recognized in slice for their fusiform shape, position amid different glomeruli, and dendrites extending between neighboring glomeruli (Shipley and Ennis, 1996).

IDENTIFICATION AND BASIC PROPERTIES OF THE CURRENT

In a first series of experiments, carried out using perforated patch recordings in slice at 34°C, hyperpolarizing steps were delivered from a holding potential of $−40$ mV, which is close to the predicted $K^+$ equilibrium potential in our experimental conditions ($E_K = −37.7$ mV for $[K^+]_o = 32.5$ mM), to potentials ranging from $−60$ to $−130$ mV (10 mV increments, 10 s interval). The inward current obtained in response is shown in Figure 1A; a fraction of this current could be suppressed by two organic compounds known as selective HCN channels blockers, i.e., ZD7288 30 μM (BoSmith et al., 1993) and S-16257, a.k.a. ivabradine, 10 μM; (Bois et al., 1996; Bucchi et al., 2002), Figure 1B; the $h$-current in DA-PG cells has been the object of another study (Pignatelli et al., 2013), and will be not further discussed in this paper.

The current activated by hyperpolarization remaining after suppression of the $h$-current, was suppressed by $Ba^{2+}$ (Figure 1C), a classical blocker of $K_h$ channels (Hagiwara et al., 1978; French and Shoukimas, 1985); for its potassium and voltage-dependence, reversal potential and sensitivity to $Ba^{2+}$ this component was identified as potassium inward rectifier ($K_{IR}$) current (Hibino et al., 2010). The $I/V$ relationship of the current evoked by hyperpolarization in a group of 81 cells in the presence of 0.6 μM TTX, 100 μM Cd$^{2+}$ and 10 μM ivabradine is shown in Figure 1D; here, and in the following experiments, for the inherent difficulties, the leakage component of the current evoked by hyperpolarization was not subtracted.

**Barium sensitivity**

The $Ba^{2+}$ dependent block of $I_{KIR}$ was evaluated from the decrease of steady-state current amplitude at $−120$ mV in the presence

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**FIGURE 1** | General properties. (A–C) Representative currents obtained in response to hyperpolarizing pulses in 32.5 mM external $K^+$ solution: (A) EC1 saline with 1 mM kynurenic acid, 10 μM bicuculline (BL1 mix), 0.6 μM TTX, 100 μM Cd$^{2+}$; (B) same solution as in A plus 10 μM ivabradine; (C) same solution as in B plus 2 mM Ba$^{2+}$. Voltage steps from a holding potential of $−40$ mV with hyperpolarizing steps ranging from $−60$ to $−130$ mV in 10 mV increments. (D) $I/V$ relationship of peak (green dots) and steady-state (blue dots) current; mean current amplitude of 81 cell recordings. Vertical error bars represent standard error; EC2 saline, with BL1 and BL2 mixes of blockers. (E) Instantaneous $I/V$ curve during application of a 220 mV/s ramp protocol (from $−180$ to $+40$ mV, 0.23 V/s) in a DA PG cell perfused with the solution described in (A), after subtraction of the ohmic leak; the red dot ($−41.3$ mV) marks the observed reversal potential, the green dot the Nernstian equilibrium potential in the experimental conditions used ($[K^+]_o = 32.5$ mM). All the experiments shown in this figure were performed in slice, perforated patch configuration, at 34°C.
of increasing external Ba\(^{2+}\) concentrations. In Figure 2A is represented the percentage of the current inhibition as a function of external Ba\(^{2+}\) concentrations ranging from 1 µM to 10 mM. The data could be interpolated by a logistic equation with the form:

\[
y = \frac{I_{\text{max}}}{1 + \left(\frac{[\text{Ba}^{2+}]_o}{K_d}\right)^H}
\]

where \(I_{\text{max}}\) is the asymptotic value of the current block, \(K_d\) the external Ba\(^{2+}\) concentration causing 50% block, and \(H\) is the slope of the dose-response curve (Hill coefficient). The fit of the Ba\(^{2+}\) block of peak \(I_{\text{Kir}}\) gave a \(K_d\) of 0.21 ± 0.10 mM and a \(H\) value of 0.69 ± 0.23 (\(n = 5, -120 \text{ mV}\)).

**Voltage-dependence of the steady-state block by Ba\(^{2+}\) and Cs\(^{+}\)**

Ba\(^{2+}\) and Cs\(^{+}\) have been found to block the \(I_{\text{Kir}}\) channel through an interaction which is thought to occur via a deep binding site, located approximately half-way along the channel (Standen and Stanfield, 1978; Shieh et al., 1998; Alagem et al., 2001). As normally occurs for deep-site blockers, Ba\(^{2+}\) and Cs\(^{+}\) block is highly voltage dependent (Hagiwara et al., 1978; Harvey and Ten Eick, 1989; Alagem et al., 2001). The effect induced by 1 mM Ba\(^{2+}\) in DA-PG cells is shown in Figures 2B,C. The time required for the blocking reaction to reach steady state was calculated by fitting the exponential decay of the currents to the function:

\[
I = A \exp(-t/t_{\text{blk}}) + C
\]

where \(A\) is the current amplitude, \(t\) is the independent variable, \(C\) is the current amplitude at the steady-state, and \(t_{\text{blk}}\) is the blocking time constant, whose voltage dependence is shown in Figure 2F.

We also tested the effects of 1 mM Cs\(^{+}\), another classical blocker of this channel for which the approach to steady-state block following a voltage step is much faster than for Ba\(^{2+}\) (Hagiwara et al., 1976, 1978; Shioya et al., 1993). The results, shown in Figures 2D–F, are in good agreement with those reported in literature (Hagiwara et al., 1976, 1978).

**Reversal potential**

The \(I_{\text{Kir}}\) channels are selective for K\(^{+}\) ions, and consequently the reversal potentials of the inward rectifying current for different extracellular K\(^{+}\) concentrations should always follow the Nernstian equilibrium potential for potassium (Figures 1E, 3A). When the [K\(^{+}\)]\(_o\) was changed from 2.5 to 10 and 32.5 mM, the reversal potentials progressively shifted toward more positive potentials (−105.12 ± 3.67 mV, \(n = 15\), for 2.5 mM; −56.67 ± 9.78 mV, \(n = 9\), for 10 mM; −36.78 mV ± 1.89, \(n = 27\), for 32.5 mM); the reversal potentials in the different experimental conditions are represented in Figure 3B, where they are compared to the theoretical Nernstian equilibrium potentials for K\(^{+}\) ions (black triangles). The plot of the reversal potentials against the logarithm of [K\(^{+}\)]\(_o\) gives a linear relationship (\(r^2 = 0.93\)) with a slope of −61.9 mV, close to the theoretical value of −61.0 mV predicted by the Nernst equation (Figure 3C).

**K\(^{+}\) and voltage dependence of the \(I_{\text{Kir}}\)**

Besides the selectivity to K\(^{+}\) ions, another typical characteristic of this current is a voltage-dependence of the \(g_{\text{Kir}}\) conductance (\(g_{\text{Kir}}\)) on the K\(^{+}\) reversal potential; then, in DA-PG cells we further examined the dependence of \(g_{\text{Kir}}\) from membrane potential for different external K\(^{+}\) concentrations.

The conductance-voltage relationship showed the typical sigmoidal profile, increasing at negative potentials and with a point

![Image](https://www.frontiersin.org)
FIGURE 3 | Potassium sensitivity. (A) Effect of changing \([K^+]_o\) on membrane current. Average currents (\(n = 8\)) at the indicated external potassium concentrations in response to voltage ramps from \(-170\) to \(+20\) mV from a holding potential of \(-40\) mV, 0.22 V/s; perforated patches; the bathing solution included B11 and B12. (B) Box charts showing the reversal potentials at different \([K^+]_o\) (same color code as in (A)); black arrow heads to the right of each box mark the expected reversal potentials predicted by the Nernst equation. In the box charts, here and in the following, the square in the center of the box represents the mean value, the horizontal line crossing the box indicates the median, the range of the box represents standard error and the whiskers define the 10–90% range of data sample. (C) Plot of the reversal potential for the inwardly rectifying current against the logarithm of \([K^+]_o\). The linear regression fit (black dash line) has a slope of \(-61.9\) mV, close to the theoretical value of \(-61\) mV predicted by the Nernst equation (red line). (D) \([K^+]_o\)- and voltage-dependence of chord conductance \(g_{Kir}\); the chord conductance was calculated using the equation \(g_{Kir} = \frac{I_{Kir}}{V_m - E_K}\), where \(I_{Kir}\) is the steady state current. \(g_{Kir}\) plotted as a function of voltage-clamp test potentials at 2.5, 10, and 32.5 mM \([K^+]_o\). (E) Data in (D) replotted as a function of the driving force. Data points were fitted by Boltzmann curve using a least-squares method; \(n\) for 2.5, 10, and 32.5 mM was 7, 5, and 12, respectively.

Effect of \(I_{Kir}\) on membrane potential and input resistance
If the \(I_{Kir}\) is active at rest, then it should be expected that the block of the current with \(Ba^{2+}\) should influence both input resistance and resting potential; in effect, \(Ba^{2+}\) (2 mM) induces a rapid and strong depolarization of DA-PG cells (Figures 4A,C), paralleled by an increase of the firing frequency before its block in depolarization (Figures 4A,B). The \(K_p\) current is not essential to the pacemaker process, as the injection of hyperpolarizing current (40 pA at the time marked of half-activation approximately centered at \(E_K\) (Figure 3D). Plotting the conductance for different \([K^+]_o\) as a function of the driving force \((V_m - E_K)\), the midpoints were approximately aligned at the zero of the abscissa axis, with minima and maxima at the same voltage levels. This confirms that \(K_p\) conductance in DA-PG cells has a voltage-dependence which is function of \(E_K\), in analogy to what has been found for \(I_{Kir}\) in several other preparations (Hestrin, 1981; Leech and Stanfield, 1981; Harvey and Ten Eick, 1988).
by a downward arrow in the representative experiment shown in Figure 4A) resumes completely the activity.

To find a parameter accounting for the “resting” membrane potential in a cell characterized by autorhythmicity, we have calculated the potential at which the cell was staying most of the time, that we have defined “prevailing membrane potential,” using the method illustrated in Figure 4C: frequency count histograms of the digitized membrane potential were obtained at 10 s intervals, and the distributions were fitted by an exponentially modified Gaussian function (Kalambet et al., 2011) with the form:

$$f(x) = y_0 + \frac{A}{t_0} e^{\frac{1}{2}(\frac{x-x_0}{\sigma})^2} - \frac{x-x_0}{\sigma} \int_{-\infty}^{x} e^{-y^2} \frac{1}{\sqrt{2\pi}} dy$$  

(4)
where

\[ z = \frac{x - x_c}{w} - \frac{w}{t_0} \]

and \( y_0 \) is the offset, \( A \) is the amplitude, \( x_c \) is the center of the peak (i.e., the prevailing potential, red dot in Figure 4C), \( w \) is the width of the peak and \( t_0 \) is the modification factor (skewness, \( t_0 > 0 \)).

Using this method, we measured the variation of the prevailing membrane potential for two different external \( \text{Ba}^{2+} \) concentrations (0.3 and 2 mM). In a group of cells, we measured a depolarization from \(-59.1 \pm 4.1 \) to \(-45.94 \pm 4.0 \text{ mV} \) with 0.3 mM \( \text{Ba}^{2+} \) (Figure 4E; \( n = 14, p = 0.000025, t \)-test for paired data), and from \(-52.3 \pm 3.7 \) to \(-62.2 \pm 4.9 \text{ mV} \) with 2 mM \( \text{Ba}^{2+} \) (Figure 4E; \( n = 7, p = 0.0006, t \)-test for paired data).

Next, we tested the variations of the input resistance in response to hyperpolarizing current pulses in presence of 0.3 and 2 mM \( \text{Ba}^{2+} \) (Figures 5A,B). In these conditions, for both concentrations we observed an increase of the membrane impedance (Figures 5D,E). In \( \text{Ba}^{2+} \) 0.3 mM the membrane impedance changes from \( 1079.6 \pm 163.9 \) to \( 1260.0 \pm 186.5 \text{ M} \Omega \) (Figure 5C; \( n = 12, p = 0.00033, t \)-test for paired data), and in \( \text{Ba}^{2+} \) 2 mM the mean value changes from \( 1061.6 \pm 202.0 \text{ M} \Omega \) to \( 1621.2 \pm 284.2 \text{ M} \Omega \) (\( n = 10; \) Figure 5C; \( p = 0.0018, t \)-test for paired data).

**Effect of temperature**

As for the other K currents, also the Kir kinetics is sensitive to thermic conditions. The temperature at which electrophysiological recordings are obtained influence the current kinetics (Figure 6A), and therefore in this study all recordings were made in controlled temperature conditions.

\( Q_{10} \) at the different voltages, measured using Equation 1, is substantially stable, with a mean value of \( 1.22 \pm 0.008 \) (\( n = 9; \) Figure 6B), a value which is typical for inward rectifying K-conductances (Leech and Stanfield, 1981; Mitsuiye et al., 1997; Paajanen and Vornanen, 2003).

**PHARMACOLOGY**

**Blockers**

Although the involvement of Kir channels has been demonstrated in numerous common disorders, including hypertension, cardiac arrhythmias and pain, their pharmacology is virtually limited to \( \text{Ba}^{2+}, \text{Cs}^{+} \), and few poorly selective cardiovascular and neuroactive drugs with off-target activity toward these channels (Bhave et al., 2010; Hibino et al., 2010; Lüscher and Slesinger, 2010).

**Tertiapin.** Tertiapin, a toxin from the honey bee (Apis mellifera), is a remarkable exception, as it is a rather selective blocker of Kir1.1 and Kir3.1 – 3.4 channels (Jin and Lu, 1998; Dobrev et al., 2010; Hibino et al., 2010; Lüscher and Slesinger, 2010).

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**FIGURE 5 | Effect of different concentrations of Ba2+ on input resistance.**

(A,B) Sample tracings showing the response to the injection of 40 pA in current-clamp conditions for the indicated external Ba2+ concentrations. (C) Increase of input resistance at the indicated external Ba2+ concentrations. \(+17.8 \pm 3.2\% , n = 12, \text{ and } 58.7 \pm 14.2\% , n = 10 \text{ in } 0.3 \text{ and } 2 \text{ mM external } \text{Ba}^{2+}\) with respect to controls. ** and *** indicate significance levels of 0.01 and 0.001, respectively. (D) Family of tracings obtained in response to hyperpolarizing current pulses as indicated in (A); green and blue traces are taken at the beginning and at the end of a 5′ test; Ba2+ was applied after 2′. (E) Time course of the variation of input resistance for the experiment shown in (D); the dashed line marks the time of application of Ba2+ 2 mM; green and blue dots mark the resistance of the traces with the same color in (D).
Quinacrine is a molecule developed in the 1920s as an anti-malarial agent, based upon the aminoacridine ring structure; more recently, it has been shown to inhibit different voltage-gated potassium channels, are of no interest in our case, but the latter (G protein-coupled Kir, a.k.a. GIRK, channels) are present in the periglomerular layer of the MOB (Karschin et al., 1996), and therefore it was of some importance to test the efficacy of the drug in our cells.

The oxidation-resistant form of the drug, tertiapin-Q, was ineffective when tested alone at concentrations ranging from 100 nM to 3 μM (not shown). However, GIRK channels become activated only following the binding of ligands to their cognate G protein-coupled receptors, which causes the dissociation of the βγ subunits of a pertussis toxin-sensitive G protein which subsequently bind to and activate the GIRK channel (Walsh, 2011). Therefore, we tested the effect of tertiapin after activation of Kir current with oxotremorine, a metabotropic cholinergic receptor activator (see also below). In these conditions, tertiapin completely abolished the current increment promoted by the muscarinic receptor activation (Figure 7A), suggesting that functional GIRK channels are actually present in DA-PG cells.

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**Quinacrine.** Quinacrine is a molecule developed in the 1920s as anti-malarial agent, based upon the aminoacridine ring structure; more recently, it has been shown to inhibit different voltage-gated potassium channels, are of no interest in our case, but the latter (G protein-coupled Kir, a.k.a. GIRK, channels) are present in the periglomerular layer of the MOB (Karschin et al., 1996), and therefore it was of some importance to test the efficacy of the drug in our cells.

The oxidation-resistant form of the drug, tertiapin-Q, was ineffective when tested alone at concentrations ranging from 100 nM to 3 μM (not shown). However, GIRK channels become activated only following the binding of ligands to their cognate G protein-coupled receptors, which causes the dissociation of the βγ subunits of a pertussis toxin-sensitive G protein which subsequently bind to and activate the GIRK channel (Walsh, 2011). Therefore, we tested the effect of tertiapin after activation of Kir current with oxotremorine, a metabotropic cholinergic receptor activator (see also below). In these conditions, tertiapin completely abolished the current increment promoted by the muscarinic receptor activation (Figure 7A), suggesting that functional GIRK channels are actually present in DA-PG cells.
We tested the effects on the $I_{Kr}$-current amplitude of 5-10 min applications of 5-HT (50 μM), dopamine (100 μM, + 1 mM ascorbic acid), quinpirole (D2 agonist, 30 μM), SKF 38393 (D1 agonist, 15 μM) noradrenaline (NA; 100 μM, + 1 mM ascorbic acid), phenylephrine ($\alpha_1$ agonist, 10 μM), clonidine ($\alpha_2$ agonist, 10 μM), histamine (10 μM), oxotremorine (muscarnic agonist, 10 μM) and baclofen (GABAB agonist, 10 μM); the results, illustrated in Figure 9.

NA. The MOB receives a rich noradrenergic projection from the locus coeruleus (LC): approximately 40% of LC neurons (an estimated 400–600 out of 1600 cells) project to the rat OB (Shipley et al., 1985).

NA, acting via $\alpha_1$ receptors, has been reported to inhibit rectifying and non-rectifying leak potassium currents (Inokuchi et al., 1992; Vaughan et al., 1996; Hayar et al., 2001; Nai et al., 2010). We tested the NA (100 μM) on DA-PG cells in slice at 34°C observing a 24.6% reduction in the amplitude of the current activated by hyperpolarization: the current evoked at $-100 \text{ mV}$ decreased from $-17.51 \pm 1.62 \text{ pA/pF}$ in control conditions to $-13.20 \pm 1.23 \text{ pA/pF}$ in the presence of NA ($n = 11; p < 0.001$; Two-Way ANOVA; Figure 9A). Next, we tried to further characterize this effect identifying the subtype of $\alpha$-receptor involved. Clonidine ($\alpha_2$ agonist, 10 μM) was ineffective (from $-15.1 \pm 1.4 \text{ pA/pF}$ in control conditions to $-14.1 \pm 1.7 \text{ pA/pF}$ with clonidine; $n = 4; p = 0.4$—Two-Way ANOVA; Figure 9A), whereas phenylephrine ($\alpha_1$ agonist, 10 μM) induced a 24.1% inhibition (from $-12.9 \pm 1.3 \text{ pA/pF}$ in control conditions to $-9.8 \pm 0.92 \text{ pA/pF}$ with phenylephrine; $n = 8; p < 0.05$—Two-Way ANOVA; Figure 9A), an inhibition almost identical to that of NA.

If the $I_{Kr}$ current deeply influences the resting potential, then we should expect that any modification of the amplitude of the current is paralleled by a variation of the membrane potential. In particular, in this case, a reduction of a hyperpolarizing current should be reflected in a depolarization of the cell in current-clamp experiments; this is exactly what can be observed (Figure 9B):
phenylephrine 10 μM induces a depolarization of 8.1 ± 3.0 mV ($n = 7; p < 0.05$—t-test for paired data).

**ACh.** Cholinergic fibers from the horizontal limb of the diagonal band of Broca project to all bulbar layers, with the heaviest density occurring in the GL and EPL (Ichikawa and Hirata, 1986; Zaborszky et al., 1986; Matsutani and Yamamoto, 2008); the OB itself appears to be devoid of intrinsic cholinergic neurons (Godfrey et al., 1980; Le Jeune and Jourdan, 1991; Butcher et al., 1992; Ichikawa et al., 1997), although this view has been more recently challenged (Krosnowski et al., 2012). The M2 agonist oxotremorine (10 μM) increases from $-10.8 ± 1.1$ pA/pF (CTL) to $-12.4 ± 1.1$ pA/pF the amplitude of the current ($n = 13; p < 0.01$; t-test for paired data), an effect which is paralleled by a 4.5 ± 0.8 mV hyperpolarization in current-clamp conditions ($n = 9; p < 0.001$; t-test for paired data).

**5-HT.** Serotonin (50 μM) induces a decrease of the $K_{ir}$ current amplitude: at $-100$ mV the hyperpolarization-activated inward current is reduced from $-22.8 ± 6.3$ pA/pF (CTL) to $-19.4 ±
5.3 pA/pF (n = 18, p < 0.01; Two-Way ANOVA), to which correspond a depolarization of 12.8 ± 3.2 mV (n = 8; p < 0.001; t-test for paired data) in current-clamp conditions.

**Histamine.** In voltage-clamp conditions, histamine (10 μM) induces a significant reduction of the Kᵦ current amplitude, which at −100 mV decreases from −19.0 ± 2.0 pA/pF (CTL) to −14.1 ± 1.8 pA/pF (test; p < 0.05, Two-Way ANOVA; n = 8), an effect which is paralleled by a 10.9 ± 2.4 mV depolarization (p = 0.0013, n = 10; t-test for coupled data).

**DA.** The presence of autoreceptors is an hallmark of dopaminergic neurons, and therefore it was of interest to verify if their activation could modify the Iₖ₉. Dopamine (100 μM) induces an increase of the Kᵦ current: in slice, at 34°C there is a nearly 17% increase of the current amplitude, from −16.9 ± 2.9 pA/pF (CTL) to −19.9 ± 2.3 pA/pF (current measured in response to a step to −100 mV; n = 9; p < 0.05; Two-Way ANOVA). The effect is exactly mimicked by the D2 agonist quinpirole: 30 μM promotes an average increase 17%, from −15.5 ± 0.8 pA/pF (CTL) to −18.3 ± 1.2 pA/pF (current measured in response to a step to −100 mV; n = 9; p < 0.05; Two-Way ANOVA); on the contrary, the D1 agonist SKF 38393 (Sibley et al., 1982) remains ineffective (15 μM, n = 4; Figure 9A).

**GABA.** Kir3 channel family (GIRK) has been shown to be functionally regulated by GABAR receptors in numerous systems (Sodickson and Bean, 1996; Lüscher et al., 1997; Tabata et al., 2005; David et al., 2006), including dopaminergic neurons (Lacey et al., 1988). We therefore tested the GABAR agonist baclofen (Bowery et al., 1980) 10 μM on the Kᵦ current, without observing any effect (from −30.5 ± 4.6 pA/pF to −29.5 ± 5.0 pA/pF; n = 11; p > 0.5; Two-Way ANOVA; not shown).

**DISCUSSION**

Two hyperpolarization-activated currents with inward rectifying properties are present in TH-GFP+ neurons.

The first is an h-current (Iₜₛₘₜₜ or Iₛ in cardiac tissue), a mixed cation current with a reversal potential substantially positive to Eₖ (Hibino et al., 2010). Iₛ has a relatively slow activation kinetics, is insensitive to Ba²⁺, can be selectively blocked by drugs like ivabradine or ZD728, and does not show a voltage sensitivity dependent on [K⁺]ₒ (Biel et al., 2009). This current has been the object of a previous study (Pignatelli et al., 2013), and will not be further discussed here.

A second type of hyperpolarization-activated current is characterized by fast kinetics, is permeable primarily to K⁺, is blocked by extracellular Ba²⁺ and Cs⁺, has a voltage-dependence itself dependent on extracellular K⁺ concentration, and is identified as a classical inward rectifier potassium current (Kᵦ). Sensitivity to Ba²⁺, insensitivity to selective h-current blockers, fast kinetics of activation and reversal potential, all suggest that the second hyperpolarization-activated current observed in TH-GFP+ neurons and described in this study belongs to this class.

Under physiological conditions, Kᵦ channels generate a large K⁺ conductance at potentials negative to Eₖ, but permit a small current flow also at potentials positive to Eₖ (Hibino et al., 2010); as a result, the Kᵦ conductance has a tonic hyperpolarizing influence on the resting membrane potential (V₉ₜₜₜ), controlling excitability and affecting the repolarizing phase of the action potentials in excitable cells (Constanti and Galvan, 1983; Hume and Uehara, 1985; Day et al., 2005). In this study, we show that the Kᵦ current plays a key role in controlling V₉ₜₜₜ in DA-PG cells, neurons that due to their strategic positioning at the entry of the bulbar circuitry and for direct connection with both the sensory input and projection neurons- are pivotal elements in the operation of glomerular circuits, and we show that the Kᵦ in these cells is finely tuned by a variety of neurotransmitters.

**WHICH POPULATION OF Kᵦ CHANNELS?**

Of the seven main types of Kᵦ channels, at least two (KIR2.x and 3.x) are present in the MOB. Of the 2.x family, KIR2.1 is highly expressed in periglomerular cells (Prüss et al., 2005), as well as KIR2.2 (a.k.a. IRK2/KCNJ12; (Karschin et al., 1996); also KIR2.3 is weakly expressed in the glomerular layer (Inanobe et al., 2002; Allen Brain Atlas, 2013). Quinacrine, which differentially inhibits the Kᵦ channels (KIR2.3 > KIR2.1 > KIR6.2; (Lopez-Izquierdo et al., 2011), suppresses a large (46%) fraction of hyperpolarization-activated inward current. However, the presence of KIR6.x (a.k.a. K_ATP) channels can probably be excluded: these channels are thought to be octomers composed of four pore-forming Kᵦ subunits, and four auxiliary proteins, the sulfonylurea receptors (SURx) believed to be responsible for the channel (Hibino et al., 2010). SURx proteins are not detected in the MOB (Allen Brain Atlas, 2013), and therefore the more likely target of the action of quinacrine are 2.x Kᵦ channels, whose presence in the MOB would be confirmed by our data.

The presence of KIR3.x channels (G-protein-coupled Kᵦ, a.k.a. GIRK, channels) has been reported in the periglomerular layer of the MOB (Karschin et al., 1996); the sensitivity of a fraction of the hyperpolarization-activated inward current to tertiapin, a rather selective blocker of KIR3.1–3.4 channels (Jin and Lu, 1998; Kitamura et al., 2000; Ramu et al., 2008), would confirm this finding.

In conclusion, in control conditions, DA-PG cells display an inward rectifying current at hyperpolarizing potentials around Eₖ. A first component is sustained by Ba²⁺-sensitive KIR2.x channels, which are constitutively active and which are well known to contribute to the resting K⁺ conductance in many cells (Hibino et al., 2010). On the other hand, this background activity could receive the contribution also of KIR3.x channels opening in response to G-protein activation by different neuromodulators, as discussed below.

**PHARMACOLOGY**

Many neuromodulators such as NA, ACh, and 5-HT, play important functions in many sensory systems. As it occurs to other brain functions, sensory perception must be finely tuned according to task demands, qualities of sensory stimuli -such as strength or signal-to-noise ratio- and global physiological state. In this context, it is rather interesting that Iₖᵦ, a current playing such an important role in the resting membrane potential of cells strategically placed at the entry of the bulbar circuitry, can be modulated in both directions by a variety of neurotransmitters.
all released in the region where DA-PG cells reside. The responses induced by neurotransmitters shown and discussed in this work are due to the direct activation of receptors on bulbar DA-PG cells, since all recordings were made in conditions of block of synaptic transmission.

**NA**

In this work, we show that in mouse DA-PG cells, NA and the α1 agonist phenylephrine significantly reduce the IK\( \text{ir} \) and depolarize the cell.

Although the role of NA in olfactory function is one of the best studied in the OB (Trombley, 1994; Ciombor et al., 1999; Devore and Linster, 2012; Zimnik et al., 2013) to name a few, its effects at cellular, network and behavioral levels are somewhat discordant (Ennis and Hayar, 2008); it is worth noting that these inconsistencies have been ascribed at least in part to the absence of information pertaining to glomerular modulation by NA (Linster et al., 2011), a gap to fill which this work provides a first contribution.

The _locus caeruleus_ neurons have been proposed to influence external signal processing so that salient stimuli are enhanced and the activity more related to tonic, vegetative functions is suppressed (Aston-Jones et al., 2000). In the OB, the NA release in response to LC activation should bring the DA-PG neurons to a more excited state for the inhibition of the K\( \text{ir} \) conductance. This, for the known presynaptic inhibitory effect of DA (Koster et al., 1999; Ennis et al., 2001), would reduce the release of glutamate from the olfactory nerve terminals, improving the signal-to-noise ratio of the information coming from the olfactory epithelium, an effect that would be in line with the postulated general role of the LC on sensory stimuli perception (Aston-Jones et al., 2000).

An additional role of NA on dopaminergic cells might be of some interest: a significant fraction of the interneurons added in adulthood to the glomerular layer of the MOB are dopaminergic (Pignatelli et al., 2009), and noradrenaline signaling enhances newborn cell survival (Bauer et al., 2003; Bovetti et al., 2011).

**ACh**

In the MOB, ACh, acting on both nicotinic and muscarinic receptors, has a complex effect (for a review see Devore and Linster, 2012). Overall, the resulting effect of cholinergic modulation is excitatory (Elagoubi and Gervais, 1992) and the multiple action of ACh seems to be orchestrated toward an enhancement of specificity and temporal precision of mitral cell responses to odors (Elagoubi and Gervais, 1992; Mandairon et al., 2006; Tsuno et al., 2008; D’Souza and Vijayaraghavan, 2012).

In this work we show that in DA-PG cells the activation of M2 muscarinic receptors increases the amplitude of a K\( \text{ir} \) current, an effect which is paralleled by a 4.5 mV hyperpolarization in current-clamp conditions. A similar effect has been reported in a variety of preparations, ranging from mammal atrial myocytes (Sakmann et al., 1983), to thalamic reticular neurons (McCormick and Prince, 1986), spinal motoneurons (Chevallier et al., 2008), interneurons of striatum (Calabresi et al., 1998), neocortex (Xiang et al., 1998), and hippocampus (McQuiston and Madison, 1999).

M2-type muscarinic receptors are described in the glomerular layer associated to PG-DA neurons (Crespo et al., 2000; Allen Brain Atlas, 2013). In a previous paper (Pignatelli and Belluzzi, 2008), we showed that the activation of M2 metabotropic cholinergic receptor in PG-DA neurons induced a hyperpolarization mediated by a K-conductance, which in the present work is now identified as a K\( \text{ir} \).

**5-HT**

Projections from the dorsal and median raphe nuclei -one of the most prominent neuromodulatory systems in the brain- provide a dense serotonergic innervation of the MOB, and in particular of the glomerular region (Araneda et al., 1980; McLean and Shipley, 1987). Earlier studies have shown that 5-HT2 receptor mRNA and protein are heavily expressed in the glomerular layer (Morilik et al., 1993) and activation of 5-HT(2C) receptors mediates excitation in about one third of glomerular neurons, not better identified (Hardy et al., 2005); this study further develops this observations showing that serotonin produces excitatory modulation of DA-PG cells by reducing the IK\( \text{ir} \) amplitude, thereby depolarizing the cell for 12.8 ± 3.2 mV.

A similar action on IK\( \text{ir} \) is described also in rat motoneurons (Kjaerulff and Kiehn, 2001).

**Histamine**

The MOB receives histaminergic inputs primarily from the caudal tuberal and postmammillary magnocellular hypothalamus (Auvinen and Panula, 1988; Panula et al., 1989) via the olfactory peduncle (Brunjes, 2013), and previous studies have shown that in an unidentified fraction of periglomerular cells, H1-receptor activation causes a block of a potassium current (Jahn et al., 1995). Here we show that the IK\( \text{ir} \) in DA-PG cells is reduced and that this action results in a depolarization of the cells.

**DA**

Dopamine (100 μM) induces an increase of the K\( \text{ir} \) current via D2R; the D2R agonist quinpirole (30 μM) perfectly replicates the effect of dopamine. Further experiments using receptor protection with D1R selective antagonists might exclude more definitively a contribution from D1R, although a direct activation of D1R with SKF 38393 (15 μM) was completely ineffective. We did not investigate the pathways involved.

**CONCLUDING OBSERVATIONS**

In the present study we have shown that (i) DA-PG cells contain a large inward rectifier current whose block produces significant depolarizations, nominating this conductance as one of the main players controlling the resting membrane potential (and consequently excitability) in these cells, and that (ii) this current is subject to a complex modulation.

Bulbar DA-PG cells, the largest and one of the most conserved populations of DA neurons in the CNS, are pivotal neurons in the operation of glomerular circuits, the site where odor information is initially processed. It is therefore of some interest that their excitability is profoundly dependent upon the K\( \text{ir} \) current, and that this -in turn- is target of numerous neurotransmitters that can finely modulate its amplitude, a process that ultimately impacts all subsequent odor processing in the olfactory system.

In this context, it is increasingly evident that in the bulb as a whole there is an enormous and still largely hidden layer
“molecular computation” (Bhalla, 2014), which multiplies tremendously the degrees of freedom of the bulbar network in signal processing.

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