

# Electroolfactogram Responses from Organotypic Cultures of the Olfactory Epithelium from Postnatal Mice

Giulietta Pinato, Juraj Rievaj, Simone Pifferi, Michele Dibattista, Lara Masten and Anna Menini

Neurobiology Sector, International School for Advanced Studies, SISSA, Italian Institute of Technology, SS 14 Km 163.5, 34012 Basovizza, Trieste, Italy

Correspondence to be sent to: Anna Menini, Neurobiology Sector, International School for Advanced Studies, SISSA, SS 14 Km 163.5, 34012 Basovizza, Trieste, Italy. e-mail: [menini@sisssa.it](mailto:menini@sisssa.it)

## Abstract

Organotypic cultures of the mouse olfactory epithelium connected to the olfactory bulb were obtained with the roller tube technique from postnatal mice aged between 13 and 66 days. To test the functionality of the cultures, we measured electroolfactograms (EOGs) at different days in vitro (DIV), up to 7 DIV, and we compared them with EOGs from identical acute preparations (0 DIV). Average amplitudes of EOG responses to 2 mixtures of various odorants at concentrations of 1 mM or 100  $\mu$ M decreased in cultures between 2 and 5 DIV compared with 0 DIV. The percentage of responsive cultures was 57%. We also used the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) to trigger the olfactory transduction cascade bypassing odorant receptor activation. Average amplitudes of EOG responses to 500  $\mu$ M IBMX were not significantly different in cultures up to 6 DIV or 0 DIV, and the average percentage of responsive cultures between 2 and 5 DIV was 72%. The dose–response curve to IBMX measured in cultures up to 7 DIV was similar to that at 0 DIV. Moreover, the percentage of EOG response to IBMX blocked by niflumic acid, a blocker of Ca-activated Cl channels, was not significantly different in cultured or acute preparations.

**Key words:** electroolfactogram, IBMX, olfaction, organotypic culture, transduction

## Introduction

The olfactory system allows the detection and perception of a large number of odorant molecules. The initial events in olfaction occur in the olfactory epithelium (OE), in the nasal cavity, where the olfactory sensory neurons (OSNs) are located. OSNs are bipolar neurons with a dendritic process ending with several cilia and an axon projecting to the olfactory bulb (OB). Odorant molecules bind to odorant receptors in the ciliary membrane of OSNs, initiating the olfactory transduction cascade. The binding of odorant molecules to odorant receptors activates a G protein, which stimulates adenylate cyclase to produce cyclic adenosine 3',5' monophosphate (cAMP). The increased concentration of cAMP opens cyclic nucleotide-gated (CNG) channels allowing a depolarizing influx of Na and Ca ions into the cilia. The increase in intraciliary Ca concentration opens Ca-activated Cl channels that further contribute to depolarization. Action potentials evoked by odor-induced depolarization propagate through the axons of OSNs to the OB, where odorant information is further processed (for reviews, see Schild and Restrepo 1998; Menini 1999; Firestein 2001; Buck 2004; Menini et al. 2004; Lledo et al. 2005).

Several experimental models have been used to study olfactory transduction, coding, and regeneration, from invertebrates to mammals (Ache and Young 2005), including in vivo as well as in vitro preparations. For in vitro experiments, acute preparations are usually employed: dissociated OSNs (Kleene and Gesteland 1983; Boccaccio et al. 2006), slices of the OE (Hegg et al. 2003) or of the OB (Nickell et al. 1996; Pinato and Midtgaard 2005), and semi-intact preparations of the OE and of the OB (Ma et al. 1999; Lin et al. 2007). Several types of culture methods for OSNs have also been developed from embryonic, newborn, or adult rats (Noble et al. 1984; Pixley and Pun 1990; Chuah et al. 1991; Ronnett et al. 1991; Trombley and Westbrook 1991; Vargas and Lucero 1999). Organotypic cultures of the mammalian OE, where OSNs are cultivated in their native environment, have been developed not only by using fetal tissue (Farbman 1977; Gonzales et al. 1985; Gong et al. 1996; Goetze et al. 2002) but also postnatal tissue (Michel et al. 1999; Josephson et al. 2004). Organotypic cultures have several advantages over acute preparations or living animals: whereas cells are maintained in their native environment,

organotypic cultures allow easier visualization, long-lasting monitor of the activity, delivery of substances such as gene constructs or small interfering RNAs (siRNA), and measurement of the induced functional modifications.

The organotypic culture preparations of OE from postnatal mice developed by Michel et al. (1999) or by Josephson et al. (2004) were rather different. Josephson et al. cultured pieces of OE and OB explanted from 1- to 4-day-old mice. Using cell-specific markers, they identified various long-lived cell types, including the presence of mature OSNs marked by the olfactory marker protein (OMP) up to 28 days *in vitro* (DIV). They also tested the functionality of the cultures by measuring the electroolfactograms (EOGs) in response to puffs of vapor from an odorant mixture solution and measured EOG responses from 13 of 30 cultures tested corresponding to a percentage of about 43% (13/30) responsive cultures (Josephson et al. 2004). The organotypic culture model from postnatal mice developed by Michel et al. (1999) consisted of the explanted half olfactory cavities including the turbinates and the epithelium connected to the corresponding OB. Michel et al. showed that those explants after several DIV had a structural morphology similar to that observed *in situ*: the OE structure and all the bulbar layers were preserved, and anti-OMP immunoserum specifically labeled the sensory neurons up to at least 10 DIV. However, electrophysiological experiments that would allow verifying the functional integrity of the OSNs maintained *in vitro* have not been investigated yet.

The purpose of our study was to obtain organotypic cultures of the OE connected to OB and test their functionality by measuring the EOGs in response to odorants or to 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase. We compared these measurements with controls obtained from acute preparations of OEs connected to OB. These measurements are of primary importance to contribute to the assessment of whether, and to which extent, these cultures can be used as a chronic model to address questions related, for example, to proteins involved in the signal transduction cascade or in the coding process, employing gene or siRNA delivery (Li et al. 2006).

## Materials and methods

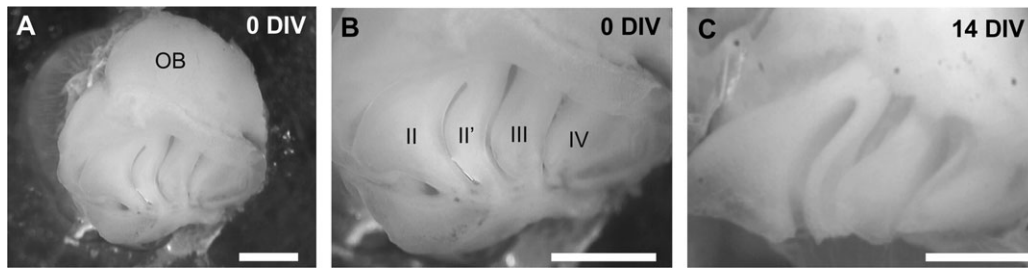
### Preparation of organotypic cultures

Olfactory organotypic cultures were prepared from BALB/c mice aged between 13 and 66 days. Mice were anesthetized with CO<sub>2</sub> inhalation and decapitated, in accordance with the regulations of the Italian Animal Welfare Act and the local authority veterinary service. The cultures were prepared with a technique similar to that developed by Michel et al. (1999) and based on the Gähwiler's roller tube technique (Gähwiler 1981), with some modifications as described in the following. The head was split midsagittally and placed in mammalian Ringer's solution containing (in millimoles): 140 NaCl,

5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 D-Glucose, 1 sodium pyruvate, and pH 7.4 with NaOH. The cranial bones and the septum were removed, and each brain hemisphere was detached, leaving the OB intact. The turbinates with the OE connected to the OB were placed on a glass cover slip of 12 × 24 mm in a 20- $\mu$ l drop of chicken plasma reconstituted according to the manufacturer's instructions from filtered and lyophilized plasma (30-0390L Cocalico Biologicals, Reamstown, PA, or P3266 Sigma, St Louis, MO). The plasma was then coagulated by carefully adding 30  $\mu$ l bovine thrombin (activity 200 U/ml, 2374 Merck). Cover slips with the explants were kept under the hood at room temperature in a covered petri dish to prevent the tissue from drying out. After waiting about 40 min for a complete coagulation, the cover slips with the olfactory explants were inserted into 15-mm culture tubes containing 2 ml of growth medium. Tubes were capped and placed in a roller drum rotating at 20 turns/h inside an incubator at 37 °C. (The glass cover slips were previously cleaned by immersion in a 0.5 M HCl solution for 24 h, washed with deionized water, placed in ethanol for 30 min, washed again, and dried at 150 °C.) The growth medium was changed every 2 days and contained: MEM with Glutamax (Gibco, 41090), 25 mM HEPES, 25 mM D-glucose, 2.5 mM MgCl<sub>2</sub>, 0.5 mM ascorbic acid sodium salt, 0.5 mg/l insulin, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) (Gibco), 20% horse serum (Gibco, Grand Island, NY), and pH 7.4.

### Electroolfactograms

Cultures at different DIV, or acute preparations, were transferred to the recording chamber, completely immersed and perfused with mammalian Ringer's solution, at room temperature (20–22 °C). EOGs were measured at the surface of the OE (Ottoson 1955; Scott and Scott-Johnson 2002). The recording electrodes were made from glass capillaries pulled with a Narishige PP83 puller (Narishige, Tokyo, Japan), gently broken at the tip, fire polished, and filled with mammalian Ringer's solution (electrical resistance of about 1 M $\Omega$ ). The electrode was connected via an Ag/AgCl wire to the head stage of a MultiClamp 700B patch-clamp amplifier (Axon Instruments, Union City, CA). The bath electrode consisted in an Ag/AgCl pellet placed in the recording chamber. Stimuli, consisting of a mixture of odorants (Sigma) or of IBMX (Sigma), were delivered by a multipipe system placed close to the epithelium. The recording pipette was placed close to the surface of the OE with a micromanipulator (Luigs and Neumann, Retingen, Germany). EOG recordings were typically measured by placing the electrode in the middle of turbinate II' or III (see Figure 1B, using the nomenclature of Ressler et al. 1993), and the position of the electrode was adjusted to measure negative EOGs in response to the stimuli. We defined a response as a negative deflection of more than twice the magnitude of the peak-to-peak noise of the recording (the smallest response we



**Figure 1** Comparison of an acute explant of the mouse OE connected to the OB with an organotypic culture. **(A)** Photomicrograph of the OE preparation connected to the OB acutely isolated (0 DIV). **(B)** and **(C)** The olfactory turbinate system in the 0 DIV preparation (same OE as in A) and in a 14 DIV culture. Roman numerals indicate individual turbinates using the nomenclature of Ressler et al. (1993). Scale bar in A, B, C: 2 mm.

considered was  $-50 \mu\text{V}$ ). Electrical signals were passband filtered at 0.1 Hz–1 kHz, digitized at 10 kHz (Digidata 1200, Axon Instruments), stored in a personal computer, and analyzed with pClamp 8.2 (Axon Instruments) or with Igor Pro software (WaveMetrics, Lake Oswego, OR).

For odorant stimulation, we used 2 odorant mixtures. Mixture 1 was composed of 3 odorants: cineole, acetophenone, and amylacetate, each present at 1 mM; mixture 2 was composed of 17 odorants: amylacetate, 2-heptanone, S-(+)-carvone, R-(–)-carvone, isoamylacetate, cineole, acetophenone, eugenol, octanal, R-(+)-limonene, 1-octanol, nonanal, 1-heptanal, citral, geraniol, (–)-menthone, and decanal, at 100  $\mu\text{M}$  each. All odorants were prepared in dimethylsulfoxide (DMSO) at 0.5 M and diluted to the final concentration in Ringer's solution. The total concentration of DMSO in the mixtures was never more than 0.6% and had no effect on EOGs (data not shown). All odorants and DMSO were from Sigma.

The IBMX stock solution was prepared in Ringer's solution at 1 mM and diluted to final concentrations. Niflumic acid (NFA; Sigma) was prepared in DMSO at 200 mM as a stock and diluted to the final concentration in Ringer's solution.

To compare different treatments or populations, we used analysis of variance (ANOVA). Significance of differences between individual data points was determined using unpaired *t*-tests. *P* values  $<0.05$  were considered significant. Data are reported as mean  $\pm$  standard deviation and the total number of responsive OEs (*N*).

## Results

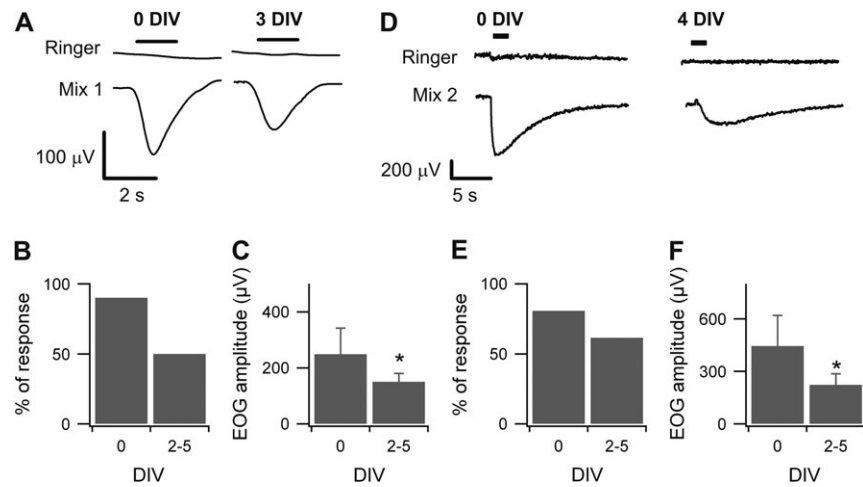
We set up a preparation by explanting one-half of the entire olfactory system from cranial bones. It included all turbinates, covered by olfactory neuroepithelium, and the corresponding hemisphere of OB, as illustrated in the photomicrograph in Figure 1A. The overall morphology of the OE at 0 DIV and at 14 DIV appeared largely similar (Figure 1B,C).

To assess the functionality of cultured mouse OEs, we recorded the EOG in response to the exposure of odorants

or to the phosphodiesterase inhibitor IBMX and compared these results with controls performed in acute preparations. The EOG is an extracellular field potential originating from the summated activities of many OSNs near the recording electrode at the surface of the epithelium and provides a good quantitative test of the neurons' functionality (Ottoson 1955; Scott and Scott-Johnson 2002; Nickell et al. 2006). EOG responses were recorded from turbinates II' or III (Figure 1B).

### EOG responses to odorants

In a first set of experiments, we tested the physiological response to odorants. Figure 2 shows representative recordings of EOG responses to 2 different odorant mixtures, whose composition was selected to increase the probability that a large number of OSNs respond to the stimulus. Odorant mixture 1 was composed of 1 mM cineole, amylacetate, and acetophenone. Very high odorant concentrations (1 mM) were included in this mixture to increase the probability of obtaining responses also from OSNs expressing odorant receptors with a low sensitivity to the selected odorants. Indeed, it is well known that about 1000 different odorant receptors are expressed in various neurons and that each OSN has a specific dose–response curve for various odorants (Firestein 2001; Mombaerts 2004). For example, it has been shown that the odorant concentration necessary to activate half of the maximal current in individual neurons ranges from  $3 \times 10^{-6}$  to  $9 \times 10^{-5}$  M (Firestein et al. 1993) and, more recently, that 100  $\mu\text{M}$  was not sufficient to reach saturation in dose–response curves measured with EOG recordings from mouse OEs (Munger et al. 2001). Recordings in Figure 2A show that the shapes of the EOGs from cultured OEs at different DIV were quite similar to those of EOGs from acute preparations, that is, it was characterized by a steep negative deflection of the polarization that reached a peak and then repolarized. We measured EOGs in response to the odorant mixture 1 in 10 acute preparations from different mice and found 9 responsive OEs, corresponding to 90% functional preparations, with an average response of  $-249 \pm 94 \mu\text{V}$  ( $N = 9$ ). Eight cultured OEs, ranging from 2 to 5 DIV, obtained from different mice were tested with



**Figure 2** EOG responses to odorants in mouse acute isolated and cultured OEs. **(A)** Recordings from an acute OE (0 DIV, first column) and a 3 DIV OE (second column). For each OE, Ringer alone was first applied for 2 s as a control for perfusion artifacts (upper traces), and then odorant mixture 1 composed of 1 mM acetophenone, amylacetate, and cineole in Ringer was applied for 2 s (lower traces). The time of application of the stimulus is indicated with the black horizontal bars. **(B)** Percentage of responsive preparations at 0 DIV or between 2 and 5 DIV, as indicated. **(C)** Average amplitude of EOG responses at 0 DIV or at 2–5 DIV was significantly different ( $t$ -test:  $P < 0.05$ ). The same type of experiments was repeated with odorant mixture 2 composed of 17 odorants each at 100  $\mu$ M: amylacetate, 2-heptanone, S-(+)-carvone, R-(–)-carvone, isoamylacetate, cineole, acetophenone, eugenol, octanal, R-(+)-limonene, 1-octanol, nonanal, 1-heptanal, citral, geraniol, (–)-menthone, and decanal. **(D)** The percentage of responsive preparations and the average EOG amplitudes at various DIV were plotted in **(E)** and **(F)**, respectively. As in **(C)** the average amplitude of EOGs at 0 DIV and at 2–5 DIV was significantly different ( $t$ -test:  $P < 0.05$ ).

odorants, and 4 epithelia (50%) responded with a negative EOG to the applied stimulus (Figure 2B). The average EOG response to odorant mixture 1 in cultures between 2 and 5 DIV was  $-150 \pm 30 \mu\text{V}$  ( $N = 4$ ), significantly lower than the average amplitude measured at 0 DIV ( $t$ -test:  $P < 0.05$ ) (Figure 2C).

It could be of interest to note that we tested 3 more epithelia that remained in culture up to day 8, 10, and 14, and we found that all 3 responded to the odorant mixture, although we did not systematically investigate responses in long-living cultures.

We performed a second set of experiments using a different mixture, composed of 17 odorants, each present at 100  $\mu$ M (see odorant composition in the legend of Figure 2 or Materials and methods), to increase the probability to obtain a response from more OSNs that could express odorant receptors nonsensitive to mixture 1. As shown in Figure 2D, the shape of the EOGs was similar to that previously recorded with mixture 1 (Figure 2A), both in acute and cultured OEs. Moreover, also with odorant mixture 2, we measured a reduction in the percentage of cultures responding to odorants between 2 and 5 DIV compared with 0 DIV (Figure 2E) and a significant decrease in the average amplitude of EOGs (Figure 2F). Indeed, the average EOG response to odorant mixture 2 in cultures between 2 and 5 DIV was  $-225 \pm 63 \mu\text{V}$  ( $N = 8$ ), significantly lower than  $-445 \pm 174 \mu\text{V}$  ( $N = 28$ ) measured at 0 DIV ( $t$ -test:  $P < 0.05$ ).

Therefore, for both mixtures, the percentage of cultures responding to odorants was lower than that for acute preparations. Moreover, also the average EOG amplitude of the

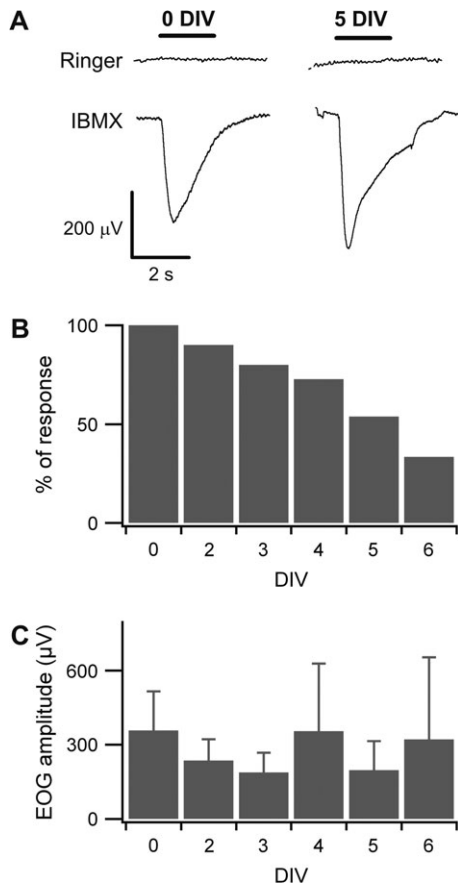
responsive cultures was significantly lower than that measured at 0 DIV.

It would be interesting to determine dose–response relations in response to odorants; however, previous experiments with EOG measurements could not determine useful dose–response curves because saturation was never effectively achieved (Munger et al. 2001). We therefore did not attempt to measure dose–response relations in response to odorants, but we obtained dose–response relations in response to IBMX, as described in detail in the following paragraphs.

### EOG responses to IBMX

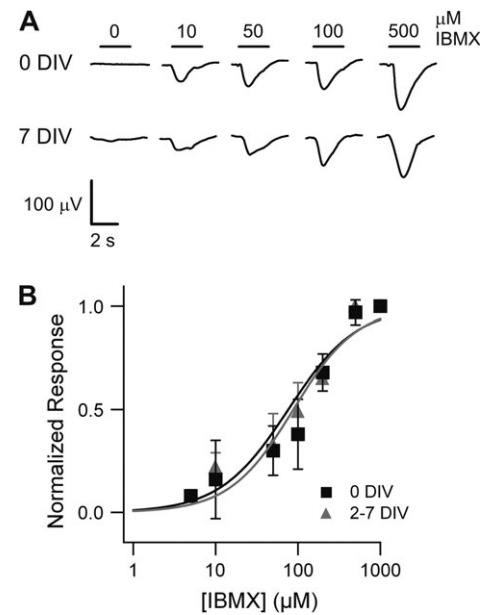
IBMX is widely used to activate the cyclic nucleotide second messenger cascade bypassing the activation of odorant receptors in OSNs (Firestein, Darrow, and Shepherd 1991; Firestein, Zuffall, and Shepherd 1991; Chen et al. 2000). IBMX is an inhibitor of PDE, and because in the cilia of functional OSNs there is an endogenous activity of both adenylate cyclase and PDE, the inhibition of PDE causes an increase in the intraciliary cAMP concentration due to basal production of cAMP by adenylate cyclase. The increased cAMP concentration causes the opening of CNG channels and the depolarizing influx of Na and Ca ions in the cilia (Schild and Restrepo 1998; Firestein 2001). We investigated the functionality of OSNs by measuring EOG responses to the application of IBMX. Figure 3A shows representative recordings of EOG responses to IBMX in an acute preparation and after 5 days in culture. As previously observed for the responses to odorants (Figure 2), also the shapes of the





**Figure 3** (A) EOG responses to IBMX application. Recordings from a 0 DIV (first column), and from a 5 DIV OE (second column). For each OE, Ringer alone was first applied for 2 s as a control for perfusion artifacts (upper traces), then 500  $\mu\text{M}$  IBMX in Ringer was applied for 2 s (lower traces). The time of application of the stimulus is indicated with the black horizontal bars. (B) Percentage of responsive preparations at various DIV as indicated. (C) Average amplitude of EOGs in 500  $\mu\text{M}$  IBMX in responsive acute preparations at various DIV was not significantly different (1-way ANOVA:  $P = 0.36$ ) (0 DIV,  $N = 10$ ; 2 DIV,  $N = 6$ ; 3 DIV,  $N = 4$ ; 4 DIV,  $N = 5$ ; 5 DIV,  $N = 7$ ; and 6 DIV,  $N = 5$ ).

EOGs measured in response to IBMX from cultured OEs were similar to those of EOGs from acute preparations. We tested the response to 500  $\mu\text{M}$  IBMX, a concentration giving saturation of the dose–response curve as shown in Figure 4B, in 10 acute OEs from different mice, and we found a response in every OE, with an average value of  $-358 \pm 158 \mu\text{V}$  ( $N = 10$ ). We analyzed the percentage of OEs responding to stimulation with IBMX at 0 DIV and after various days in culture (Figure 3B). At 0 DIV all OEs responded; between 2 and 4 DIV, we measured a response to IBMX in about 80% of the cultures; whereas after 5 DIV, the percentage of responsive cultures decreased to about 33% at 6 DIV. Therefore, a decrease in responses appears to be correlated to the longer time in vitro. The analysis of the average EOG amplitudes (Figure 3C) of the responsive cultures shows that the average amplitude was not significantly different at various DIV up to at least 6 DIV (1-way ANOVA:  $P > 0.05$ ).



**Figure 4** The dose–response relations for IBMX. (A) Comparison of EOG responses to different concentrations of IBMX from an acute OE (upper traces) and from a 7 DIV OE (lower traces). Black horizontal bars indicate the time of application of IBMX in Ringer. Numbers represent the IBMX concentrations (in micromoles). (B) EOG amplitudes induced by various concentrations of IBMX were normalized to the maximal response. Symbols plot the average responses from 4 acute (squares) and 6 cultured (triangles) OEs tested between 2 and 7 DIV. Lines were the best fit of the Hill equation to the data with  $K_{1/2} = 76 \mu\text{M}$ ,  $n = 1.3$ , for acute and  $K_{1/2} = 88 \mu\text{M}$ ,  $n = 1.1$ , for cultured epithelia.

It could be of interest to note that we tested some additional epithelia that remained in culture up to day 13 and found that some of them responded to IBMX, although we did not systematically investigate responses in long-living cultures.

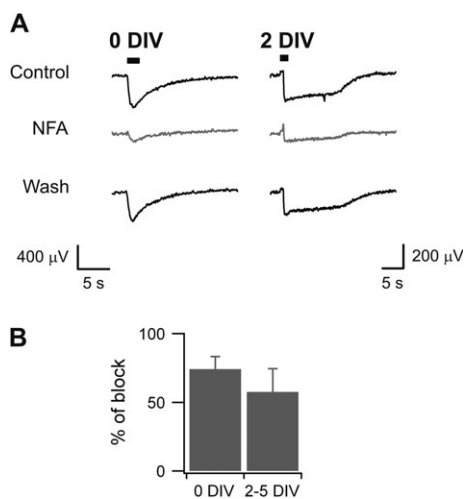
#### IBMX induces similar dose–response relations in both acute and organotypic preparations

A further important test of the functional properties of OSNs is the measurement of the dose–response relations. Various concentrations of cAMP in OSNs were produced applying different concentrations of IBMX ranging from 5  $\mu\text{M}$  to 1 mM. We therefore measured the EOGs in response to various concentrations of IBMX on the same epithelium. Figure 4 shows EOG recordings for different concentrations of IBMX in an acute (Figure 4A, upper traces) and in a 7 DIV OE (Figure 4A, lower traces). Several dose–response relations were measured both in acute and in cultured OEs. The average normalized peak responses at various IBMX concentrations were plotted versus the IBMX concentration (Figure 4B), and data were fitted with the Hill equation:  $V/V_{\text{max}} = c^n/(c^n + K_{1/2}^n)$ , where  $V$  is the peak EOG response measured at the concentration  $c$ ,  $V_{\text{max}}$  is the maximal peak EOG response,  $K_{1/2}$  is the IBMX concentration

producing 50% of the maximal response, and  $n$  is the Hill coefficient. The best fit gave  $K_{1/2} = 76 \mu\text{M}$ ,  $n = 1.3$  for measurements from 4 fresh epithelia and  $K_{1/2} = 88 \mu\text{M}$ ,  $n = 1.1$  for 6 epithelia in culture. These experiments demonstrate that in cultured OEs the dose–response relation for IBMX was very similar to that measured in acute OEs.

### NFA reduces the EOG amplitude in both acute and organotypic preparations

To further investigate the pharmacology of olfactory signaling in cultured and acute preparations, we analyzed in more detail a commonly used blocker of the response. Indeed, it has been recently reported that the EOG response is primarily caused by a depolarizing Cl current (Nickell et al. 2006) that can be blocked by NFA, a well-known blocker for Ca-activated Cl channels involved in olfactory transduction (Kleene 1993; Kurahashi and Yau 1993; Lowe and Gold 1993; Zhainazarov and Ache 1995; Reisert et al. 2003; Pifferi et al. 2006; Boccaccio and Menini 2007). We therefore analyzed the effect of NFA on EOG recordings in organotypic cultures stimulated with IBMX. Perfusion for 5 min of 300  $\mu\text{M}$  NFA in Ringer caused a great reduction of EOG responses both in acute preparations and in cultured OEs (Figure 5A). The block was largely reversible after extensive wash with Ringer's solution for 20–30 min. On average, NFA reduced the EOG amplitude by  $74 \pm 9\%$  ( $N = 11$ ) in acute preparations and by  $58 \pm 17\%$  ( $N = 5$ ) in cultured OEs (up to 5 DIV); the difference was not significant ( $t$ -test:



**Figure 5** Reduction of EOG response amplitude by NFA. **(A)** EOG recordings from a 0 DIV (first column) and from a 2 DIV OE (second column) in response to 500  $\mu\text{M}$  IBMX. Filled horizontal bars indicate the time of application of IBMX. A control EOG was first recorded, and then 300  $\mu\text{M}$  NFA was added to the bath for 5 min. The EOG in response to IBMX was recorded again in the presence of NFA (middle traces), finally, the recovery of the EOG response to IBMX was tested after washing out NFA by 20- to 30-min perfusion of Ringer alone (lower traces). **(B)** Average percentage of EOG amplitude blocked by NFA at 0 DIV and between 2 and 5 DIV was not significantly different (unpaired  $t$ -test:  $P = 0.08$ ; 0 DIV,  $N = 11$ ; 2–5 DIV,  $N = 5$ ).

$P > 0.05$ ) (Figure 5B). These results indicate that the Ca-activated Cl component of olfactory transduction in responsive organotypic cultures (at least up to 5 DIV) is similar to that measured in acute preparations.

### Discussion

Our data show that it is possible to record EOG in response to odorants and to IBMX from organotypic cultures of the OE connected to its OB obtained from postnatal mice. To the best of our knowledge, EOG responses to odorants from organotypic cultures of OEs from postnatal mice have previously been reported only by Josephson et al. (2004). The olfactory organotypic culture system of Josephson et al. (2004) was different from that employed in our study because it was composed of pieces of OE and OB instead of the entire OE connected to the corresponding OB (Josephson et al. 2004). Moreover, they used a different way to apply odorants to the OE cultures: they recorded EOG in response to puffs of vapor from a 16 mM odorant mixture solution containing ethyl butyrate, eugenol, (+) carvone, and (–) carvone and they used a salt solution without calcium and magnesium. In contrast, we applied the stimuli dissolved in solution and used also other compounds to block or activate the transduction cascade (Chen et al. 2000; Lin et al. 2007). The percentage of cultures responding to odorants in Josephson et al. (2004) was 43%, whereas in our work, the total percentage of cultures responding to odorant mixtures was 57%. Therefore, a large reduction in the number of cultures responding to odorants was observed in both types of cultures. We did not detect any evident morphological difference between responding and nonresponding cultures. It is possible that damage of axonal connections between OE and OB could cause nonresponsive cultures, but we did not investigate the cause of the reduction of responses in cultures, which will open an interesting area for further studies.

We further investigated the EOG responses of organotypic cultures by using IBMX instead of odorants. The relationship between the number of DIV and the ability to respond to IBMX was investigated in detail, and we found that the percentage of cultures responding to IBMX decreased from about 80% between 2 and 4 DIV to about 33% at 6 DIV. We did not examine the causes of the reduction in the percentage of responsive cultures, which remain to be further investigated.

Our data showed that dose–response relations in response to IBMX were similar in acute and in cultured OEs (up to at least 7 DIV). Because IBMX induces an electrophysiological response bypassing the activation of odorant receptors and G proteins, the similarity of dose–response relations in response to IBMX indicates that the transduction cascade after activation of adenylyl cyclase is functional both in acute and in responsive organotypic preparations. Therefore, our results indicate that, although not every culture is functional,

at least those responding to IBMX appear to have a dose–response similar to acute preparations. The responsive OEs are likely to be useful in studies in which the function of some protein involved in the second part of the transduction cascade has been modified by using molecular biology techniques. For example, previous work has used differences in the dose–response to IBMX between wild-type and knockout mice for the subunit CNGA4 to show that the CNGA4 subunit contributes to the high cAMP sensitivity of the native olfactory channel (Munger et al. 2001). In their study, Munger et al. (2001) used IBMX because it induced response in all olfactory neurons, whereas useful dose–response relationships could not be determined for odors because saturation was never effectively achieved. The possibility of using of RNA interference (RNAi) on cultured OEs, instead of producing knockout mice, and the subsequent electrophysiological measurements, could be a powerful experimental tool to measure the functional consequences of modifying proteins that may be involved in olfactory transduction and signaling. Therefore, although further detailed studies of each step of the transduction cascade will be necessary to fully validate the use of this model, our EOG recordings show the ability of OE organotypic cultures to maintain their functionality over time *in vitro* and that this model is likely to be useful to help identifying the contribution and the identity of several proteins involved in the transduction cascade.

Finally, in this study, to further investigate the olfactory signaling in cultures, we measured the percentage of EOG response to IBMX blocked by NFA, a blocker of Ca-activated Cl channels, and found that the culture conditions did not significantly modify it. This property could be useful to help establishing the molecular identity of the Ca-activated Cl channel. Indeed, although recent work has suggested that bestrophin-2 is a candidate olfactory Ca-activated Cl channel (Pifferi et al. 2006), the molecular identity of this channel has not been definitely established yet. These cultures may be appropriate for using RNAi techniques to reduce the expression of a particular gene, such as VMD211 or other genes, and to analyze the effect the gene has on a function measured by EOG recordings. Indeed, this culture system is appropriate for the delivery of genes or of other substances and for the assessment of functional properties. The use of RNAi on cultured OEs could be a powerful experimental tool to measure the functional consequences of inactivating proteins that may be involved in olfactory transduction and signaling.

A further new area of investigation will also be the measurement of electrophysiological responses of neurons in the OB connected to the OE in this organotypic cultures.

## Funding

European Commission (ERG 11-505935); Italian Institute of Technology (IIT 2007); Italian Ministry of Education Research and University (COFIN 2006).

## Acknowledgements

We thank Anna Boccaccio for comments on the manuscript, Beatrice Pastore for technical help, and Manuela Schipizza-Lough for checking the English.

## References

- Ache BW, Young JM. 2005. Olfaction: diverse species, conserved principles. *Neuron*. 48:417–430.
- Boccaccio A, Lagostena L, Hagen V, Menini A. 2006. Fast adaptation in mouse olfactory sensory neurons does not require the activity of phosphodiesterase. *J Gen Physiol*. 128:171–184.
- Boccaccio A, Menini A. 2007. Temporal development of cyclic nucleotide-gated and Ca<sup>2+</sup>-activated Cl<sup>-</sup> currents in isolated mouse olfactory sensory neurons. *J Neurophysiol*. 98:153–160.
- Buck LB. 2004. Olfactory receptors and odor coding in mammals. *Nutr Rev*. 62:S184–S188.
- Chen S, Lane AP, Bock R, Leinders-Zufall T, Zufall F. 2000. Blocking adenylyl cyclase inhibits olfactory generator currents induced by "IP(3)-odors". *J Neurophysiol*. 84:575–580.
- Chuah MI, David S, Blaschuk O. 1991. Differentiation and survival of rat olfactory epithelial neurons in dissociated cell culture. *Brain Res Dev Brain Res*. 60:123–132.
- Farbman AI. 1977. Differentiation of olfactory receptor cells in organ culture. *Anat Rec*. 189:187–199.
- Firestein S. 2001. How the olfactory system makes sense of scents. *Nature*. 413:211–218.
- Firestein S, Darrow B, Shepherd GM. 1991. Activation of the sensory current in salamander olfactory receptor neurons depends on a G protein-mediated cAMP second messenger system. *Neuron*. 6:825–835.
- Firestein S, Picco C, Menini A. 1993. The relation between stimulus and response in olfactory receptor cells of the tiger salamander. *J Physiol*. 468:1–10.
- Firestein S, Zufall F, Shepherd GM. 1991. Single odor-sensitive channels in olfactory receptor neurons are also gated by cyclic nucleotides. *J Neurosci*. 11:3565–3572.
- Gahwiler BH. 1981. Organotypic monolayer cultures of nervous tissue. *J Neurosci Methods*. 4:329–342.
- Goetze B, Breer H, Strotmann J. 2002. A long-term culture system for olfactory explants with intrinsically fluorescent cell populations. *Chem Senses*. 27:817–824.
- Gong Q, Liu WL, Srodon M, Foster TD, Shipley MT. 1996. Olfactory epithelial organotypic slice cultures: a useful tool for investigating olfactory neural development. *Int J Dev Neurosci*. 14:841–852.
- Gonzales F, Farbman AI, Gesteland RC. 1985. Cell and explant culture of olfactory chemoreceptor cells. *J Neurosci Methods*. 14:77–90.
- Hegg CC, Greenwood D, Huang W, Han P, Lucero MT. 2003. Activation of purinergic receptor subtypes modulates odor sensitivity. *J Neurosci*. 23:8291–8301.
- Josephson EM, Yilma S, Vodyanoy V, Morrison EE. 2004. Structure and function of long-lived olfactory organotypic cultures from postnatal mice. *J Neurosci Res*. 75:642–653.
- Kleene SJ. 1993. Origin of the chloride current in olfactory transduction. *Neuron*. 11:123–132.
- Kleene SJ, Gesteland RC. 1983. Dissociation of frog olfactory epithelium. *J Neurosci Methods*. 9:173–183.

- Kurahashi T, Yau KW. 1993. Co-existence of cationic and chloride components in odorant-induced current of vertebrate olfactory receptor cells. *Nature*. 363:71–74.
- Li CX, Parker A, Menocal E, Xiang S, Borodyansky L, Fruehauf JH. 2006. Delivery of RNA interference. *Cell Cycle*. 5:2103–2109.
- Lin W, Margolskee R, Donnert G, Hell SW, Restrepo D. 2007. Olfactory neurons expressing transient receptor potential channel M5 (TRPM5) are involved in sensing semiochemicals. *Proc Natl Acad Sci USA*. 104:2471–2476.
- Lledo PM, Gheusi G, Vincent JD. 2005. Information processing in the mammalian olfactory system. *Physiol Rev*. 85:281–317.
- Lowe G, Gold GH. 1993. Nonlinear amplification by calcium-dependent chloride channels in olfactory receptor cells. *Nature*. 366:283–286.
- Ma M, Chen WR, Shepherd GM. 1999. Electrophysiological characterization of rat and mouse olfactory receptor neurons from an intact epithelial preparation. *J Neurosci Methods*. 92:31–40.
- Menini A. 1999. Calcium signalling and regulation in olfactory neurons. *Curr Opin Neurobiol*. 9:419–426.
- Menini A, Lagostena L, Boccaccio A. 2004. Olfaction: from odorant molecules to the olfactory cortex. *News Physiol Sci*. 19:101–104.
- Michel V, Monnier Z, Cvetkovic V, Math F. 1999. Organotypic culture of neuroepithelium attached to olfactory bulb from adult mouse as a tool to study neuronal regeneration after ZnSO<sub>4</sub> neuroepithelial trauma. *Neurosci Lett*. 271:195–198.
- Mombaerts P. 2004. Genes and ligands for odorant, vomeronasal and taste receptors. *Nat Rev Neurosci*. 5:263–278.
- Munger SD, Lane AP, Zhong H, Leinders-Zufall T, Yau KW, Zufall F, Reed RR. 2001. Central role of the CNGA4 channel subunit in Ca<sup>2+</sup>-calmodulin-dependent odor adaptation. *Science*. 294:2172–2175.
- Nickell WT, Kleene NK, Gesteland RC, Kleene SJ. 2006. Neuronal chloride accumulation in olfactory epithelium of mice lacking NKCC1. *J Neurophysiol*. 95:2003–2006.
- Nickell WT, Shipley MT, Behbehani MM. 1996. Orthodromic synaptic activation of rat olfactory bulb mitral cells in isolated slices. *Brain Res Bull*. 39:57–62.
- Noble M, Mallaburn PS, Klein N. 1984. The growth of olfactory neurons in short-term cultures of rat olfactory epithelium. *Neurosci Lett*. 45:193–198.
- Ottoson D. 1955. Analysis of the electrical activity of the olfactory epithelium. *Acta Physiol Scand Suppl*. 35:1–83.
- Pifferi S, Pascarella G, Boccaccio A, Mazzatenta A, Gustincich S, Menini A, Zucchelli S. 2006. Bestrophin-2 is a candidate calcium-activated chloride channel involved in olfactory transduction. *Proc Natl Acad Sci USA*. 103:12929–12934.
- Pinato G, Midtgaard J. 2005. Dendritic sodium spikelets and low-threshold calcium spikes in turtle olfactory bulb granule cells. *J Neurophysiol*. 93:1285–1294.
- Pixley SK, Pun RY. 1990. Cultured rat olfactory neurons are excitable and respond to odors. *Brain Res Dev Brain Res*. 53:125–130.
- Reisert J, Bauer PJ, Yau KW, Frings S. 2003. The Ca-activated Cl channel and its control in rat olfactory receptor neurons. *J Gen Physiol*. 122:349–363.
- Ressler KJ, Sullivan SL, Buck LB. 1993. A zonal organization of odorant receptor gene expression in the olfactory epithelium. *Cell*. 73:597–609.
- Ronnett GV, Parfitt DJ, Hester LD, Snyder SH. 1991. Odorant-sensitive adenylylate cyclase: rapid, potent activation and desensitization in primary olfactory neuronal cultures. *Proc Natl Acad Sci USA*. 88:2366–2369.
- Schild D, Restrepo D. 1998. Transduction mechanisms in vertebrate olfactory receptor cells. *Physiol Rev*. 78:429–466.
- Scott JW, Scott-Johnson PE. 2002. The electroolfactogram: a review of its history and uses. *Microsc Res Tech*. 58:152–160.
- Trombley PQ, Westbrook GL. 1991. Voltage-gated currents in identified rat olfactory receptor neurons. *J Neurosci*. 11:435–444.
- Vargas G, Lucero MT. 1999. A method for maintaining odor-responsive adult rat olfactory receptor neurons in short-term culture. *Chem Senses*. 24:211–216.
- Zhainazarov AB, Ache BW. 1995. Odor-induced currents in *Xenopus* olfactory receptor cells measured with perforated-patch recording. *J Neurophysiol*. 74:479–483.

Accepted January 17, 2008