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SHORT COMMUNICATION

A Method for Maintaining Odor-responsive Adult Rat Olfactory Receptor Neurons in Short-term Culture

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Abstract

We report a culture system requiring the addition of freshly made ascorbic acid to the medium, that supports the short-term survival of adult rat olfactory receptor neurons. The cultured neurons exhibit typical voltage-gated currents and are responsive to application of odorants.

Introduction

Olfactory receptor neurons (ORNs), the sensory components of the olfactory neuroepithelium, are unique in their ability to undergo cellular turnover and replacement throughout life. Due to this unique characteristic, several culture systems have been developed to maintain and study these cells *in vitro*. Differentiation and survival of dissociated rat ORNs have been documented for olfactory cell cultures derived from embryonic (Chuah and Farbman, 1983; Calof and Chikaraishi, 1989; Chuah *et al.*, 1991) or newborn rats (Noble *et al.*, 1984; Pixley and Pun, 1990; Ronnett *et al.*, 1991; Trombley and Westbrook, 1991; Pixley, 1992). However, the differentiation and survival of ORNs in those culture systems required plating onto a feeder layer of CNS astrocytes (coculture), addition of growth factors or contact with the olfactory bulb. Survival of dissociated cells from adult mammalian olfactory mucosa has also proven to be difficult. Primary cultures of ORNs from adult mice required induction of chemical injury to the olfactory epithelium by $ZnSO_4$ to activate progenitor mitogenic activity *in situ* prior to culture (Sosnowski *et al.*, 1995; Liu *et al.*, 1998), while primary cultures from adult rats required coculture with a supportive bed layer of cortical glia (Grill and Pixley, 1997). Also, addition of brain-derived neurotrophic factor to the culture medium was necessary to increase the number of bipolar cells (ORNs) in culture (Liu *et al.*, 1998). We have developed and report here a culture system that supports the short-term survival of dissociated adult rat ORNs without requiring coculture or addition of growth factors to the culture medium. The neurons in our culture system are immunoreactive for the general neuronal marker neuron-specific tubulin (NST) and olfactory marker protein (OMP), show typical voltage-gated currents and respond to odorants. While our system is not designed to study the process of neurogenesis of ORNs, it provides an efficient and convenient technique to study the physiology of adult rat ORNs, which has been done primarily on acutely dissociated ORNs.

Materials and methods

Cell Preparation and Culture

Rat ORNs were dissociated with a modification of previously described procedures (Lynch and Barry, 1991a; Ronnett *et al.*, 1991; Vargas and Lucero, 1999). Briefly, adult male Simonsen albino rats (~200 g) were deeply anesthetized with 150 mg/kg ketamine + 15 mg/kg rompum (Mallinckrodt Veterinary, Inc., Mundelein, IL) and sacrificed by decapitation. The olfactory epithelium from the nasal septum and turbinates of one rat was dissected under 100% oxygen vapor saturated with rat Ringer's [6 ml of rat Ringer's nebulized with 100% oxygen in a Respigard 2 Nebulizer System (Marquest Medical, Englewood, CO)]. The tissue was placed in 5 ml of divalent-cation-free rat ringers (in mM: 145 NaCl, 5.6 KCl, 10 HEPES, 10 glucose, 4 EGTA, pH 7.4, 300 mOsm) containing 10 mg/ml bovine serum albumin, 1 mg/ml collagenase (Gibco BRL, Grand Island, NY), 50 μg/ml deoxyribonuclease II and 44 U/ml dispase (Gibco BRL), and incubated with gentle shaking (80 r.p.m.) at 37°C for 45 min. Following incubation, the tissue was transferred to 5 ml of fresh divalent-cation-free

rat Ringer's and incubated with gentle shaking at 37°C for 5 min. The tissue was then transferred to 2 ml of fresh divalent-cation-free rat Ringer's and triturated using a fire-polished Pasteur pipette. The resulting cell suspension was filtered using a 53 micron monofilament cloth (Small Parts Inc., Miami Lakes, FL). Cells (200 µl) were plated onto Concanavalin A (10 mg/ml; Sigma type IV)-coated glass coverslips placed in 35 mm Petri dishes. Following a 20 min settling time, 2 ml of culture medium was added to each dish. The dishes were placed at 37°C in a CO_2 incubator until used (up to 4 days). The culture medium was replaced daily and consisted of Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 100 µM ascorbic acid, 1× insulin–transferrin–selenium-X (Gibco BRL), 2 mM glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin (Irvine Scientific, Santa Ana, CA). All chemicals were obtained from Sigma Chemical Company (St Louis, MO) unless stated otherwise.

Immunocytochemistry

Primary cultures of rat olfactory receptor neurons were characterized by immunostaining for NST and OMP according to a previously described procedure (Pixley, 1992). Briefly, rat ORN cultures were prepared as described above and fixed at 0, 1, 2, 3 and 4 days in culture with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 min at room temperature. Fixed cells were incubated at room temperature in blocking buffer [0.1 M phosphate-buffered saline (PBS: 0.1 M phosphate, 0.15 M NaCl) with 10% horse serum, 0.2% Triton X-100 and 0.02% sodium azide] for 1 h and overnight with the primary antibodies [monoclonal anti-β tubulin isotype III (mouse ascites fluid) 1:1500 and goat anti-OMP 1:5000, a gift from Dr F. Margolis]. The cultures were then washed with PBS and incubated for 2 h with the secondary antibodies (biotinylated horse anti-mouse IgG 1:500 and rabbit anti-goat 1:500; Vector Laboratories, Inc., Burlingame, CA). The staining was performed using the Vectastain Elite ABC kit (Vector Laboratories, Inc.) and was visualized using 3,3′-diaminobenzidine (0.5 mg/ml in 0.1 M PB; 20 s). After immunostaining, coverslips were mounted on microscope slides using Gelvatol (Harlow and Lane, 1988).

Electrophysiological recordings

Standard whole-cell voltage-clamp recording techniques (Hamill *et al.*, 1981) were performed. Electrodes (10–12 MΩ resistance) were pulled on a Flaming/Brown P87 puller from thick walled (0.64 mm) borosilicate filament glass (Sutter Instrument Co., San Rafael, CA). Coverslips with adherent cells were placed into the recording chamber and perfused with external bath solution at a rate of 1–2 ml/min. The external bath solution was maintained at 35°C and was grounded with a 3 M KCl agar bridge to a AgCl wire.

Voltage-clamp recordings were performed and digitized with a Digidata 1200 interface, a 200A patch clamp amplifier, PClamp software (Axon Instruments, Foster City, CA) and a 486-33 IBM clone computer. Na^+ and K^+ currents were evoked by depolarizing voltage pulses from a holding potential of –80 mV. The hyperpolarization-activated current, I_h, was evoked by hyperpolarizing voltage pulses from a holding potential of –50 mV. Data were sampled at 1–5 kHz and filtered off-line at 500–5000 Hz.

Intracellular calcium measurements

Ca^{+2} imaging experiments were carried out on rat ORNs dissociated and plated as described above. Cells were loaded with 5 µM Fura-2/AM (Grynkiewicz *et al.*, 1985) plus pluronic acid F127 (80 µg/ml) in filtered rat Ringer's at 16°C in the dark for 20–30 min (followed by four washes in rat Ringer's). Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was approximated from the background corrected ratio of fluorescence at 340 nm/380 nm using a two-point calibration scheme and the equation:

$$[Ca]_i = K_d(F_o/F_s)(R - R_{min})/(R_{max} - R)$$

where R is the fluorescence ratio (F_{340}/F_{380}), R_{min} and R_{max} are the fluorescence ratios of 5 µM Fura standards at the limiting low (0 µM Ca^{2+}) and high (10 mM Ca^{2+}) concentrations, K_d is the calcium dissociation constant, and (F_o/F_s) is the ratio of fluorescence intensities when excited at 380 nm at the limiting low (F_o) and high (F_s) Ca^{+2} concentrations. A Zeiss-Attofluor imaging system and software (Atto Instruments Inc., Rockville MD) was used to make the calibration curves, acquire and analyze the data. Data points were sampled at 1 Hz. Basal intracellular Ca^{+2} levels estimated using the two-point calibration method described above averaged 65 ± 4 nM (mean ± SEM, n = 50 cells). Odorants and IBMX were applied to the cells using a loop injector (100 µl).

Results and discussion

We characterized our culture system by immunostaining for a general neuronal marker antibody (NST) and for an olfactory specific marker (OMP). NST staining identifies immature as well as mature neurons while OMP staining identifies only mature ORNs (Pixley, 1992). The immunostaining was performed on cultures from day 0 (day of cell dissociation) to day 4 in culture. Figure 1 shows examples of typical immunostaining at days 0 (Figure 1A1–3) and 2 (Figure 1B1–3) in culture. At day 0, the cultures typically exhibited a large number of small round cells (suggesting that some ORNs had lost their processes during the dissociation procedure) and cells with a more characteristic ORN morphology (soma extending a dendrite that terminates in a dendritic knob). Staining for both NST (Figure 1A2) and OMP (Figure 1A3) was abundant, and observed on cells with characteristic ORN morphology as well as small round

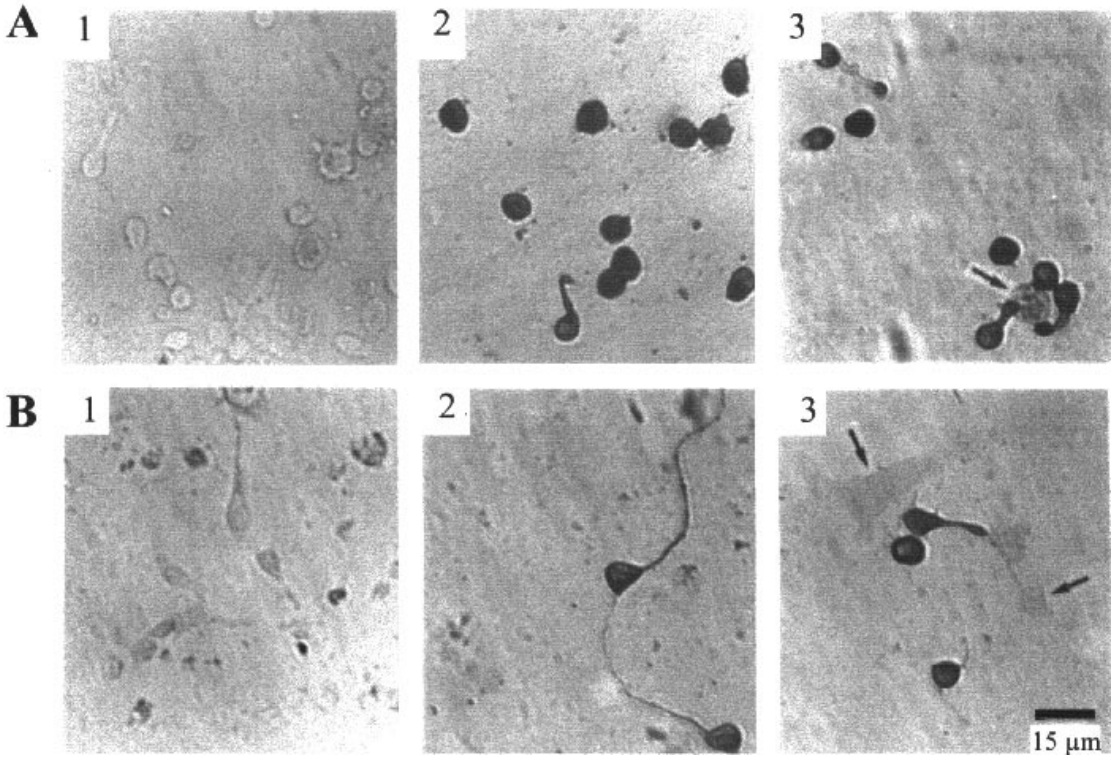

Figure 1 Characterization of rat ORN cultures. **(A)** Primary cultures of rat ORNs at day 0 in culture were immunostained for NST (2) and OMP (3). The arrow indicates a non-immunostained cell. No immunostaining was found in the controls in which primary antibodies were not included (1). **(B)** Immunostaining of rat ORNs at 2 days in culture. NST-positive (2) and OMP-positive cells (3) are still abundant in culture. Cells not immunostained are indicated by arrows. A control (no primary antibodies) is shown in (1).

cells (Figure 1A2 and 1A3). By day 1 in culture, only small changes in the cultures were observed: some cells had started to extend short processes. NST and OMP staining was observed in small round cells, cells with ORN morphology and cells starting to extend processes (data not shown). At day 2 in culture, a large number of cells had extended processes. Some cells extended only one process (unipolar) while others extended two processes (bipolar). The processes were typically long, usually extending to nearby neurons. NST (Figure 1B–2) and OMP (Figure 1B–3) staining was still abundant: round cells, cells with ORN morphology, unipolar and bipolar cells were immunostained. These positively stained cells were present at day 3 and 4 in culture; however, the total cell density was reduced (data not shown). Larger round cells and cells with atypical morphology (flat large cells) were not immunostained (indicated by arrows). Controls in which the primary antibodies were not included showed no staining for NST nor OMP on either day 0 (Figure 1A1) or day 2 in culture (Figure 1B1).

To evaluate the yield of neurons from our dissociation procedure and the survival of those neurons under our culture conditions, we quantified the total number of NST- and OMP-immunoreactive neurons present from day 0 to day 4 in culture. The number of immunoreactive neurons

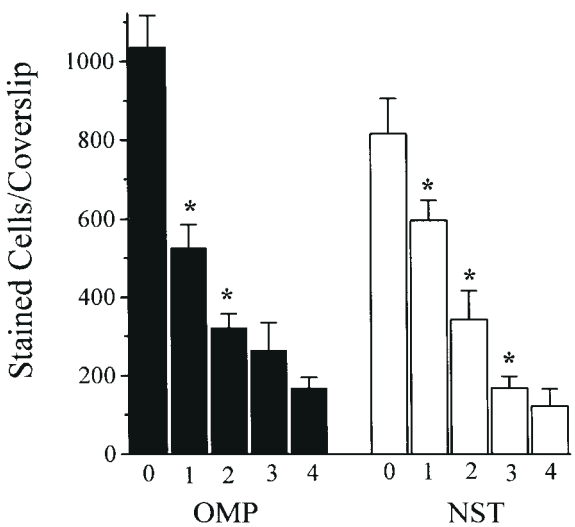

Figure 2 Total number of OMP- and NST-immunoreactive cells per coverslip at each day in culture (day 0 to day 4). The total number of immunoreactive cells was calculated as the average number of immunostained cells present in a total of 12 randomly selected 2.4 mm^2 areas (six areas in two coverslips studied) times the number of such areas in the 113 mm^2 coverslip (47 areas). No significant differences were observed between the total number of NST- and OMP-immunoreactive cells at each day in culture studied (Student's *t*-test, $P < 0.05$). Significant decreases in the number of OMP- and -NST-immunoreactive cells were observed up to 2 and 3 days in culure respectively (Student's *t*-test, $P < 0.05$).

was obtained for six random areas (2.4 mm^2) at ×10 magnification for two different coverslips from each day in culture (day 0 to day 4). At day 0, 814 ± 89 neurons/coverslip (mean ± SEM, n = 12 areas) and 1036 ± 80 neurons/coverslip (mean ± SEM, n = 12 areas) were immunoreactive for NST and OMP respectively (Figure 2). No significant difference was observed between the number of NST- and OMP-immunoreactive cells (Student's t-test, $P > 0.05$). Significant decreases in the number of both NST- and OMP-immunostained neurons were observed up to day 3 and day 2 in culture respectively. After these time points, the number of cells remained stable and no further decreases were observed (Figure 2). No significant differences were observed between the total number of immunostained neurons for NST and OMP at each day in culture (Student's t-test, $P > 0.05$). This suggests that the majority of the cells present in our cultures are mature or well differentiated ORNs, since the OMP protein is a specific marker of mature ORNs and does not appear in the basal or stem cells. Therefore, our culture conditions are able to support the short-term survival of well differentiated or mature ORNs. In the search for these optimal culture conditions, we found that daily addition of freshly made ascorbic acid to the culture medium was critical not only for the survival of cells but also for process extension. At 24 h after the dissociation procedure, a significant decrease (~50%) in the number of cells that normally survived in culture was observed when ascorbic acid was omitted from the culture medium. The

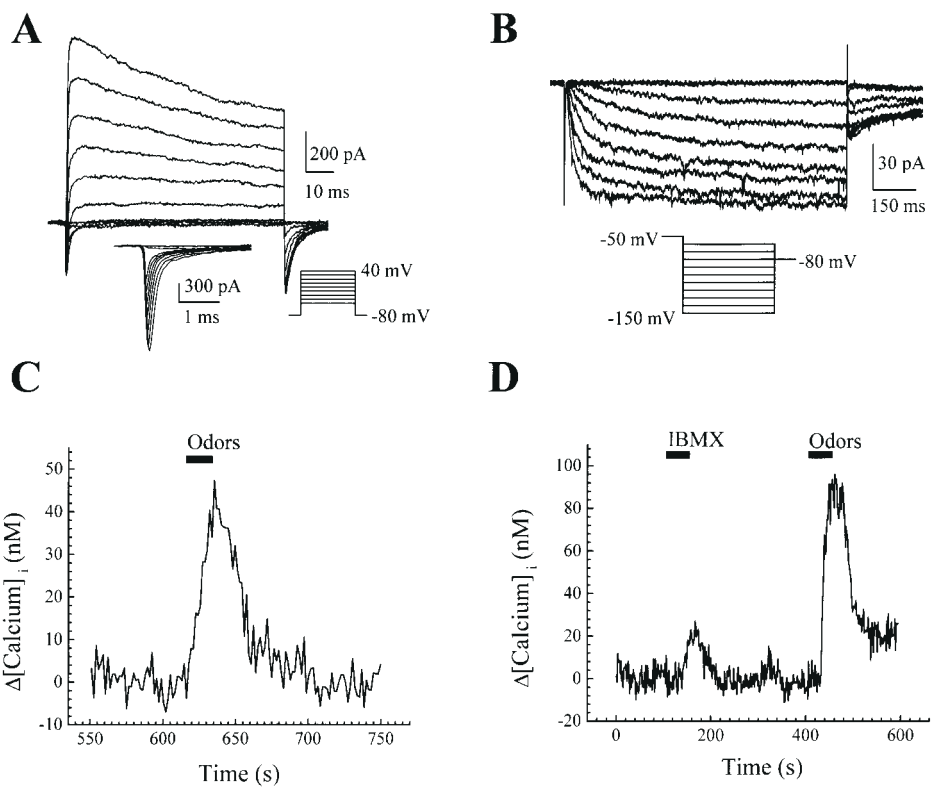

Figure 3 Cultured rat ORNs show voltage-gated currents and respond to odors. **(A)** Both fast transient inward Na^+ currents and slowly inactivating outward K^+ currents are present in cultured rat ORNs. Currents were elicited by depolarizing voltage steps up to 40 mV from a holding potential of –80 mV. These recordings were obtained from a cell on day 1 in culture. **Insert:** in a different cell (day 2 in culture), inward Na^+ currents are easily observed when outward K^+ currents are blocked by addition of 5 mM TEA to the internal solution. The external bath solution was, in mM, 25 mM K^+ rat Ringer's: 120 NaCl, 25 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4, 300 mOsm. Internal patch solutions were, in mM, (a) KF internal solution: 125 KF, 15 KCl, 5 $MgCl_2$, 11 EGTA, 10 HEPES, 2 NaATP, 1 glutathione, pH 7.2, 310 mOsm (cell shown in A) and (b) TMA oxide internal solution: 62.5 TMA oxide, 62.5 KH_2PO_4, 15 KCl, 5 $MgCl_2$, 11 EGTA, 10 HEPES, 2 NaATP, 1 glutathione, 5 TEA, pH 7.2, 300 mOsm (cell shown in insert). Voltage protocol applies to both traces. **(B)** A voltage-gated hyperpolarization-activated current, I_h, is present in cultured rat ORNs. I_h was activated by hyperpolarizing voltage pulses to –150 mV from a holding potential of –50 mV. The external solution was 25 mM K^+ rat Ringer's and the internal patch solution was KF internal solution (described above). The Cs^+-insensitive current was subtracted from raw current traces. These recordings were obtained from a cell on day 2 in culture. **(C)** Cultured rat ORNs respond to odors. The application of an odor cocktail (50 μM r-carvone, 50 μM s-carvone and 50 μM amyl acetate in rat Ringer's) to cultured rat ORNs (day 2 in culture) produced an increase in $[Ca^{2+}]_i$. A linear baseline drift in the $[Ca^{2+}]_i$ was subtracted from the record (the y-axis represents relative changes in $[Ca^{2+}]_i$ rather than absolute concentrations). The bath solution was, in mM, rat Ringer's: 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4, 300 mOsm. Stock solutions (500 μM) of odors were prepared and diluted to 50 μM with rat Ringer's on the day of the experiments. **(D)** Cultured rat ORNs are responsive to both 100 μM IBMX and odors. The application of 100 μM IBMX and an odor cocktail (50 μM r-carvone, 50 μM s-carvone and 50 μM amyl acetate in rat Ringer's) to cultured rat ORNs (day 1 in culture) elicited a Ca^{2+} influx. Data were baseline corrected. The bath solution was rat Ringer's. IBMX was made fresh as a 10 mM stock solution in ethanol and diluted to 100 μM with rat Ringer's on the day of the experiments.

cells that initially survived in the absence of ascorbic acid failed to extend processes and died within 48–72 h in culture.

Voltage-gated currents, including inward Na^+ currents, outward K^+ currents and the hyperpolarization-activated current, I_h, are present in acutely dissociated rat ORNs (Lynch and Barry, 1991a,b; Rajendra *et al.*, 1992b). We found, using whole-cell voltage-clamp recordings, that these conductances were present in every cultured ORN tested (>200 cells) regardless of the day in culture. Figure 3A shows an example of a typical family of currents activated by depolarizing voltage steps up to 40 mV from a holding potential of –80 mV. Fast transient inward Na^+ currents followed by slowly inactivating K^+ currents were activated. The outward K^+ currents were sensitive to internal appliction of 5 mM tetraethylammonium (TEA, insert in Figure 3A). I_h was also present in the cultured rat ORNs (Figure 3B). It was activated by hyperpolarizing voltage steps to –150 mV from a holding potential of –50 mV and was reversibly blocked by external application of 5 mM CsCl (data not shown). The patterns of these voltage-gated currents are similar to the ones described previously in the acutely dissociated adult rat ORNs (Lynch and Barry, 1991a,b; Rajendra *et al.*, 1992b); therefore, our system is suitable for the study of the electrophysiological properties of ORNs. We did not observe any changes in the properties of the voltage-gated currents studied over the 1–4 days in culture; however, we found that our success rate in obtaining stable recordings was greater with cultures on days 2 and 3.

Since ORNs are the sensory transducers of odorant stimuli, we used Ca^{2+}-imaging techniques to study whether cultured rat ORNs respond to odors. Cultured ORNs were loaded with the Ca^{2+} indicator dye, Fura-2, and an odor cocktail (50 μM r-carvone, 50 μM s-carvone and 50 μM amyl acetate in rat Ringer's) was applied. Figure 3C shows the results of a representative experiment. Application of the odor cocktail produced an increase in $[Ca^{2+}]_i$, as expected from the activation of odorant receptors. The percent of cultured ORNs responsive to the application of odors was consistent throughout all the days in culture. On days 0, 1, 2, 3 and 4 in culture, 10/38 (26%), 48/180 (27%), 22/82 (27%), 14/45 (31%) and 8/34 (24%) cells tested responded to odors respectively. We also tested the effect of 100 μM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor. Application of 100 μM IMBX to the cultured ORNs allowed basal levels of cAMP to rise, resulting in the activation of cyclic nucleotide gated (CNG) channels and subsequent increase in $[Ca^{2+}]_i$, as shown in Figure 3D. When an odor cocktail was applied to the same cell, an odorant-elicited Ca^{2+} influx was also observed (Figure 3D). These experiments indicate that all of the elements necessary for the odorant transduction cascade are conserved in the cultured ORNs, making our culture system suitable for the study of such events.

We have reported a culture system that supports the short-term survival of adult rat ORNs without the addition of growth factors or coculture. While our system does not support neurogenesis like previously reported culture systems (Grill and Pixley, 1997; Liu *et al.*, 1998), it provides a simple and convenient alternative to the use of acutely dissociated ORNs for the study of every aspect of olfactory signal transduction, including odor sensitivity, desensitization, adaptation, neurotransmitter modulation and membrane excitability. Compared with the acute isolation of cells, short-term cultures reduce the number of animals sacrificed and time spent on everyday isolation. Both, the number of cultured ORNs present after the cultures have stabilized and the overall health of the cultures support the realization of physiological experiments.

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