Primary Culture of Lobster (*Homarus americanus*) Olfactory Sensory Neurons

Ruben Stepanyan, Bettye Hollins, S. Erin Brock and Timothy S. McClintock

Department of Physiology, University of Kentucky, Lexington, KY 40536-0298, USA

Correspondence to be sent to: Dr Timothy S. McClintock, Louis Boyarsky Associate Professor of Physiology, Department of Physiology, University of Kentucky, Lexington, KY 40536-0298, USA. e-mail: mcclint@uky.edu

Abstract

Lobster olfactory sensory neurons have contributed to a number of advances in our understanding of olfactory physiology. To facilitate further study of their function, we have developed conditions allowing primary culture of the olfactory sensory neurons in a defined medium. The most common cells in the culture were round cell bodies with diameters of 10–15 µm that often extended fine processes, features resembling olfactory sensory neurons. We discovered that acetylcholinesterase acted as a growth factor for these cells, improving their survival in culture. We also confirmed previous evidence from spiny lobsters that poly-D-lysine was a superior substrate for olfactory cells of this size and morphology. We then identified olfactory sensory neurons in the culture in two ways. Almost half the cells tested responded to application of a complex odorant with an inward current. An even more rigorous test was made possible by the development of an antiserum to OET-07, an ionotropic glutamate receptor homolog specifically expressed by *Homarus americanus* olfactory sensory neurons. It labeled a majority of the round cells in the culture, unequivocally identifying them as olfactory sensory neurons.

Key words: acetylcholinesterase, crustacea, glutamate receptor, smell

Introduction

We have been investigating the molecular biology of olfactory sensory neurons (OSNs) in the American lobster, *H. americanus*. The lobster olfactory system has properties that have made it especially advantageous for studies of olfaction. Most of the work has focused on two species, *H. americanus* and *Panulirus argus*. Much is known about the behavioral relevance to lobsters of defined mixtures of odorants, thereby facilitating investigation of olfactory discrimination, orientation behavior, adaptation, and social interactions (Borroni and Atema, 1988; Zimmer-Faust *et al.*, 1988; Bovalenta *et al.*, 1993; Atema, 1995; Gomez and Atema, 1996a,b; Derby, 2000). Many aspects of the physiology of the OSNs are also understood, including electrophysiological properties, olfactory transduction mechanisms, the regulation of transductory ion channels by products of phosphatidylinositol 3-kinase and neuromodulation via presynaptic inhibition by histamine and GABA (Anderson and Ache, 1985; Bayer *et al.*, 1989; McClintock and Ache, 1989a,b,c; Michel *et al.*, 1991; Fadool and Ache, 1992, 1994; Michel and Ache, 1992; Boekhoff *et al.*, 1994; Hatt and Ache, 1994; Fadool *et al.*, 1995; Wachowiak and Ache, 1998; Wachowiak and Cohen, 1998, 1999; Zhainazarov *et al.*, 2001). Lobsters have also become a model for the continuous regeneration of OSNs that is characteristic of olfactory systems in most animal phyla. Cells in the lobster olfactory organ are continuously

replaced and are anatomically segregated by age. New segments (called annuli) containing the OSN clusters and auxiliary cells are formed at the proximal end of the olfactory organ and lost from the distal tip, leading to a continuous age distribution along the rostro-caudal axis of the organ (Steullet *et al.*, 2000). Our work on the molecular biology of lobster OSNs has focused primarily on components of the olfactory transduction pathways and receptors for neurotransmitters that modulate OSN excitability (McClintock *et al.*, 1997; Xu *et al.*, 1997, 1998; Xu and McClintock, 1999; Hollins and McClintock, 2000; Hollins *et al.*, 2003). Further studies would benefit from a primary culture system to test the function of these proteins with greater control of the local environment and easier assessment of responses of individual cells. We therefore sought to develop conditions for primary culture of OSNs from *H. americanus*.

Primary culture systems for several types of crustacean neurons have been developed previously (reviewed by Toullec, 1999). The most relevant studies established culture conditions for OSNs from the spiny lobster, *Panulirus argus* and stomatogastric neurons of *H. americanus* (Graf and Cooke, 1990; Fadool *et al.*, 1991). Crustacean neurons typically survive for several days in simple media. Survival and growth of neurites can be further improved by carefully controlling conditions that include temperature, humidity,

substrata and growth factors (reviewed by Toullec, 1999). In the absence of knowledge about endogenous growth factors for crustacean neurons, many investigators have used hemolymph, tissue extracts, or mammalian serum to attempt to improve survival and neurite outgrowth. We also looked for inspiration in work on other invertebrates, noting that acetylcholinesterase (AChE) is a growth factor for molluscan neurons, whose survival in culture is improved by AChE (Srivatsan and Peretz, 1997). We hypothesized that AChE might also be beneficial for crustacean neurons in culture. Herein we describe defined media conditions employing AChE as a growth factor and poly-D-lysine (PdL) as a substratum for the primary culture of *H. americanus* OSNs.

Materials and methods

Animals

Adult American lobsters (*H. americanus*) were obtained from commercial vendors. Lobsters were held for <2 weeks in filtered, aerated artificial seawater at 4°C.

Tissue preparation and culture media

The dispersal of individual cells from the olfactory organ and starting culture conditions were derived primarily from Fadool *et al.* (1991). The olfactory organ, which occupies the end of the lateral branch of the first antenna, was removed and submerged for 5 min in 10% Listerine in an antibiotic lobster saline (ALS). ALS consists of lobster saline (460 mM NaCl, 13 mM KCl, 13 mM CaCl$_2$, 10 mM MgCl$_2$, 1.7 mM D-glucose, 10 mM HEPES, pH 7.4) plus 100 U/ml penicillin, 75 U/ml streptomycin and 0.25 µM fungizone. The region of the olfactory organ containing aesthetasc hairs was cut into sections three annuli long. Sections at the proximal and distal end that contain only a few aesthetasc hairs were discarded. The remaining sections were bisected and the ventral hemisections that contain the clusters of OSN somata were transferred to fresh ALS as a wash step. Hemisections were gently agitated on a shaker (22 cycles/min) in ALS containing 1 mg/ml collagenase for 20 min and then in calcium free lobster saline (same as ALS but lacking the CaCl$_2$) containing 1 mg/ml trypsin for 10 min. Hemisections were then washed once with ALS and transferred to fresh ALS. OSN somata clusters of the aesthetasc sensilla were collected from the hemisections by aspiration into a 5 ml syringe through a sterile 26-gauge needle and then injected into 35 mm Falcon culture dishes or 24-well plates containing culture medium. Passage twice through the 26-gauge needle was sufficient to separate many individual cells from the clusters. Culture medium was prepared by mixing Leibowitz-15 (L-15) media (Sigma No. L4386) containing 0.3 g/l L-glutamine with an equal volume of buffered double-strength lobster saline (pH 7.4) supplemented with 120 mM D-glucose, 1% BME (basic minimal essential) vitamins, and 0.1 mg/ml gentamicin (all from

Sigma). The cultures were placed in an incubator at saturation humidity at 5°C and not moved for 2 h to aid the adhesion of cells to the substratum. Additive treatments to the culture medium such as fetal bovine serum (FBS; Life Technologies) and AChE (acetylcholine acetylhydrolase, EC3.1.1.7) were applied at 2 h after plating and maintained through subsequent feeding with media. FBS was introduced by the addition of 0.2 ml of 10% FBS to wells containing 0.3 ml of media to give a final dilution of 4% FBS. AChE was introduced by the addition of 0.1 ml of the appropriate stock concentration to wells containing 0.4 ml of media. One half of the culture medium was exchanged on the fourth day. Cultures in 35 mm dishes were used for electrophysiology. Cultures in 24-well plates were used to evaluate culture conditions. Cultures in eight-well chamber slides (Lab-Tek Permanox; Nalge Nunc International Corp.) were used for immunocytochemistry.

To prepare a PdL substratum (Sigma, No. P7280), 0.5 ml of a 0.02 mg/ml PdL solution was applied to each well of a 24-well plate, or 2 ml was applied to each 35 mm dish. After 5 min, the solution was aspirated and rinsed with two volumes of sterile distilled H$_2$O. The plates and dishes were allowed to dry by storing them overnight at 4°C. AChE purified from fetal bovine serum (GIBCO BRL) by procainamide affinity column chromatography according to Ralston *et al.* (1985) was the kind gift of Dr Malathi Srivatsan. It was stored in aliquots at 100 U/ml at –80°C in phosphate-buffered saline (PBS). Working solutions were prepared fresh each day by dilution of a frozen aliquot.

Quantification and statistical analyses

The experimental design to investigate culture conditions involved preparing dissociated cells on four separate days, using two olfactory organs on each day. On each day equal volumes of dissociated cell suspensions were injected into wells of a 24-well plate. Each experimental condition was represented by six wells in each plate. Assessment of cell survival and neurite outgrowth was done in a longitudinal experimental design. Cell numbers were estimated by counting adherent round cell bodies in 10 fields of view per well at 200× magnification using Hoffman modulation contrast optics. During these counts the numbers of cell bodies with neurites were also counted. We summed the 10 sample counts and calculated the number of cells per well based on the area in the 10 fields of view and the total area of the well. For calculating cell survival, the numbers were normalized to the data at 2 h. The numbers at each time point were converted to percentage values by dividing by the number of cells that were in that same well at 2 h after plating. To track the prevalence of round cells with neurites, the percentage of cells with neurites in each well was calculated by dividing the number of cells with neurites by the number of total cells in that well on each day. The length of neurites was not measured, though we set a length of one radius of the cell body as a criterion that must be met or

exceeded by at least one neurite in order for the cell to be counted as having a neurite. The experimental design for investigating AChE effects involved cultures prepared on two different days, again with six wells per treatment condition. To use percentage data in analysis of variance (ANOVA) an arcsine transformation (Zar, 1974) was done. ANOVA and Tukey's multiple comparisons test were performed using a plug-in to Excel (Microsoft).

Electrophysiology

Whole-cell patch clamp recording was performed on an inverted microscope at room temperature. All recordings were made on cells at days 3–5 in culture. Custom Patch Glass (No. G85150T-4; Warner Instrument Corp.) was used to construct patch electrodes. Electrode tips were fire-polished and pipettes having a tip diameter of ~1 μm as measured by the bubble number method (Mittman *et al.*, 1987) were used for recording. The composition of the pipette solution was 200 mM KCl, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 570 mM glucose and 10 mM Na-HEPES (pH 7.0). Currents were acquired on an Axopatch 200B amplifier using pClamp8 software (Axon Instruments, Foster City, CA). Data were digitized at 2 kHz and filtered at 1 kHz. Voltage-activated currents were evoked in cells held at –60 mV. Inward current amplitude measurements were made at peak amplitudes. Sustained outward current amplitudes were measured by averaging the current amplitude during the 20–40 ms period of 40 ms voltage steps. Odorants and vehicle solutions were applied by rapidly switching microperfusion streams (SF-77B Perfusion Fast-Step; Warner Instrument Corp.) on cells held at –60 mV. A complex odorant mixture was prepared from TetraMarin (TET) (Tetrawerke, Melle, Germany), a commercial flake fish food, by homogenization of 2 g dry flakes in 60 ml saline, followed by low speed centrifugation and filtration of the supernatant. Aliquots of TET were frozen at –20°C. A fresh aliquot was thawed each day and TET was applied to cultured OSNs as a 1:100 dilution in lobster saline.

Antisera, immunoblotting, and immunocytochemistry

A rabbit antiserum was generated against the C-terminal region of lobster OET-07 (Genbank accession No. AY098942) and affinity-purified by ProSci Incorporated (Poway, CA). The antigen used was a cocktail of two non-overlapping peptides, TGEGFDIAPVANPW and REYPT-NDVDKTNFN. As a control, a pre-absorbed antibody solution was prepared by incubating the affinity-purified antibody solution (20 μl) with 1 mg of each peptide in 1 ml of PBS for 4 h at 4°C followed by centrifugation at 11 600 × g for 30 min.

Western blotting and the isolation of membrane fractions from lobster tissues for these blots were performed as described previously (McClintock *et al.*, 1997), with the following modifications. Membrane pellets were resuspended by vortexing in 100 mM NaCl and 50 mM HEPES

(pH 7.4) before being diluted into 1× Laemmli sample buffer. Ten micrograms of membrane protein was loaded in each lane of a 10% SDS/PAGE gel. Electrotransfer was done onto Immobilin-P membranes (No. IPVH00010; Millipore Corp., Billerica, MA) at 25 V for 90 min. The affinity-purified OET-07 antiserum was used at a dilution of 1:10 000 and the blocking solution contained 10% normal goat serum instead of albumin. The chemiluminescence detection system used was SuperSignal (No. 34080, Pierce, Rockford, IL).

To prepare tissue sections of the olfactory organ, the region of the lateral branches of the first antenna that bears aesthetascs was cut into segments 15–20 annuli in length. These were placed in 4% paraformaldehyde in lobster saline at room temperature for immediate dissection and removal of the exoskeleton. Removal of the exoskeleton improves the quality of sections prepared from this tissue. The tissue was then embedded in 15% gelatin. The gelatin block was overlaid with 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and allowed to stand at 4°C for 48 h. Sagittal sections of 30 μm thickness were cut with a vibratome and collected in PBS. Prior to incubation with antisera, tissue slices were treated with 2% peroxide in 50% methanol in PBS for 1 h at room temperature, then washed thrice for 10 min in PBS. If necessary, sections brought to this point were stored in PBS at 4°C.

In preparation for staining, vibratome slices containing four to six tissue sections were distributed in 12-well plates. The dissociated cells from the olfactory organ described above were washed in lobster saline, fixed in 4% paraformaldehyde in lobster saline for 5 min, quenched for 10 min in 5 mM NH_4Cl followed by three 5 min washes in PBS. Cells and sections were blocked for 2 h at room temperature in 10% normal goat serum in PBS containing 0.3% Triton X-100 and 0.05% Tween-20 (blocking buffer). The affinity purified antibody solution and the pre-absorbed antibody solution were diluted 1:500 in the blocking buffer. These solutions were applied overnight at 4°C. Three 5 min washes were done in wash buffer (PBS containing 0.3% Triton X-100 and 0.5% Tween-20) and then the samples were incubated in Cy3-conjugated goat anti-rabbit immunoglobulin (diluted 1:600 in blocking buffer) for 1 h at room temperature. Three final 5 min incubations in wash buffer were done. The slides were then prepared for epifluorescence microscopy by mounting cover slips over 90% glycerol in PBS.

Results

Survival and neurite outgrowth depend on substrata and serum.

Platings of freshly dissociated cells from the olfactory organ of both *H. americanus* and *P. argus* used for patch-clamp studies are dominated by round cells whose size and electro-

physiological signatures identify them as OSNs (Anderson and Ache, 1985; Bayer *et al.*, 1989; McClintock and Ache, 1989a,b). Primary cultures of *P. argus* olfactory organ contain many cells with morphologies similar to these freshly dissociated cells (Fadool *et al.*, 1991). We therefore used cell size and morphology as a preliminary indicator of successful plating and survival of *H. americanus* OSNs. We found that the majority of the cells in the culture were round somata with diameters in the 10–15 μm range (Fig. 1). Many of these somata send out fine processes over the period of culture. Examples of cells with processes can be seen in Figure 1. To make these cultures, we used sections of 25 annuli from the mature zones of two lobster olfactory organs. This tissue contains ~315 000 OSNs. When counted at 2 h after plating, we obtained an average of 30 320 adherent round cell bodies, a yield of 9.6%. An average well in a 24-well plate contained 884 adherent round cells at 2 h after plating.

We first tested whether a PdL substratum and addition of fetal bovine serine (FBS) would have an effect on survival and neurite outgrowth of the round cells we believed to be OSNs. Over a culture period of 6 days only plating on PdL in the 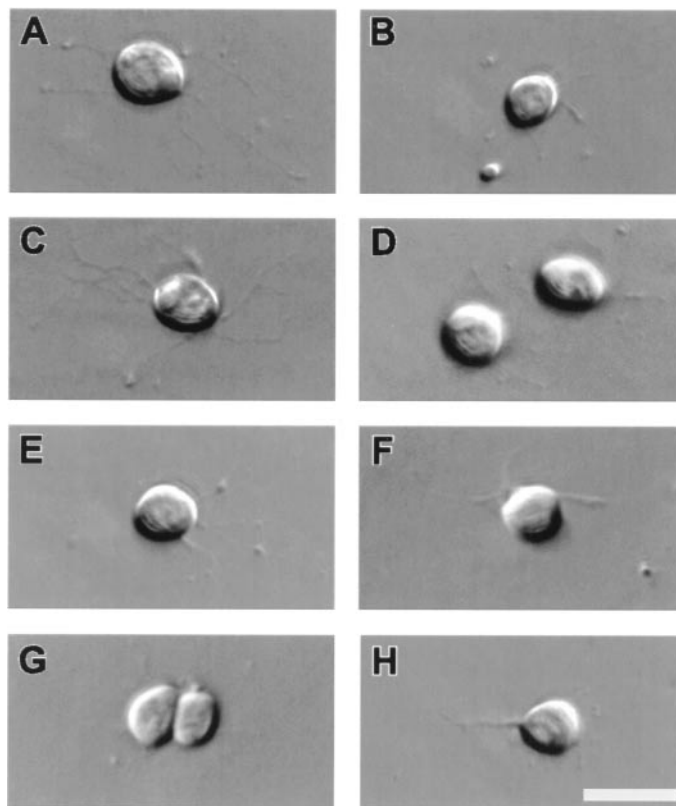absence of FBS gave significantly better cell survival than control plating on Falcon tissue culture plastic (Fig. 2A; ANOVA, $F = 5.06$, $P < 0.005$, 3 df). The number of cells with neurites was significantly better than the control condition only when FBS was added (Fig. 2B; ANOVA, $F = 8.22$, $P < 0.0001$, 3 df). However, FBS did not increase the number of cells with neurites when added to cultures plated on PdL. The duration in culture also had a significant effect on both cell survival and neurite outgrowth ($F > 8$, $P < 0.0001$, 5 df in both cases). Cell numbers declined slowly over the first 3 days and then more rapidly thereafter. Cell numbers on days 4–6 were significantly lower than on days 1–3, and day 6 had significantly fewer cells surviving than
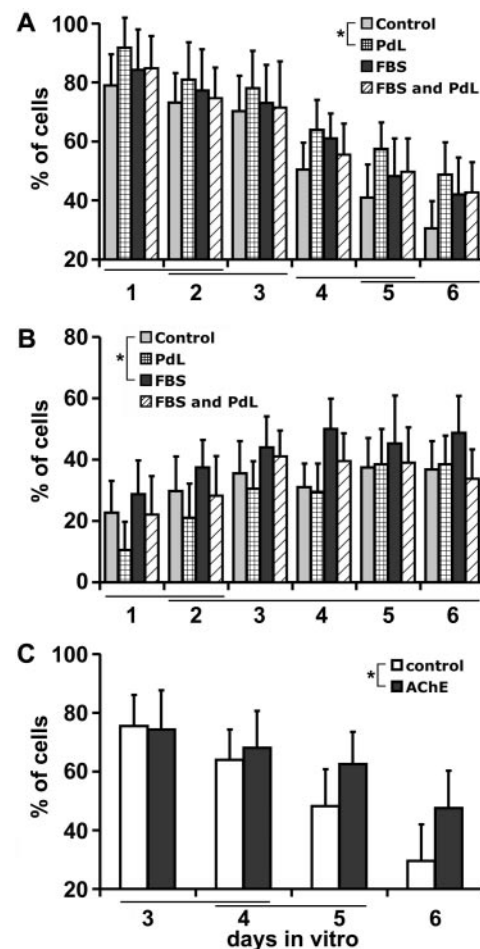

Figure 1 The appearance of round cells in primary culture from the *H. americanus* olfactory organ under Hoffman modulation contrast optics. Most cells have round or slightly elongated somata, and many have one or more neurites. Images are from cultures at day 4 **(A–F, H)** or day 5 **(G)**. Scale bar = 25 μm.

Figure 2 Poly-D-Lysine (PdL) substratum, fetal bovine serum (FBS) and acetylcholinesterase (AchE) affect cell survival or neurite outgrowth. **(A)** PdL increased the number of cells surviving through 6 days in culture compared to plating on tissue culture plastic. These percentage values were calculated using the number of adherent round cells in each well at 2 h after plating as the initial 100% value. **(B)** The proportion of cells with neurites was increased by adding FBS, but only if FBS was used on cells plated on tissue culture plastic rather than on PdL. These percentage values use the total number of cells in each well at each time point as the 100% value. **(C)** AChE improved long-term survival in culture. These percentage values were calculated as in panel (A). Error bars are standard deviations. *A statistically significant effect (see text for details). Underlining below days *in vitro* depicts the results of Tukey's multiple comparisons tests: days sharing an underline did not differ.

days 4 and 5. The percentage of surviving cells with neurites increased rapidly, becoming significantly different from day 1 at day 3 and maintaining a significant difference through day 6, even though the number of cells was declining. These data reflect true outgrowth of neurites rather than differential survival of cells with neurites since in 15 of 16 culture plates the actual number of cells with neurites increased from day 1 through day 3 or day 4. We detected no interaction effects between the treatments and the days in culture.

Acetylcholinesterase improves cell survival

To attempt to enhance cell survival at later time points, we tested whether AChE would slow the decline in cell number at days 4–6 compared to day 3. Preliminary tests with solutions containing 0.05, 0.1, or 0.5 U/ml AChE suggested that the 0.1 U/ml concentration was most effective. We tested this concentration in a more complete experimental design and found a significant effect of AChE on cell survival (Fig. 2C; ANOVA, $F = 4.30$, $P < 0.05$, 1 df). This improvement was due largely to improved survival through days 5 and 6 in culture. Day 5 was different from day 1 and day 6 was different from all the other days. All subsequent experiments were therefore done with culture media containing AChE at 0.1 Us/ml.

The majority of round cells were OSNs

To confirm that the cells in the cultures were indeed OSNs, we took two approaches. First, we tested whether the electrophysiological properties of the round cells were consistent with known properties of lobster OSNs. Lobster OSNs typically have a rapidly activating inward current and a more slowly activating sustained outward current (McClintock and Ache, 1989c; Fadool *et al.*, 1993). Under voltage clamp, most round cells in our cultures showed this pattern of voltage-activated currents (Fig. 3A,B). We recorded from 21 cells that had both inward and outward currents. The fast inward currents tended to be small. The average peak inward current at 0 mV (where this current was largest) was –323 ± 40 pA, with –840 pA being the largest observed. The average sustained outward current amplitude in these cells was 1132 ± 96 pA at 20 mV. We also recorded from six cells that showed large sustained outward currents but no discernible inward current. Another electrophysiological property of OSNs is responsiveness to odorants. We screened 11 cells with a complex odorant (TET) and found that four of them responded with a small inward current (Fig. 3C). The odor-evoked current averaged –157 ± 105 pA, with a range from –298 to –49 pA. These data were consistent with the conclusion that these cells were OSNs, but we sought more definitive evidence. We were fortunate to have recently identified a transcript, OET-07, that is expressed only by *H. americanus* OSNs (Hollins *et al.*, 2003). We developed an antiserum to OET-07 and we used it as a definitive method of identifying OSNs in our cultures. The antiserum labels a single band of 115 kDa in olfactory organ
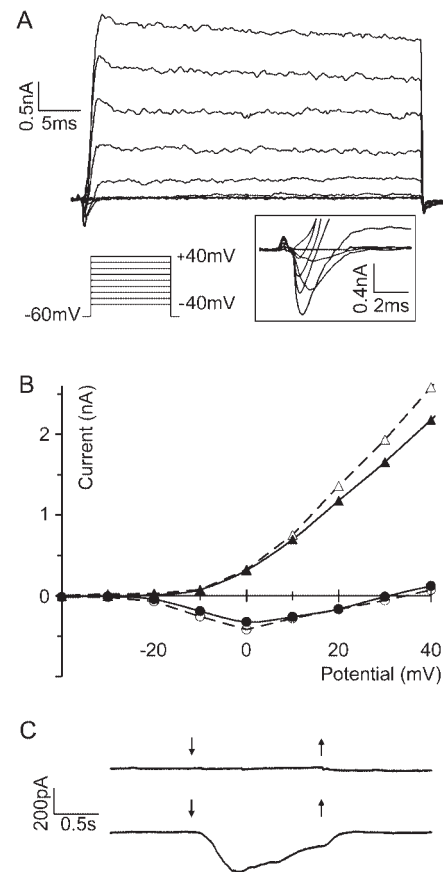

Figure 3 Putative OSNs in culture have voltage-dependent ionic currents and respond to odorants. **(A)** Total membrane currents of a putative *H. americanus* OSN after 3 days *in vitro*. Macroscopic currents evoked by depolarizing voltage steps included a small rapidly activating inward current and a large outward current. The inset on the left shows the voltage protocol. The inset on the right is an expanded scale of the initial current response of a different cell with a larger inward current to show the detail of a rapidly activating inward current. **(B)** The current–voltage relationship of the peak inward current (open circles) and the average sustained outward currents (open triangles) of the cell shown in (A). The dotted lines refer to this cell. Closed symbols and solid lines are average currents for all 21 cells that had both inward and outward currents. **(C)** A response of a cultured cell to a 100-fold dilution of TET extract (lower trace). Application of lobster saline did not evoke a response in the same cell (top trace).

lanes on immunoblots (Fig. 4). This is near the calculated size of 106.4 kDa for the OET-07 polypeptide. This band was eliminated by preabsorption of the antiserum with antigen (Fig. 4B). To confirm the cellular specificity of expression of OET-07 in the olfactory organ, we tested the antiserum on tissue sections. We observed strong labeling of the OSN cell bodies and weaker labeling of olfactory nerve bundles (Fig. 5). There was little or no immunoreactivity in OSN dendrites and no evidence of labeling in other cell types in the olfactory organ. This is consistent with our previous *in situ* hybridization results (Hollins *et al.*, 2003). OET-07 immunoreactivity was widespread throughout the cytoplasm of the cell soma, with at least one intense punctate

A

E N SA B L D

218 —
131 —
86 —

43 —

B

E N SA B L D

218 —
131 —
86 —

43 —

Figure 4 OET-07 immunoreactivity was detected in a single band of ~115 kDa found only in the olfactory organ. **(A)** Immunoblot performed with a 1:10 000 dilution of the antiserum. **(B)** Preabsorption of the antiserum with the antigen eliminated immunoreactivity. E, eye and eyestalk; N, olfactory organ; SA, second antenna; B, brain; L, pereiopod; D, dactyl.

spot within the cytoplasm of most somata (Fig. 5G). Whether there was also cell surface expression of OET-07 in the somata was equivocal, even in confocal images (not shown). We therefore judged the OET-07 antiserum sufficient to reliably identify OSNs in our dissociated cell cultures. When reacted against the cultures, we observed immunoreactivity in round cells but not in flattened cells. The majority of round cells were immunoreactive for OET-07 (Fig. 6A,B). Immunoreactive cells averaged 58 ± 13% of the round cell bodies in the culture. The distribution of immunoreactivity inside the cells appeared similar to that of the OSN somata in tissue sections (Fig. 6G).

Discussion

We have established defined medium culture conditions that allow the maintenance of *H. americanus* OSNs in culture for at least 6 days. These conditions involved culture at 5°C in humidified air on a PdL substratum in L-15 media supplemented with vitamins, gentamicin and AChE. A majority of the round cells surviving in these cultures were identified as OSNs by virtue of their expression of OET-07, which is expressed by all mature OSNs. The vast majority of the OET-07 immunoreactive cells were probably mature OSNs. This conclusion is supported by the fact that we used only the mature zone of the olfactory organ, in which all OSNs are believed to be mature (Steullet *et al.*, 2000). We qualify our conclusion only because we have not yet rigorously tested whether OET-07 can be expressed by immature OSNs. It is therefore possible, though unlikely, that a few of the cells immunoreactive for OET-07 in our cultures were immature OSNs. The cells in our cultures that were of the
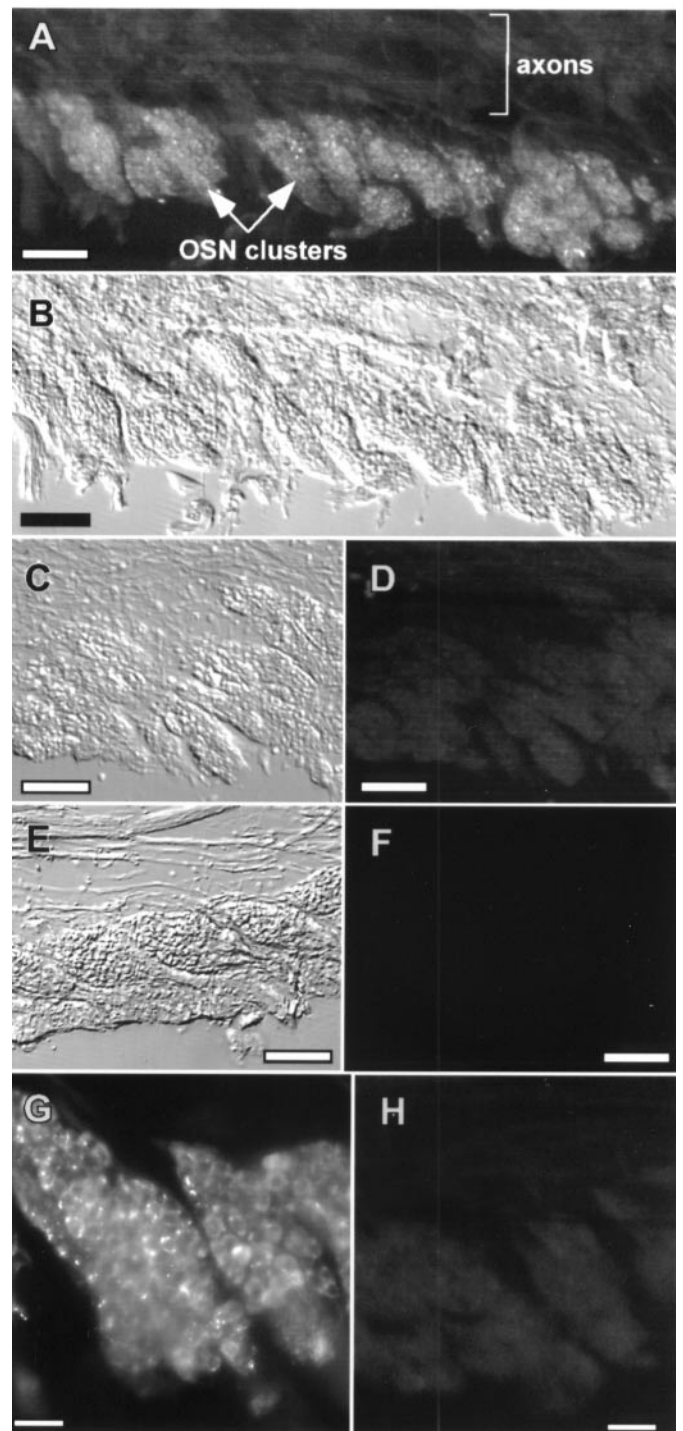
Figure 5 The antiserum to OET-07 labels only OSN somata and axons. **(A, B)** Fluorescence and Hoffman modulation contrast images of a section of the olfactory organ treated with the OET-07 antiserum. The clusters of OSN somata were strongly labeled. The bundles of axons in the antennular nerve were labeled less strongly. Little, if any staining was apparent in the dendrites of the OSNs. **(C, D)** Labeling was greatly reduced when antiserum preabsorbed with the OET-07 antigen was used. **(E, F)** Omission of the primary antiserum completely eliminated the labeling. **(G)** Labeling in OSN somata includes widespread signal of moderate intensity throughout the cytoplasm and one or more punctate spots of high intensity in most somata. **(H)** The residual labeling with preabsorbed antiserum is weak and indistinct. Scale bars = 100 μm (A–F); 25 μm (G, H).
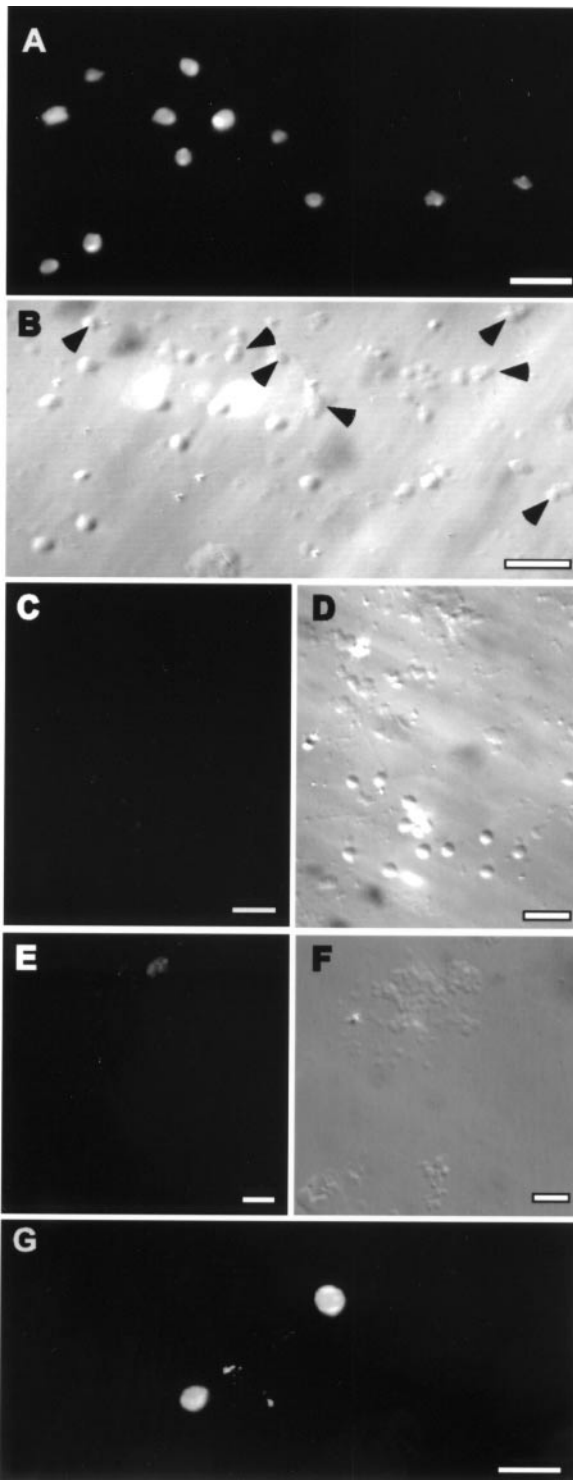
Figure 6   Immunoreactivity for OET-07 identified cultured cells as OSNs. **(A, B)** Comparing fluorescence and Hoffman modulation contrast images of cells in culture indicates that most cells were immunoreactive for OET-07. Arrowheads point to some of the cells that lacked immunoreactivity. **(C, D)** Preabsorbed antiserum gave virtually no labeling. **(E, F)** Omission of the primary antiserum eliminated labeling. **(G)** An image of a group of cells that appear to be the remnants of a cluster of OSNs. OET-07 immunoreactivity was diffuse throughout the cytoplasm, with one or more punctate spots of high intensity in most cells. Scale bars = 100 µm (A–F); 25 µm (G).

appropriate size and morphology yet negative for OET-07 expression could have had several identities. They could have been auxiliary cells, collar cells, glial cells, epithelial cells, rare immature OSNs, or neurons that innervate other types of sensilla on the olfactory organ (Cate and Derby, 2001, 2002; Hollins *et al.*, 2003). We suspect, however, that auxiliary cells, epithelial cells and glial cells accounted for flattened multipolar cells seen in the culture. Cells with this morphology were much less frequent than the round cells. We hypothesize that many of the round cells lacking immunoreactivity to OET-07 were neurons from other types of sensilla. No specific markers for these types of neurons are yet available, though we have identified a calcyphosine that is a potential marker for broad classes of sensory neurons in the olfactory organ (Hollins *et al.*, 2003). We are currently developing antisera to the calcyphosine and to proteins expressed specifically by outer auxiliary cells and collar cells (Hollins *et al.*, 2003), so we hope to be able to identify additional cell types in our cultures in the future.

The yield of adherent round cells in our cultures was ~10% of the number of OSNs in the starting tissue. Immunoreactivity for OET-07 indicated that only slightly more than half the round cells were mature OSNs. The actual yield of OSNs in our cultures was therefore 5–6% of the OSNs in the tissue. Pilot experiments in which dissociated cells were collected by centrifugation and resuspended prior to plating gave lower yields than the direct plating method we adopted from the work of Fadool *et al.* (1991). We also confirmed their evidence that PdL is a good substrate for lobster OSNs. Survival of the round cells in our culture was better on the PdL substrate and when AChE was included as a growth factor. The improved survival on PdL could have arisen in two ways. Either a greater fraction of cells successfully attached to PdL than to tissue culture plastic or PdL had a direct effect on cell survival. Cell numbers after 1 day appeared greater on PdL, consistent with greater attachment, but the rate at which cell numbers declined also appeared to be less on PdL. We have not attempted to discriminate between these two possibilities and the interpretation that both contributed to increased survival is consistent with the data. The improved survival provided by AChE was simpler to interpret. Out to 3 days, survival was not improved by AChE, but as more time passed cell numbers experienced a slower decline when AChE was present. This effect is consistent with the neurotrophic effects of AChE in other species. In addition to its role in neurotransmitter clearance at cholinergic synapses, AChE has secreted forms that are potent neuroactive growth factors for both vertebrates and invertebrates (Layer *et al.*, 1993; Small *et al.*, 1995; Koenigsberger *et al.*, 1997; Srivatsan and Peretz, 1997; Bigbee *et al.*, 2000). The neurotrophic activity of AChE depends not on the site that catalyses hydrolysis of acetylcholine, but rather on a secondary anionic site in the molecule (Srivatsan and Peretz, 1997). The cellular receptor responsible for mediating this activity has

not been identified. AChE is a highly abundant protein in the serum and cerebrospinal fluid of vertebrates and the hemolymph of invertebrates. It is therefore possible that its effect in our cultures mimicked an endogenous neurotrophic effect.

Odor responses are typically absent in freshly dissociated OSNs from *P. argus* (Anderson and Ache, 1985) and *H. americanus* (T.S. McClintock, unpublished observations). The capacity for isolated *P. argus* OSNs to respond to odorants appears after time *in vitro* (Fadool *et al.*, 1993). The same appears to be true for *H. americanus*, though responsive neurons were less frequent in our cultures: 36 versus 64–89% in *P. argus*. Many reasons, both intrinsic and extrinsic to the OSNs, could account for this difference. Perhaps the most likely reason was that ~40% of the round cells in our cultures were not mature OSNs. Using morphological criteria to select cultured cells for stimulation by odors would therefore result in the inclusion of cells other than OSNs and these are unlikely to respond. A similarly large set of extrinsic and intrinsic factors could account for the small size of odor responses in cultured *H. americanus* OSNs. No single factor has yet emerged as a leading explanation. The size and frequency of odor responses in cultured *H. americanus* OSNs may limit some types of functional experiments, but for many experiments these odor responses should be sufficient.

The voltage-dependent ionic currents of the round cells in our cultures contained a small rapidly activating inward current followed by a larger sustained outward current. This was similar to both freshly dissociated and cultured OSNs from *P. argus* (McClintock and Ache, 1989c; Fadool *et al.*, 1993). One small difference was that the voltage-dependence of the *H. americanus* currents was shifted 5–10 mV in the depolarizing direction. This difference is also apparent in freshly dissociated *H. americanus* OSNs, so it is unlikely to result from culture conditions (T.S. McClintock, unpublished observations). The reason for the difference between the two species is not clear. Similarities between the two lobsters are much more prevalent than their differences, however. They include electrophysiological and neurochemical properties such as sensitivity to histamine, similar odorant sensitivity and the same olfactory transduction pathways (Bayer *et al.*, 1989; McClintock and Ache, 1989a,b; Fadool *et al.*, 1995; McClintock *et al.*, 1997; Munger *et al.*, 2000). These similarities help to explain why the culture conditions established by Fadool *et al.* (1991) proved to be an efficient starting point for establishing conditions for culture of *H. americanus* OSNs.

In summary, we have established conditions that use a defined medium to maintain odor responsive *H. americanus* OSNs in culture for at least a week. This will provide sufficient time for several types of experimental manipulations, including the modification of transcription and translation. We have also developed an antiserum that recognizes a putative ionotropic glutamate receptor subunit (OET-07)

expressed only by OSNs and found that this antiserum was completely specific for OSNs. This antiserum allowed us to demonstrate convincingly that the defined media conditions allowed culture of bona fide OSNs. We expect both the culture system and the antiserum to be useful in future experiments to further understand the functions of lobster OSNs.

Acknowledgements

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