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Chronic Odorant Exposure Upregulates Acquisition of Functional Properties in Cultured Embryonic Chick Olfactory Sensory Neurons

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Neuronal development and differentiation is modulated by activity-dependent mechanisms that stimulate endogenous neurogenesis and differentiation to promote adaptive survival of the organism. Studies on bird odor imprinting have shown how sensory stimuli or environmental influences can affect neonatal behavior, presumably by remodeling the developing nervous system. It is unclear whether these changes originate from the sensory neurons themselves or from the brain. Thus, we attempted to address this by using an *in vitro* system to separate the peripheral neurons from their central connections. Olfactory neurons from embryonic day 17 *Gallus domesticus* chicks were isolated, cultured, and exposed to 100 μM amyl acetate or phenethyl alcohol in 12-hr bouts, alternated with periods of no-odor exposure. On days 4 and 5 *in vitro*, cells were immunostained for olfactory marker protein, neuron-specific tubulin, and olfactory GTP-binding protein, and tested for odorant sensitivity using calcium imaging. While odorant exposure did not result in a significant increase in the overall number of neurons, it promoted neuron differentiation: a larger proportion of odorant-exposed cells expressed olfactory marker protein and the olfactory GTP-binding protein. When cell responsiveness was tested using calcium imaging, a greater proportion of odorant-exposed cells responded to stimulation with 100 μM amyl acetate or phenethyl alcohol. Thus, odorant exposure during development modulated the developmental trajectories of individual neurons, resulting in changes in protein expression associated with odorant signaling. This suggests that the neuronal changes in the periphery have an important contribution to the overall long-term functional changes associated with odor imprinting.

Key words: domestic chicken; odor imprinting; peripheral olfactory system; calcium imaging

INTRODUCTION

Imprinting is a process wherein an animal develops a long-term behavioral change resulting from a (pre)natal exposure to an environmental stimulus; a classic example is visual maternal imprinting in birds (Bateson, 1966). The extended duration of this behavior suggests permanent alterations in the nervous system. This phenomenon is present in chemical senses as well in salmon (Dittman et al., 1997; Harden et al., 2006), zebrafish (Gerlach et al., 2008), and mammals (Wells and Hepper, 2006; Todrank et al., 2011). The acquired behavioral changes suggest that long-term modifications must occur in the neurons that mediate these behaviors (Dittman et al., 1997). In the olfactory system, it is known that *in utero* exposure to odors triggers measurable changes in the glomeruli of the olfactory bulb (Todrank et al., 2011). Because it is well established that the olfactory epithelium and the olfactory bulb communicate with each other to modulate development (Gong and Shipley, 1995), it can be assumed that the neurons in the periphery may exhibit long-term changes as well (Nevitt et al., 1994; Harden et al., 2006).

SIGNIFICANCE

Birds are an interesting model for the study of development because they are warm-blooded like mammals, but the embryos are exposed to the external environment which can influence development. This study demonstrates how the development of the nerve cells in the olfactory system can be guided by stimulation with odorants. This unique setting provides an interesting framework for understanding the interaction between the environment ("nurture") and the naturally occurring developmental patterns in the nervous system ("nature"), and helps us understand the underlying mechanisms of the development and adaptation of neural systems to different environments.

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In avian species, odor imprinting *in ovo* mediates adaptive behaviors such as selection of suitable nesting habitats (Caspers and Krause, 2011) or diets (Bertin et al., 2010, 2012). The olfactory system of chicks originates from the nasal placodes that appear during embryonic day 3 (E3). Olfactory receptor proteins are first expressed in the olfactory epithelium at E5 and continuing through E8 (Lalloue et al., 2003; Lalloue and Ayer-Le Lievre, 2005). By E13, the first signs of olfactory function can be measured (Lalloue et al., 2003). There is evidence of *in ovo* functionality in these embryos: embryos exposed to novel odors at E18 show instinctive responses to odor stimulation (Hagelin et al., 2013).

However, the precise origin of the changes in the olfactory system is unclear. *In ovo* exposure to odorants at E15 through E18 results in changes in glomerular organization in the chick olfactory bulb prior to hatching (Gomez and Celii, 2008). It is unclear whether these changes first occur in the periphery and are relayed to the brain, or whether the changes first occur in the olfactory bulb and are transmitted to the periphery in a retrograde fashion (for review, see Mackay-Sim et al., 2015). We attempted to address this by isolating the peripheral olfactory system and determining whether long-term odor exposure could alter the cellular properties of the olfactory sensory neurons (OSNs).

We opted to employ a primary cell culture system. OSNs regenerate throughout the adult life span through a process that recapitulates embryonic development (Oley et al., 1975; Kiyohara and Tucker, 1978; Farbman, 1994). *In vitro*, the olfactory system continuously generates OSNs from non-neuronal precursors (Coon et al., 1989; Vannelli et al., 1995; Gomez et al., 2000b) and expresses morphological and physiological characteristics reminiscent of the olfactory system *in vivo* (Gomez et al., 2000b; Gomez and Celii, 2008). We have shown that the chick embryonic olfactory epithelium can be grown in culture (Gomez and Celii, 2008) to attain the characteristic morphology, molecular marker expression profile, and physiological responsiveness of *in situ* avian olfactory neurons (Jung et al., 2005). We selected the bird olfactory system as a model because in addition to the known odor-elicited modulation of posthatching behaviors (Bertin et al., 2010, 2012), it has been suggested that the neurons of the bird olfactory system may be a primary site where odor information is processed prior to being relayed to the brain (Jung et al., 2005). This increased the likelihood of observing cellular changes of odor-induced modulation of neuronal structure and function at the periphery.

MATERIALS AND METHODS

Materials

Unless otherwise indicated, all reagents, chemicals, and secondary antibodies were obtained from Sigma Chemical Co, St. Louis, MO.

Cell Cultures

All procedures were conducted in accordance with the University of Scranton Institutional Animal Care and Use Committee. Fertile chicken eggs were obtained from a commercial source (Charles River SPAFAS, Voluntown, CT) and incubated in a forced air incubator at 38 °C for 17 days. For each day of dissection, two to three chicks (depending on embryo viability) were removed from the eggs and immediately decapitated. Olfactory turbinates that were readily identifiable (Jung et al., 2005) were dissected, tissue from the individual embryos was combined and cut into small pieces using Vannas scissors, and cells were enzymatically dissociated using 0.5% porcine trypsin for 15 min. The tissue was pelleted by centrifugation for 5 min, resuspended in culture medium (Iscove's modified Dulbecco's medium + 10% fetal bovine serum + 1% penicillin/streptomycin), plated on 22 × 22 mm #1 coverslips in six-well plates (two plates per dissected embryo), and grown in a humidified 37 °C incubator at 5% CO_2. The day of dissection was designated day 0.

Odorant Mixtures

Odorants were mixed in culture medium (for cell treatments) or in Ringer's solution (for calcium imaging studies) by vigorous mixing and sonication; each odorant was mixed at 100 μM. Amyl acetate (AA) and phenethyl alcohol (PEA) were mixed individually, while the avian odor mix (Mix AV) contained AA, cineole, ethyl vanillin, eugenol, geraniol, limonene, and octanal; these odorants were selected based on previous publications showing that they triggered physiological and/or behavioral responses in chicks (see Jung et al., 2005; Gomez and Celii, 2008).

Odorant Treatments

For cell culture treatments, cells were allowed to grow for 1 day to allow the culture to establish. On the morning of day 2, cells were exposed to odorants by exchanging the culture media with media supplemented with the corresponding odorant mix; for controls, the culture media was exchanged with fresh media. Cells were exposed to odorants for 12 hr to prevent desensitization due to adaptation of the OSNs to prolonged odor exposure (Wang et al., 1993); at the end of the exposure period, the culture media would be replaced with fresh media. Cells were unexposed to odors for at least 12 hr prior to testing. On each day of testing, coverslips with cells were removed for immunocytochemistry or calcium imaging. A single trial consisted of a complete set of data from at least one coverslip from each day of testing (day 2 to day 6).

Immunocytochemistry and Antibody Characterization

Cells were fixed in 4% paraformaldehyde for 10 min and stored in phosphate-buffered saline (PBS) + sodium azide. Cells were washed (incubated in PBS for 15 min) thrice and incubated in blocking solution (PBS + 0.3% Triton X + 0.1% normal goat serum) for 30 min. Cells were washed thrice in PBS, then incubated overnight in primary antibodies as outlined in Table I. For controls, primary antibodies were substituted with non-immune serum. Cells were then washed thrice with PBS and incubated in FITC- or TRITC-conjugated secondary antibody (1:200, made in the appropriate species) for 1 hr. Cells were washed twice with PBS and twice with deionized water. Cells

TABLE I. Antibodies Used for the Study

Name	Immunogen	Manufacturer/cat #/species	Dilution
anti-β tubulin III (neuron-specific tubulin; NST)	Synthetic peptide corresponding to amino acids 436–450 of neuron-specific βIII tubulin	Sigma Chemical Co. #T8578; mouse monoclonal, AB 1841228	1:500
anti-OMP (olfactory marker protein)	Olfactory marker protein; wide species reactivity	Wako Chemicals #544-10001; goat polyclonal; AB 664696	1:200
anti-G_{olf} (anti-GNAL C-terminal region)	Guanine nucleotide-binding protein (G protein), alpha-activating activity polypeptide, olfactory type; wide species reactivity	Aviva Systems, # ARP54634; rabbit polyclonal; AB 2046013	1:500

Note: These antibodies were identical to the ones used in a previous publication from our laboratory characterizing cultured chick olfactory neurons (Gomez and Celii, 2008).

were stained with 4,6-diamino-2-phenylindole (DAPI) for 5 min to visualize nuclei (to facilitate cell counting), mounted with Vectashield (Vector Laboratories, Burlingame, CA), and viewed using a fluorescence microscope. Images of cells under transmitted and fluorescence illumination were photographed with a Nikon TE1000 epifluorescence microscope with × 40 or × 100 objectives using a digital camera attached to the microscope with the exposure set at 500 msec.

For each coverslip, five random fields of view were photographed for *post hoc* analysis. To eliminate bias, cell counts and visual determination of immunostaining were conducted by two of the researchers working together; images were retrieved and analyzed without knowledge of which experimental sample each one originated. Data were subsequently tabulated and analyzed as follows: the number of stained and unstained cells in each photo was determined. Individual cells or individual trials were averaged and validated with ANOVA. All subsequent analyses were conducted by comparing data from the odor-treated cells vs. the control in a pairwise fashion using a two-tailed Student *t*-test, $P = 0.05$, assuming unequal variances; this test provided the most straightforward and direct comparison, assuming that the randomly selected microscope fields (for immunostaining) or the subset of cells (for calcium imaging) were representative of the entire population of cells. All statistical comparisons were made using GraphPad (La Jolla, CA).

For NST staining, the proportion of NST-stained cells for odorant-treated vs. untreated cultures was compared separately at each time point (day 1 through 6) since the total number of cells increased over time. For olfactory marker protein (OMP) and G_{olf} staining, the proportion of immunopositive cells for AA- or PEA-treated cultures at day 4 and 5 were each compared separately vs. the control values at the same time point.

Calcium Imaging

Cells were prepared for imaging as follows: the culture medium in each well was replaced with warm culture medium supplemented with 1 μM fura-2 and 20 μg/ml Pluronic F-127 (Invitrogen, Carlsbad, CA) and incubated for at least 1 hr. Coverslips with fura-2–loaded cells were then situated in a recording chamber and continuously bathed with a warm Ringer's solution superfusion and secured to the stage of an inverted fluorescence microscope. OSNs were visually identified under 360-nm illumination to monitor viability and appropriate fura-2 loading. Stimulus solutions were applied by switching the superfusion for 30 sec; the chamber configuration allowed for a complete exchange of superfusion solutions within 0.5 sec while providing a consistent level of fluid over the cells. Cells were constantly superfused with Ringer's solution (145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1mM $MgCl_2$, 1 mM Na-pyruvate, 20 mM HEPES-Na). Cells were stimulated using high-potassium solution (HiK^+; Ringer's solution with 135 mM Na^+ substituted equimolarly with K^+) or Ringer's solution supplemented with odorants (above).

Intracellular calcium concentration ($[Ca^{2+}]_i$) in individual OSNs was quantified and analyzed as follows: cells under 200 × magnification were illuminated with a Xenon lamp filtered to 340- and 380-nm excitation wavelengths controlled by a filter wheel (Sutter Instruments). Emitted light was filtered at 510 nm and recorded by a cooled Hamamatsu Orca CCD camera (12-bit resolution, Hamamatsu Corp., Bridgewater, NJ). Ratiometric image acquisition and analysis was performed using IPLabs Software (BD Biosciences, Bethesda, MD). Under these conditions, cells remained viable for several minutes up to 1 hr. Data were analyzed as described previously (Jung et al., 2005; Yazinski and Gomez, 2014). Briefly, individual cells were visually identified and selected by drawing a region of interest (ROI) to restrict calcium measurements within the individual ROIs. Immediately prior to the application of a stimulus solution, the baseline $[Ca^{2+}]_i$ was noted. If a distinct and sustained change in $[Ca^{2+}]_i$ following stimulation (within 5–20 sec) and a return of $[Ca^{2+}]_i$ toward the original baseline following solution removal (within 50 sec) were noted, this counted as a response.

For each day of the study, we tested at least two coverslips of cells from each treatment (AA-exposed, PEA-exposed, and controls). Any trials that did not complete the entire stimulus protocol on both experimental days 4 and 5 were excluded from analysis. Odorant response rates for each stimulus for odorant-treated cells were compared pairwise against the corresponding response rate of the control cells separately for each odorant treatment.

RESULTS

Cell Culture

Dissociated cells grew readily in culture and attached to the substrate within 1 day. The cultures contained cell types that could be differentiated visually under light microscopy (Fig. 1). The majority ($> 95\%$) of the cells in culture were flat, triangular cells. Interspersed among these cells were round, phase-bright cells with distinct

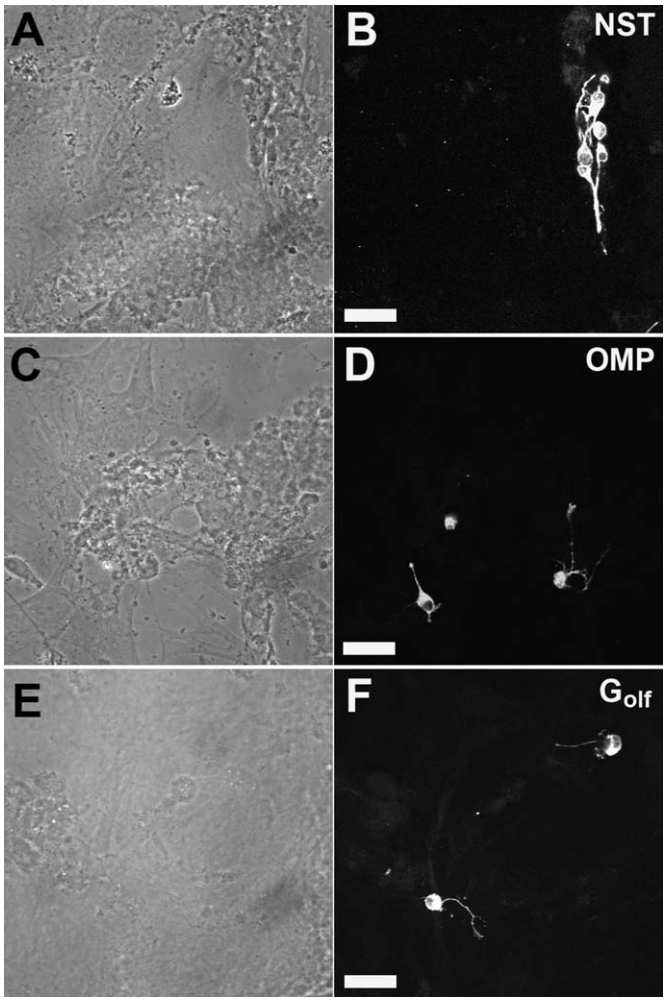

Fig. 1. Sample immunostaining images for NST (**A, B**), OMP (**C, D**), and G_{olf} (**E, F**) at day 5. Images on the left column show bright-field images, and images on the right column show the corresponding epifluorescence images. OSNs had a distinct morphology, with a rounded cell body and bipolar processes. Our preliminary studies have shown that these three marker molecules yield the most reliable and consistent results, and were thus the focus of our study.

processes emanating from opposite sides of the cell; these cells were identified as the neurons. This morphological identification of these neurons was used as the basis for identifying cells for calcium imaging studies (below).

Previous studies in our laboratory (Gomez and Celii, 2008) have used a subset of marker molecules (Table I) to stain for immature and mature OSNs. β-tubulin III (NST, Fig. 1B) stains immature and mature OSNs (Roskams et al., 1998). OMP (Fig. 1D) is a molecule known to be expressed only by mature olfactory neurons (Farbman and Margolis, 1980) and has thus been used as a canonical marker molecule, but it has recently been shown to have a modulatory role in odorant signaling (Buiakova et al., 1996; Dibattista and Reisert, 2016). G_{olf} (Fig. 1F) is an element involved in cAMP-mediated odor signal transduction (Jones and Reed, 1989) that is known to be involved in the bird olfactory system (Jung et al., 2005). While preliminary studies in our laboratory have shown that cultured avian OSNs stain positively for other known marker molecules (such as neuron-specific enolase, neural cell adhesion molecule, neurofilament 68, nerve growth factor receptor p75, glial fibrillary acidic protein, and adenylyl cyclase III), we used our three selected marker molecules because of their high specificity, consistency, and reliability in all of our studies.

Time Course of Neuronal Differentiation

Studies using other olfactory systems *in vitro* have shown that OSNs differentiate from non-neuronal cells and take 5 to 6 days to attain maturity (Yazinski and Gomez, 2014). We first attempted to determine the time course of OSN growth in the chick olfactory system by measuring NST expression. Cultures were established and grown in six-well plates, and cells were either treated with the odorant Mix AV or with no odor (controls) at day 2. We opted to use the odorant mix to maximize the potential effect of odorant exposure on OSN differentiation. On each day of the trial, a subset of coverslips was fixed and immunostained for NST; the total number of NST + cells was counted and expressed as a proportion of the total number of cells (determined using DAPI staining). We obtained data from eight separate trials, and measured data from at least 100 cells for each time point (see legend for Fig. 2).

Figure 2 shows the time course of emergence of NST + cells in both untreated and odorant-treated cells. The relative number of neurons increased steadily through day 4; after this point, the growth of the non-neuronal cells tended to outpace the rate of neuron differentiation, resulting in a plateau. This timeline of neuronal differentiation was similar to that seen in other olfactory culture systems (Yazinski and Gomez, 2014). The proportion of NST + cells in untreated vs. odorant-treated cultures at each time point was compared using a Student *t*-test. Odorant treatment did not result in upregulation of nerve cell growth: for each time point, there was no significant difference between the proportion of NST + cells in untreated vs. odorant-treated cells ($t_{10} = 1.739$; $P = 0.26$). Based on these results, we focused our assays on days 4 and 5.

Odorant Exposure Upregulates Expression of Marker Molecules in OSNs

We then focused our study using single odorant exposures on our cultures to determine whether OSN differentiation could be influenced by odorant stimulation. We therefore exposed cultures to either AA, PEA, or no odor (control) for 5 days; at days 4 and 5, coverslips were fixed and immunostained for OMP or G_{olf}. Because OSNs that express these marker molecules possessed a distinct spindle-like or bipolar morphology (Yazinski and Gomez, 2014; see also Fig. 1), we visually identified cells that displayed this morphology as OSNs and determined the proportion of these that were immunoreactive for

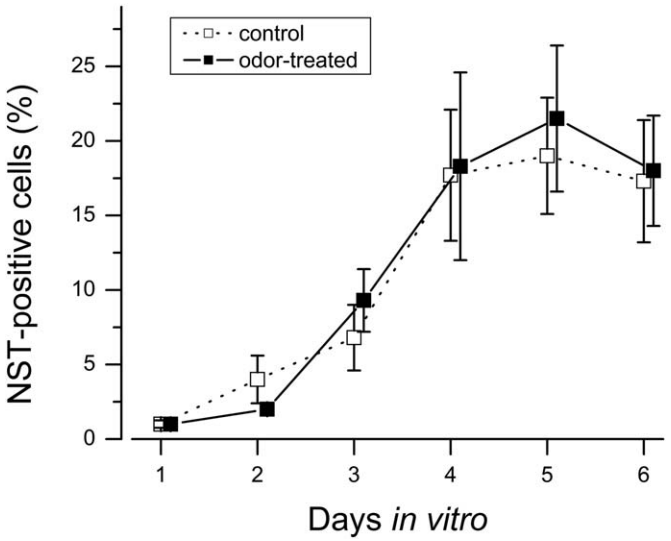

Fig. 2. The maturation timeline of neuronal differentiation was determined using NST immunoreactivity. The total number of NST + cells was counted and expressed as a proportion of total number of cells (determined using DAPI-stained nuclei). The timeline of maturation was similar to that seen in other *in vitro* studies on the development of olfactory function (Yazinski and Gomez, 2014). Note that odorant treatment did not result in an increase in the overall number of neurons. For this and all subsequent figures, data and error bars are shown as mean ± SD. The total number of cells for days 1 through 6 was increased over time; controls: 130, 220, 460, 840, 1240, and 1370; odor-treated: 123, 198, 500, 898, 1136, and 1445.

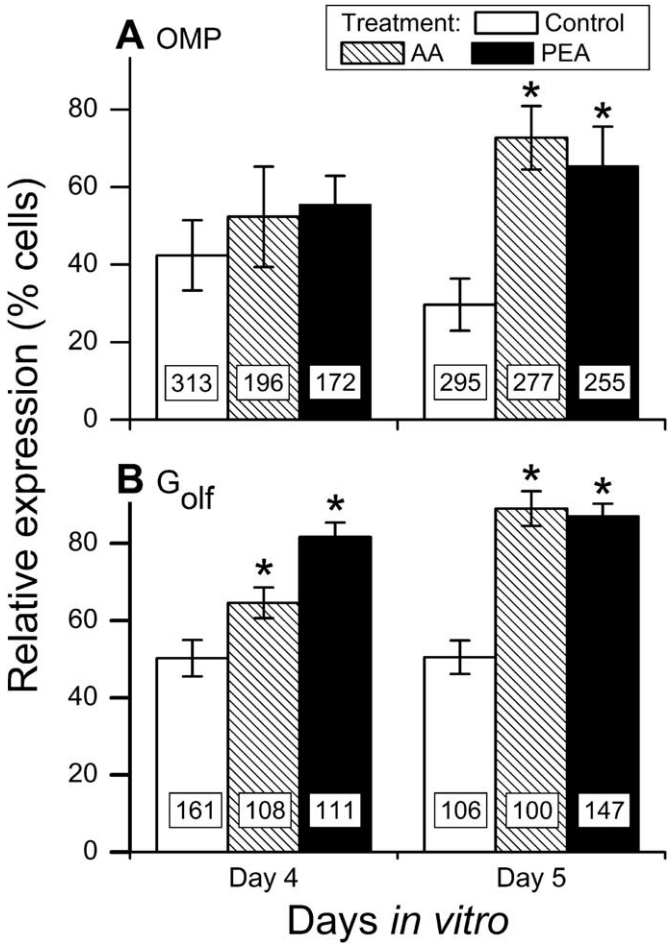

Fig. 3. Odorant exposure resulted in increased expression of marker molecules associated with olfactory maturation. Cells grown *in vitro* were treated with AA, PEA, or no odorant (control) as described in Materials and Methods, and immunostained for OMP (a marker molecule expressed by mature olfactory neurons) (**A**) or G_{olf} (a molecule associated with odorant signal transduction) (**B**) after 4 or 5 days *in vitro*. Neuron-like cells were visually identified by their distinct morphology, and their immunostaining profile was determined. After 4 days of odorant treatment, the number of cells that were G_{olf} + was significantly higher in odorant-treated cells, while the number of OMP + cells was unaffected by odorant treatment. Odorant treatment for 5 days resulted in a significant increase in the number of both OMP + and G_{olf} + cells. Asterisks denote a significant difference vs. control ($P < 0.05$). The total numbers of cells (stained and unstained) appear at the bottom of each bar.

OMP (Fig. 3A) or G_{olf} (Fig 3B). Five trials were conducted for each odorant treatment.

At day 4, about 40% to 50% of the OSN-like cells expressed OMP; odorant treatment resulted in a slight but not significant increase in OMP + cells (AA-treated: $t_8 = 1.40$; $P = 0.19$; PEA-treated: $t_8 = 2.10$; $P = 0.06$). In contrast, G_{olf} was expressed in 50% of the untreated cells and in a significantly higher proportion of the odorant-treated cells (AA-treated: $t_8 = 2.79$; $P = 0.02$; PEA-treated: $t_8 = 8.22$; $P < 0.0001$). The difference in the time course of OMP and G_{olf} expression has been seen previously (Yazinski and Gomez, 2014) and suggests that the genes that regulate expression of primary odorant signaling are modulated differently from those that regulate signal modulation. At day 5, odorant treatment resulted in significant increases in both OMP (AA-treated: $t_8 = 9.85$; $P < 0.0001$; PEA-treated: $t_8 = 6.86$; $P = 0.0001$) and G_{olf} (AA-treated: $t_8 = 13.80$; $P < 0.0001$; PEA-treated: $t_8 = 15.00$; $P < 0.001$) expression.

Odorant Exposure Elicits Changes in Responses to Odorant Stimulation in OSNs

We used fura-2 ratiometric calcium imaging to measure possible changes in OSN odorant sensitivity resulting from odorant exposure. Figure 4 shows a sample of fura-2–loaded OSNs (Fig. 4A) and the corresponding stimulus-elicited calcium changes in two of the cells (Fig. 4B). OSNs were readily identifiable based on their size, morphology, and fura-2 loading intensity (the non-OSNs were typically dimmer). Ratiometric imaging allowed us to ensure that OSNs remained viable throughout the experiment. We measured cell responses from at least 100 cells per treatment; only cells that completed our entire stimulus protocol were included. We tested each stimulus at least twice to ensure replicability of the cell responses. Our stimulus battery consisted of high K^+ (which elicits $[Ca^{2+}]_i$ changes by activating voltage-gated Ca^{2+} channels and is a good indicator of functional OSNs, Restrepo

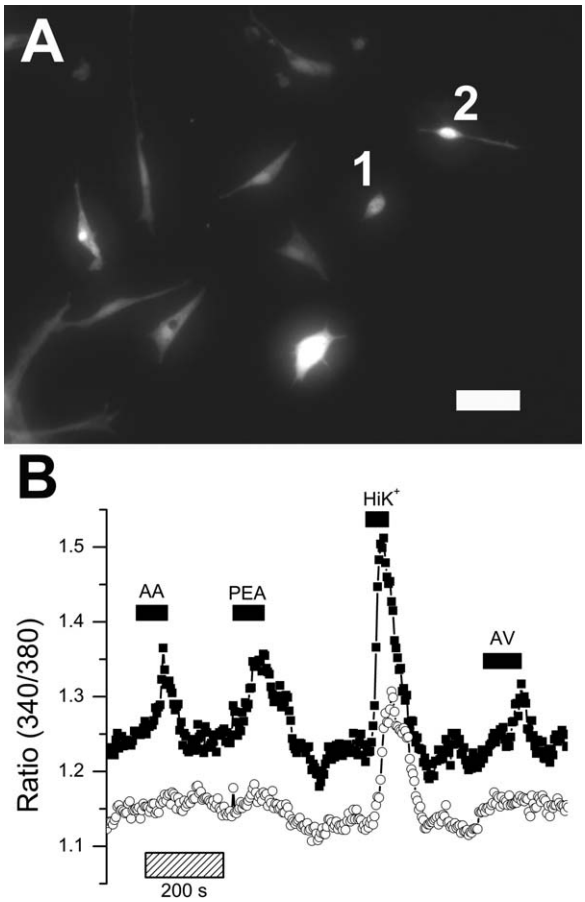

Fig. 4. **A**: A microscope field showing fura-2–loaded cells. Scale bar = 50 μm. Note that the OSNs were visually identifiable; neurons were approximately 20 μm in diameter, and cells that were excessively large or that did not have a characteristic OSN appearance were excluded from the study. **B**: The data trace for the cell in panel A marked "1" appears as filled squares, while the data trace for the cell in panel A marked "2" appears as open circles. The data traces show the single cells' responses to the application of stimulus solutions (solid bars). These cells responded to K^+-induced depolarization with an increase in $[Ca^{2+}]_i$ (HiK^+), a typical characteristic of neurons *in vitro*. Cell 1 also responded to AA, Mix AV, and PEA with increases in $[Ca^{2+}]_i$, while cell 2 only responded to HiK^+. The hatched bar indicates a time scale of 200 sec.

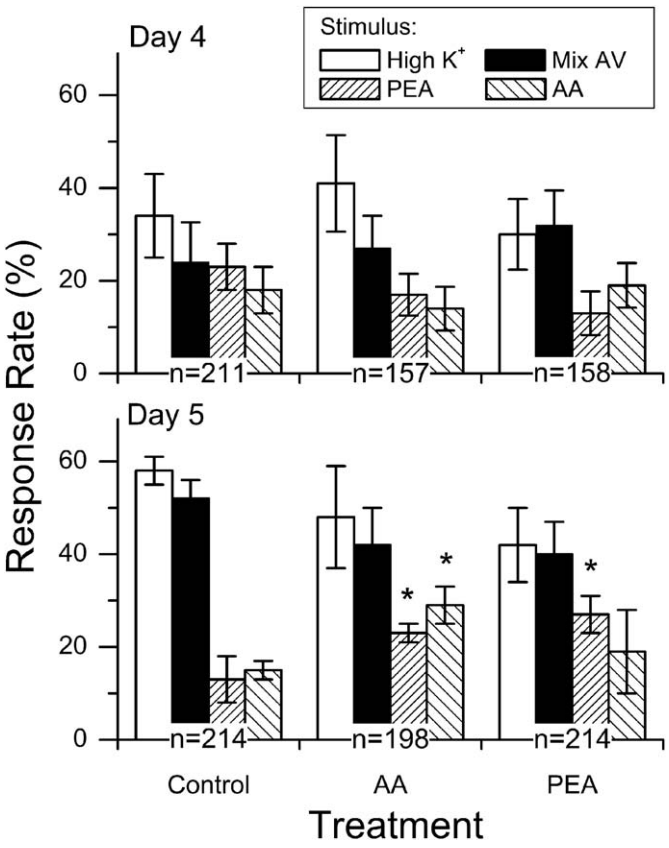

Fig. 5. Odorant treatment resulted in a significant increase in response rates in cultured olfactory cells after 5 days *in vitro*. Cells were treated with AA, PEA, or no odorant (control) as described in Materials and Methods. **A**: After 4 days *in vitro*, untreated cells developed functional neurons that were responsive to both high K^+ (HiK^+) and to odorant stimulation (Mix AV, AA, or PEA). There was no significant difference in the response rates of odorant-treated and untreated cells. **B**: After 5 days *in vitro*, the number of functional neurons (responsive to HiK^+ and to the odorant Mix AV) in both odorant-treated and untreated cells markedly increased. Interestingly, odorant treatment resulted in a significant increase in the proportion of cells sensitive to the individual odorants with which the cells were treated. There was no significant difference between the response rates in the AA- vs. PEA-treated cells. Asterisks denote a significant difference of the response rate to the specific stimulus in the odorant-treated vs. control untreated cells ($P < 0.05$). The total numbers of cells measured across all trials appear on the *x* axis below the bars.

et al., 1993, Jung et al., 2005); Mix AV, AA, or PEA (note that Mix AV contains AA but not PEA).

We obtained data from 10 complete trials of the odorant treatments. Stimulus-elicited changes in $[Ca^{2+}]_i$ were readily determined as described in Materials and Methods. In this example (Fig 4B), both OSNs responded to high K^+ depolarization, and only one cell responded to odorant stimulation. The magnitude and frequency of the $[Ca^{2+}]_i$ responses seen in our study were similar to those seen in our previously published work (Gomez and Celii, 2008; Yazinski and Gomez, 2014).

Figure 5 shows a summary of the odorant response rates for treated and untreated OSNs. At day 4 (Fig. 5A), about 40% of the OSNs were responsive to any type of stimulation. Odor treatment did not result in a significant difference in odorant response rates compared with control ($t_{18} = 0.22$, $P = 0.82$). By day 5 (Fig. 5B), odorant treatment resulted in a specific change in odorant sensitivity to only the odors used for exposure. The response rates to high K^+ and to the odor Mix AV were not significantly different between all treatment groups. Interestingly, treatment with AA resulted in a significant increase in response rates to either AA ($t_{18} = 14.15$; $P < 0.0001$) or PEA ($t_{18} = 8.58$, $P < 0.001$), while PEA treatment resulted in a significant increase in response rates to PEA ($t_{18} = 10.98$, $P < 0.001$) but not to AA ($t_{18} = 1.768$,

$P = 0.09$). These results suggest that the odorant treatments did not upregulate overall neuronal differentiation but, rather, modulated the process of OSN differentiation, resulting in specific and targeted changes in elements of odorant signal processing.

DISCUSSION

The olfactory system has long been used as a model for nerve cell differentiation because of its ability to regenerate throughout the adult life span (Graziadei and Monti-Graziadei, 1978). The induction of differentiation of neuronal phenotypes from non-neuronal basal cells is regulated by the autocrine/paracrine secretion of neurotrophins (Ensoli et al., 1998) by olfactory ensheathing cells (Boruch et al., 2001; Woodhall et al., 2001; Lipson et al., 2003); thus, olfactory ensheathing cells have been used as inducers of nerve cell differentiation and repair (Ramón-Cueto et al., 2000; Lu et al., 2002). However, these studies have not determined the mechanisms that guide the differentiation of functional attributes of the neurons. Our data suggest that the functional properties of neurons may arise from endogenous self-regulatory mechanisms related to olfactory function.

The olfactory system presents a unique challenge because individual OSNs have different sensitivity to odorant molecules (for review, see Hildebrand and Shepherd, 1997): studies on the spectral sensitivity of individual OSNs show that each OSN senses a subset of possible odorant molecules (Wetzel et al., 1999; Katada et al., 2005), and thus the perception of complex odorant mixes is encoded by a pattern of activation across the population of the different OSNs in the entire olfactory system (for review, see Buck, 1996; Laurent, 2002; Leon and Johnson, 2003). It is known that each OSN achieves this by expressing one type of olfactory receptor protein (Buck, 2004) and projects an axon to unique glomeruli in the olfactory bulb (Vassar et al., 1993, 1994; Ressler et al., 1994). It is unclear how the expression of this receptor protein is modulated. It is possible that the OSN randomly expresses one receptor type and that this information is used to guide the axons to the appropriate glomerulus (Feinstein et al., 2004; Strotmann et al., 2004), or that the projection of the axon to a glomerulus in the brain guides the OSN to express the appropriate receptor (Serizawa et al., 2003). Data from our study suggest the former is the most likely scenario. In our study, we separated the peripheral olfactory system from the connections to the central nervous system using an *in vitro* technique; our data suggest that the odorant exposure can modulate OSN differentiation in the absence of influence from the olfactory bulb.

In our study, we showed that odorants elicit a change in the population distribution of OSNs (Fig. 5). The mechanism behind odorant-mediated cell differentiation was not determined at this point; however, since odorant stimulation is known to elicit changes in $[Ca^{2+}]_i$ (Fig. 4) and the activation of downstream elements such as protein kinases (Gomez et al., 2000a) and calcium calmodulin kinases (Wei et al., 1998; Leinders-Zufall et al., 1999), these would represent likely mechanisms for regulation of gene expression (Dittman et al., 1997; Watt et al., 2004). Such genes could either modulate the expression of the olfactory receptor proteins themselves or regulate cell division that would cause an upregulation of division in the subpopulation of OSNs that were excited by the odorants; both scenarios could likely lead to the changes in population distribution seen in our study (Fig. 5). These possibilities are candidates for future studies.

One interesting observation was the differential effect of AA vs. PEA treatment, wherein AA resulted in an increased proportion of both AA- and PEA-sensitive cells, while PEA resulted only in an increased proportion of PEA-sensitive cells (Fig. 5B). This suggests that the effects of odorant treatment observed in our study are most likely mediated through the odorant receptor–induced activity in the differentiating OSNs. If the effects were mediated via a non–receptor-mediated mechanism, it would be expected that AA and PEA treatment would result in identical proportions of cells expressing molecular markers (Fig. 3) or odorant sensitivity (Fig. 5). In studies on individual odorant receptor sensitivity in mammals, it has been shown that individual receptor proteins have broad spectral sensitivity, and individual odorant molecules could potentially bind to multiple receptor types (Araneda et al., 2000; Bozza et al., 2002). The breadth of receptors expressed in the bird olfactory system as well as the sensitivity of these receptors have yet to be determined (Steiger et al., 2008). It is possible that AA binds to the receptor that binds to PEA, but not vice versa, and that AA treatment thus resulted in a concomitant increase in the sensitivity to PEA. These possibilities are potential avenues for exploration in future studies.

One important question that our study aimed to address was the precise origin of the modulation of the neuronal changes elicited by odor imprinting. In previous imprinting studies, odorant stimulation has been shown to affect cell viability and olfactory neuron turnover (Watt et al., 2004) or the cellular machinery associated with odor signal transduction in peripheral neurons (Dittman et al., 1997). The development of the olfactory system is known to be modulated by the olfactory bulb (Gong and Shipley, 1995), and removal of the olfactory bulb typically results in an increase in apoptosis, turnover, and regeneration in the peripheral olfactory system (Graziadei et al., 1979; Carr and Farbman, 1992; Schwob, 2002). In bulbectomized animals, odorant stimulation has been shown to promote survival via Bcl-2–mediated inhibition of apoptosis (Watt et al., 2004), suggesting the capacity of the peripheral system to self-modulate regeneration without the influence of the central nervous system. The results of our study suggest that this capacity for self-modulation includes regulating cellular machinery associated with odorant signaling.

While dissociated and cultured OSNs are not exactly identical to those found in live animals, they share many characteristics found in their *in vivo* counterparts, such as odorant sensitivity (Murrell and Hunter, 1999; Katada et al., 2003; Gomez and Celii, 2008), olfactory receptor

protein expression (Borgmann-Winter et al., 2009), and odor signal modulation (Gomez et al., 2000b). Therefore, our employment of the *in vitro* system was an ideal way for us to isolate phenomena that would likely be occurring exclusively at the periphery. In our approach, it was essential to keep a mixed culture without isolating neurons for a number of reasons. First, pilot studies in our laboratory have shown that purified olfactory neuron preparations do not grow or differentiate in culture (personal observations). Second, the glial-like cells have been shown to be critical in promoting neuron differentiation (Ensoli et al., 1998; Boruch et al., 2001; Lipson et al., 2003). Third, the cells that do not possess neuronal marker proteins may still demonstrate odorant sensitivity (Yazinski and Gomez, 2014), suggesting that these are OSNs in the early process of development; these cells may represent the population of cells that would be modulated by odorant exposure and subsequently develop into the OSNs that constitute the odorant-sensitive cells seen in our study (Fig. 5).

The bird olfactory system possesses unique features that were advantageous for our study. While mammalian OSNs may potentially express up to a thousand different types of odorant receptors (Buck, 1996), it is estimated that birds express a reduced complement of receptors (Leibovici et al., 1996; Steiger et al., 2008, 2009), enabling us to use single odorants for our neuron induction studies. The OSNs of birds also possess functional properties that are designed to process odorant information at the periphery (Jung et al., 2005), suggesting that OSNs in these species may have evolved mechanisms for self-regulation and modulation. Birds are also unique because they are endothermic animals that are exposed to the environment during development; thus, they have attributes that are similar to those found in mammals, but are subjected to selective pressures similar to those found in other ectothermic species. They therefore present a unique opportunity to study the modulation of neuronal differentiation by environmental factors. Thus, our study has important implications for the study of the development of the nervous system and how external factors can modulate the emergence of intrinsic properties of neuronal ensembles.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

ROLE OF AUTHORS

GO and CM contributed equally to the work and should be considered as co-primary authors. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: GO, CM, GG. Acquisition of data: GO, CM, GG. Drafting of the manuscript: GO, CM. Final authorship of the manuscript: GG. Critical revision of the manuscript for important intellectual content: GO, CM, GG. Statistical analysis: GG. Obtained funding: GG. Administrative, technical, and material support: GG. Study supervision: GG.

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