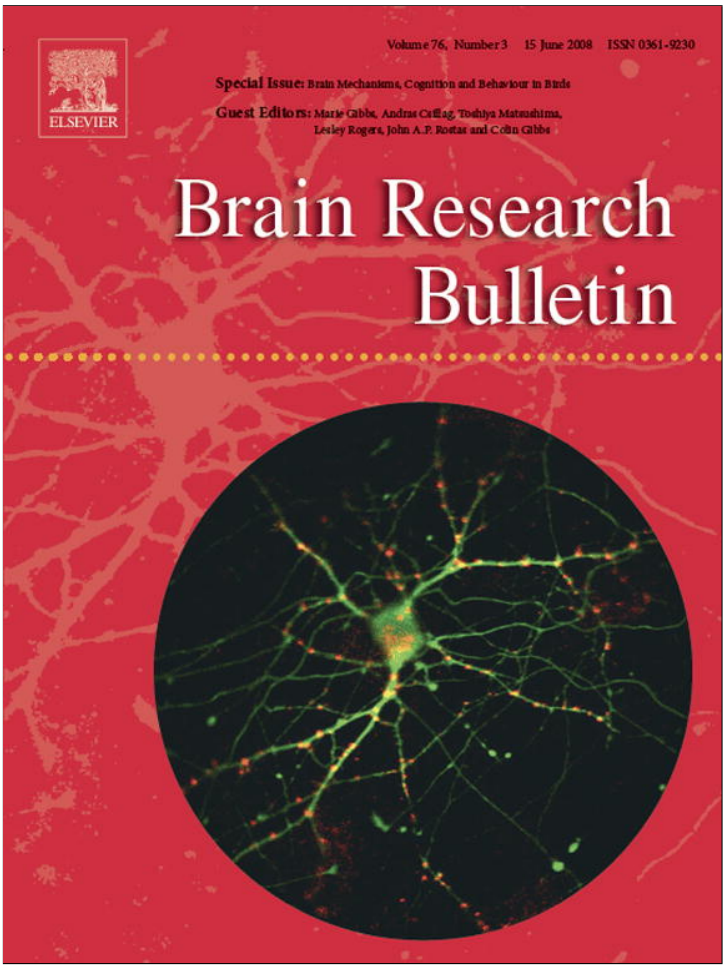

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Review

The peripheral olfactory system of the domestic chicken: Physiology and development

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ABSTRACT

Olfaction is a ubiquitous sensory system found in all terrestrial vertebrates. Birds use olfaction for several important activities such as feeding and mating; thus, understanding bird biology would also require the systematic study olfaction. In addition, the olfactory system has several unique features that are useful for the study of nervous system function and development, including a large multigene family for olfactory receptor expression, peripheral neurons that regenerate, and a complex system for sensory innervation of the olfactory bulb. We focused on physiological, anatomical and behavioral approaches to study the chick olfactory neurons and the olfactory bulb. Chick olfactory neurons displayed some properties similar to those found in mature neurons of other vertebrate species, and other properties that were unique. Since information from these neurons is initially processed in the olfactory bulb, we also conducted preliminary studies on the developmental timeline of this structure and showed that glomerular structures are organized *in ovo* during a critical time period, during which embryonic chicks can form behavioral associations with odorants introduced *in ovo*. Lastly, we have shown that chick olfactory neurons can grow and mature *in vitro*, allowing their use in cell culture studies. These results collectively demonstrate some of the features of the olfactory system that are common to all vertebrates, and some that are unique to birds. These highlight the potential for the use of the physiology and development of the olfactory system as a model system for avian brain neurobiology.

Contents

1. Introduction: why study the bird olfactory system?

The ability to detect chemical stimuli is a primitive sensory capability that is found in nearly every animal species. In terrestrial animals, the detection and identification of volatile chemicals is mediated by the olfactory system. This system had been the subject of intense study over the last several years due to its numerous complex features: a large gene family that encodes up to 1000 different types of odorant receptor proteins (in the mouse [15]), an intricate system of innervation and axonal targeting from peripheral neurons into the central nervous system [7,75], a complex mechanism for information coding and processing [16], and a highly dynamic neuronal population that regenerates throughout the adult lifespan [21,31]. The olfactory system appears to possess several structural and functional features at the periphery and in the olfactory bulb

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that are conserved across a wide range of species [50], making it an ideal system for the study of common features of neuronal function. In addition, the olfactory system can provide intact neurons for study without severely disrupting animal viability or health [45,46], and can serve as a useful model for the study of the principles governing the growth and function of nervous systems in general [45,46].

There is strong evidence that birds use olfaction to detect important cues for feeding and social interactions [22,25,33,44,83–86,105,106,125,126]. An understanding of the principles of odorant signaling in vertebrates necessitates the study of major taxonomic groups to uncover common design principles of sensory systems. Birds are particularly interesting because they possess a unique physiological and evolutionary position: they are endothermic (physiologically similar to mammals) but are cladistically more closely related to reptiles [91]. Birds may therefore provide unique insights into the influence of physiological/ecological versus evolutionary constraints on nervous system design. An understanding of the principles of odorant signaling in vertebrates necessitates the study of major taxonomic groups to uncover common design principles of sensory systems.

2. The peripheral olfactory system of birds and other vertebrates

The translation of odor molecules as an external stimulus into neuronal activation in the olfactory system occurs when odorants enter the nasal cavity (either through the normal respiratory cycle, or through voluntary sampling or "sniffing") and encounter the olfactory sensory neurons embedded in the olfactory epithelium. When these neurons are activated, their action potential outputs are transmitted to the olfactory bulb (for review, see [119]), which is a structure on the underside of the frontal lobe of the brain that receives inputs from the olfactory neurons. These inputs are processed, and the resulting neural patterns are projected to the higher regions of the cortex concerned with decoding the sense of smell.

In all vertebrates, the olfactory sensory neurons (OSNs) are embedded in the mucus-covered nasal epithelium lining the nasal cavity [76]. The avian olfactory epithelium is situated on a single spiral turbinate that occupies the nasal cavity, and on the apposed septum [5]. As in other vertebrates, the chick OSNs have a distinct morphology: they are generally goblet-shaped cells, with sensory cilia emanating from a terminal dendritic knob [13,68,73]. This characteristic morphology appears to be remarkably conserved across a variety of vertebrate species [111]. The OSN cell bodies are embedded in the olfactory epithelium, ciliated dendrites extend into the nasal cavity, and olfactory axons directly innervate glomeruli in the olfactory bulb [5,76]. The chick olfactory epithelium also has microvillar receptor neurons morphologically similar to the ciliated cells [13,68]. Although it is not known whether ciliated and microvillar cells are functionally distinct, it has been shown in fish that these two cell types have distinct functions and anatomical projections [47,77]. It is not certain whether this is the case in chicks as well.

Volatile odorant molecules that enter the nasal cavity diffuse through the mucus and bind to seven-transmembrane domain G-protein coupled receptors situated on the OSN dendrites [94]. While other vertebrates express hundreds of different types of receptors (mouse: about 1000 [15]; human, about 350 [66]), the chick has thus far been shown to express a limited complement of about 15 different odorant receptors [81,82], although it is estimated that the gene family likely encodes about 100 functional variants [2,60].

The binding of odorant molecules to the receptors initiates the odorant signal transduction cascade, beginning with the activation of G-proteins that subsequently activate second messenger producing enzymes. This leads to the formation of second messenger molecules that can directly gate cation channels and cause the OSN to depolarize (for review [111]) and generate action potentials. The best-characterized pathway for olfactory signal transduction involves receptors linked to G-proteins (G_{olf}) that activate adenylyl cyclase; this leads to the formation of cyclic AMP (cAMP; [94]), which directly gates cyclic nucleotide-gated channels [57,79,131]. A related pathway involves activation of guanylyl cyclase to produce cyclic GMP (cGMP [12]), which also activates cation channels (for review see [52]). An alternate pathway proposed to mediate olfaction for certain odorants involves odors binding to receptors that are linked to a different set of G-proteins (G_q, G_o [30]) that activate phospholipase C (PLC) leading to the formation of inositol-1,4,5-trisphosphate (IP_3), which has been shown to activate plasma membrane cation channels [29,64], or to activate an inhibitory signal that modulates cyclic nucleotide signaling [118]. While some studies suggest that cAMP is the sole second messenger molecule in mammalian [8,14,21,36] and amphibian [65,79,121] OSNs, studies on lobsters [1,29], catfish [96,97], mudpuppies [26], rats [64,88] and mice [3,63] show that OSNs from these species appear to utilize the alternate and/or additional second messenger signaling systems.

The resultant electrical activity generated by the OSNs has traditionally been assessed using extracellular recording techniques. Odor stimulation elicits large-scale DC currents across the surface of the olfactory epithelium that can be measured using an electro-olfactogram (EOG [114]). EOG recordings from the olfactory epithelium have been used to measure numerous parameters, such as spectral sensitivity, threshold, and spatial distribution of olfactory receptor expression across the entire epithelium [114]. In chicks, this recording technique has been used to measure the development of odor sensitivity of OSNs during development [58]. Action potentials from the olfactory nerve have been recorded using extracellular electrodes [54,55,123]; these action potentials monotonically increase in frequency and duration with increasing stimulus intensity and length. The electrophysiological properties of bird OSNs thus appear to be similar to those seen in other vertebrate species [27,123].

3. Calcium fluxes in olfactory sensory neurons

The activation of the signal transduction cascades (described above) and gating of second messenger-regulated cation channels in the ciliary membranes results in a depolarizing influx of calcium [80,97] and opening of voltage-gated Ca^{2+} channels on the cell soma [74,100]. The resulting increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a net result of the activation of multiple mechanisms [111]. While odorant-elicited voltage signals typically last for less that a second, the resulting change in lasts for several seconds to minutes [56,61]. The increase in $[Ca^{2+}]_i$ influences a number of cellular events such as signal termination or adaptation since many molecules involved in odorant signaling are sensitive to $[Ca^{2+}]_i$ [10,56,80].

Measurement of average cellular $[Ca^{2+}]_i$ changes are commonly used and widely accepted as a metric for an OSNs functional capability and for the study of odor signaling mechanisms [87,127]. These experiments typically employ ratiometric $[Ca^{2+}]_i$ measurements of the entire cell without resolving ciliary $[Ca^{2+}]_i$. Ratio measurements of ciliary $[Ca^{2+}]_i$ (which regulates $[Ca^{2+}]_i$ independently from the soma [61]) have not yet been conducted, although relative changes in $[Ca^{2+}]_i$ have been measured in the cilia of the salamander using confocal microscopy and fluo-3 [61]. These studies of odorant-elicited ciliary $[Ca^{2+}]_i$ regulation have shown that, although there is a difference in time-course between ciliary and

dendritic $[Ca^{2+}]_i$, increases in dendritic $[Ca^{2+}]_i$ are causally related to increases in ciliary $[Ca^{2+}]_i$ [61]. Thus, results discussed below focus on $[Ca^{2+}]_i$ regulation in the entire cell.

Odorant elicited $[Ca^{2+}]_i$ changes in OSNs exhibit characteristics common to most species. OSNs generally respond to odorants with increases (100% of odor responses in rat OSNs [39,122]), or decreases in $[Ca^{2+}]_i$ (about 1/3 of odor responses in OSNs from humans [39,93], or cats [38]). With a few exceptions [38,108] $[Ca^{2+}]_i$ increases generally do not occur when odorants are applied in the absence of extracellular Ca^{2+}, indicating that $[Ca^{2+}]_i$ increases are mediated by an influx of extracellular Ca^{2+} through second messenger-gated channels or voltage-sensitive Ca^{2+} channels [98,100]. To characterize elements of odor signal transduction, studies are typically conducted by determining how odorant-elicited $[Ca^{2+}]_i$ changes are reversibly inhibited by biochemical inhibitors of second messenger molecules and their associated subcellular components. Using this approach in vertebrates OSNs, studies have shown conflicting evidence for the involvement of the two signal transduction cascades: while it is widely accepted that cAMP is operational in all olfactory systems (for review, see [111]), the involvement of IP_3 in odor signal transduction remains controversial [14,21,121]. In rat biochemical preparations, specific odorants appear to stimulate pharmacologically distinct signaling pathways, related to the differential production of the two second messengers [11]. This pharmacological distinction is evident in odorant-elicited $[Ca^{2+}]_i$ signaling in mammalian OSNs: calcium imaging studies on rat [98,122], cat [38] and human [93,99] OSNs have shown that responses to a subset of the odorant mix are selectively and reversibly inhibited by blockers of the cyclic nucleotide-mediated signaling, and responses to a different subset of the odorant mix are selectively and reversibly inhibited by blockers of phospholipase C, indicating that different odorants selectively stimulate either cAMP- or IP_3-mediated transduction pathways [111]. In rat OSNs, it has been shown that IP_3 modulates cAMP-mediated signaling via inhibitory pathways [118]. Other studies based on mouse genetic manipulations show that ablation of cAMP signaling elements (such as cyclic nucleotide gated channels or G_{olf}) results in complete anosmia, suggesting that IP_3 is not involved in any odorant-mediated signaling pathways [8,14,36]. Mudpuppy OSNs appear to use both of these signaling pathways for odor signal transduction [26]. It is uncertain whether disparities in these results are due to methodological or species differences.

In birds, studies in our laboratory [51] have shown that newborn chick OSNs also respond to odorants with extracellular calcium-dependent changes in $[Ca^{2+}]_i$ (Fig. 1). For these studies, OSNs were isolated from the newborn chick olfactory epithelium, loaded with calcium-sensitive fluorescent dyes, and tested with odorant mixtures. The odor stimulus mixture ("avian mix") consisted of odorants that were previously shown to be detectable by birds: amyl acetate, cineole, ethyl vanillin, eugenol, geraniol, limonene, octanal, and octanol (each at 100 μM). Cell responses were measured using calcium imaging techniques [101]. Results from the study showed that chick OSNs respond to odorants (mixtures or single compounds) with increases or decreases in $[Ca^{2+}]_i$ that are selectively and reversibly inhibited pharmacological inhibitors of cAMP or IP_3 signaling. Table 1 shows a summary of the $[Ca^{2+}]_i$ signaling properties of chick OSNs in relation to those found in other terrestrial vertebrates. Many features of odor and/or calcium signaling appear to be conserved among the species studied, suggesting that these mechanisms are remarkably effective across different the habitats and lifestyles of the representative species [50]. It is also possible that the olfactory system is not under stringent

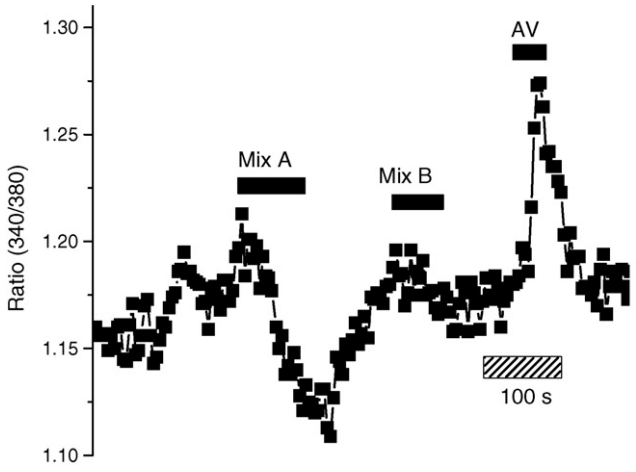

Fig. 1. Calcium responses to odor mixtures from acutely isolated chick OSNs. Higher values on the *Y*-axis indicate higher $[Ca^{2+}]_i$. Solid bars indicate the application of a stimulus, while the hatched scale bar indicates 100 s. This cell responded to a mix of seven different odorants (Mix A: citralva, citronellal, eugenol, geraniol, hedione, menthone, and phenylethylalcohol) with a decrease in $[Ca^{2+}]_i$ and to the a mixture of odorants known to be detectable by chicks (avian mix, AV) with an increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ level returned to baseline upon removal of the stimulus. This cell did not respond to a different set of odorants (Mix B: ethylvanillin, isovaleric acid, lyral, lilial, phenylethylamine, triethylamine). It is most likely that each response was caused by one of the odorants in the mixture. Odorant-elicited decreases in $[Ca^{2+}]_i$ occur less frequently than increases in $[Ca^{2+}]_i$. For most species, it is unusual for single OSNs to respond to different odorants with $[Ca^{2+}]_i$ changes of differing direction.

Table 1
A comparison of properties of odorant-elicited $[Ca^{2+}]_i$ changes in OSNs from a variety of vertebrate groups, measured using similar techniques

Calcium signaling feature	Chick [51]	Human[a] [39,93,99]	Rodent [39,98,121]	Cat [38]	Amphibian [26,108,121]
Respond with increases or decreases in $[Ca^{2+}]_i$	Increases or decreases, sometimes in the same cell	Either increases OR decreases; never both in the same cell	Increases only	Either increases or decreases only	Mostly increases; decreases are infrequent
Main source of $[Ca^{2+}]_i$ increase	Ca^{2+} influx	Ca^{2+} influx	Ca^{2+} influx	Ca^{2+} influx or Ca^{2+} stores	Ca^{2+} influx or Ca^{2+} stores
Putative second messengers used[b]	cAMP, IP_3	cAMP, IP_3	cAMP (all species), IP_3 (in rat, inhibitory role only)	cAMP, IP_3	cAMP (all species), IP_3 (in mudpuppy)
Other information		Evidence for pharmacological separation between cAMP and IP_3	Evidence for cross-talk between cAMP and IP_3 signaling pathways		

For these studies, OSNs were acutely isolated from the tissue, loaded with a calcium-sensitive fluorescent dye (usually fura-2), and tested with odorants. For pharmacological assays of second messenger involvement, commercially available biochemical activators and/or inhibitors for specific cellular components were co-applied with the odorants; reversible changes in stimulus-induced $[Ca^{2+}]_i$ changes were indicative of the components' involvement in cell signal transduction. Note that some features of $[Ca^{2+}]_i$ are remarkably conserved, but each species tested exhibits unique features.

[a] Based on studies on healthy adults only.

[b] Based on pharmacological and/or biochemical assays.

evolutionary selective pressure, allowing it to adapt as-is to other behavioral and ecological constraints imposed by the organism as a whole.

However, as is the case with other vertebrate species, the chick olfactory system has some unique features (Table 1). One interesting feature is the complex response pattern shown in Fig. 1: individual cells that would occasionally respond to one odorant with an increase in $[Ca^{2+}]_i$ and to another odorant with a decrease in $[Ca^{2+}]_i$. This pattern of response has not been observed in the other species tested (Table 1). This would imply that these OSNs are processing complex odor mixture information at the periphery, prior to propagating their signals to the olfactory bulb [40]. The prevalent hypothesis of olfactory coding states that single cells express one type of receptor, and cells which express the same receptor project to specific glomeruli ([16,75], see below). Individual chick OSNs also appear to express a single type of olfactory receptor [60]. Thus, the ability of an OSN to respond with either increase or decreases suggests that single olfactory receptors are linked to multiple signaling pathways that are differentially activated in an odorant-specific fashion [1,109]. These single cells may therefore act as complex stimulus processors [1,40], perhaps as an adaptation to compensate for the relatively narrow olfactory receptor gene complement. This would allow the entire system to detect and encode several different types of odorants with a limited number of neurons dedicated to odor processing.

4. The olfactory bulb

The axons emanating from the OSNs converge into multiple olfactory nerves that traverse the cribriform plate and terminate in glomeruli in the olfactory bulb. The vertebrate olfactory bulb is organized in concentric layers, starting with the outermost olfactory nerve layer, followed by the glomerular cell layer, and subsequent cell populations dedicated to the initial stages of signal processing (for review, see [115]). The structural organization of the olfactory bulb is another feature that is remarkably conserved across a variety of species [50]. In addition, glomerular organization for processing of olfactory inputs can be found in both vertebrate and invertebrate central structures [50], suggesting that this arrangement of neurons appears to be ideally suited for decoding and processing of odor information.

OSNs have receptor proteins, which selectively bind to odor molecules [111]. Research has shown that OSNs each express one type of receptor (out of several hundred to a thousand, depending on the species), and a single type of odor molecule can stimulate several different kinds of receptors [16]. Axons from OSNs that express the same receptor converge onto the same glomeruli in a precise and stereotypic fashion [75]. Thus, neural signals from the OSNs form a pattern of activation across the glomeruli in the bulb that is dependent on the differential odorant–receptor binding activity across the OSNs and is representative of the odorant stimulus parameters [107]. These signals are then processed and sent to the rest of the brain for subsequent processing [115].

It is possible to measure glomerular neuronal activation using a number of different techniques. Electrophysiological recordings are typically conducted on components of the olfactory bulb circuitry (such as mitral cells, granule cells, and periglomerular cells, see [116] for review). Optical imaging techniques [107] allow for the measurement of activity patterns across the entire glomerular array, and are ideal for assessing activity patterns indicative of coding of odorant information [78]. In addition, cytochemical techniques been used as a tool for creating a static map of neuronal activation in the olfactory bulb [120]. In one such approach, glomerular activity leads to the transcription of the immediate early gene cFos [43,110], and post-stimulation cFos gene expression can be used as a universal marker for this neuronal activity [69]. Activity of neurons in response to different odorant mixtures elicits differential cFos expression pattern across the entire olfactory bulb [110].

The avian olfactory bulb exhibits a high degree of structural similarity [4,5,70] and follows a similar developmental pathway (dependent on multiple tissue interactions, including the olfactory placode and olfactory nerve [128]) to those seen in other vertebrates. The outermost olfactory nerve layer, and the proximal glomerular layer initially activated by odor stimulation [71]: the olfactory bulb neurons are spontaneously active, and odor stimulation increases firing activity of neurons that is indicative of the stimulus quality, intensity, and temporal pattern [70,72]. Although it has not been systematically tested, one could assume that the avian olfactory bulb encodes odorants using an activation pattern across glomeruli, as in other vertebrates [50]. Interestingly, the anatomical features (such as olfactory bulb size [5]) of different species of birds are different, highlighting possible influences of the differences in lifestyle in the structural formation of the olfactory system [48]. These may result in functional differences in the olfactory bulb and in the higher regions of the brain among the different bird species that differ depending on the bird's behavioral repertoire [90]. This idea merits further investigation.

The olfactory bulb is similar in birds compared to other vertebrates [4,5]; other regions of the avian brain (such as the olfactory tubercle) that receive the outputs from the olfactory bulb appear to be radically different from those of other vertebrates [95]. This suggests that mechanisms for the initial steps of olfactory detection and coding are optimally served by the existing structural organization of the olfactory bulb, whereas higher order functions (such as odorant identification or behavioral responses) must naturally vary from species to species depending on their environmental and behavioral constraints.

5. Development and neurogenesis in the chick olfactory system

The olfactory system of chicks originates from the nasal placodes that appear during embryonic day 3 (E3). The placode invaginates into the underlying tissue to form the primordial nasal cavity [24], with the olfactory epithelium arising from the deeper recesses of the invagination [31]. Neuroblasts subsequently differentiate, and axonal fibers reach the prosencephalon to induce the development of the olfactory bulb at E4 [59].

Olfactory receptor proteins are first expressed in the chick olfactory epithelium at E5 through E8 [60]. Chick olfactory receptors are transiently expressed in non-olfactory tissue [82]; the significance of this phenomenon is still unclear. It is thought that olfactory receptor expression in the OSNs plays a role in guidance of the olfactory axons to their specific glomerular targets [7,59,130]. By E13, the first sign of olfactory function in OSNs is evident: electroolfactogram recordings from the chick olfactory epithelium show odor-elicited currents [58]. Over time, the developing neurons continue to acquire characteristics found in mature OSNs; by the time the chick embryos hatch, the neurons appear to be fully mature [51].

One of the most intriguing features of olfactory development and regeneration is the establishment of glomerular innervation by the OSNs (for review, see [53]). The sheer number of OSNs in the epithelium and the precise topographic organization of the innervation from these OSNs into the bulb imply the existence of an elegant guidance mechanism for axonal homing. Studies on the mouse have suggested a hierarchical model for axonal targeting,

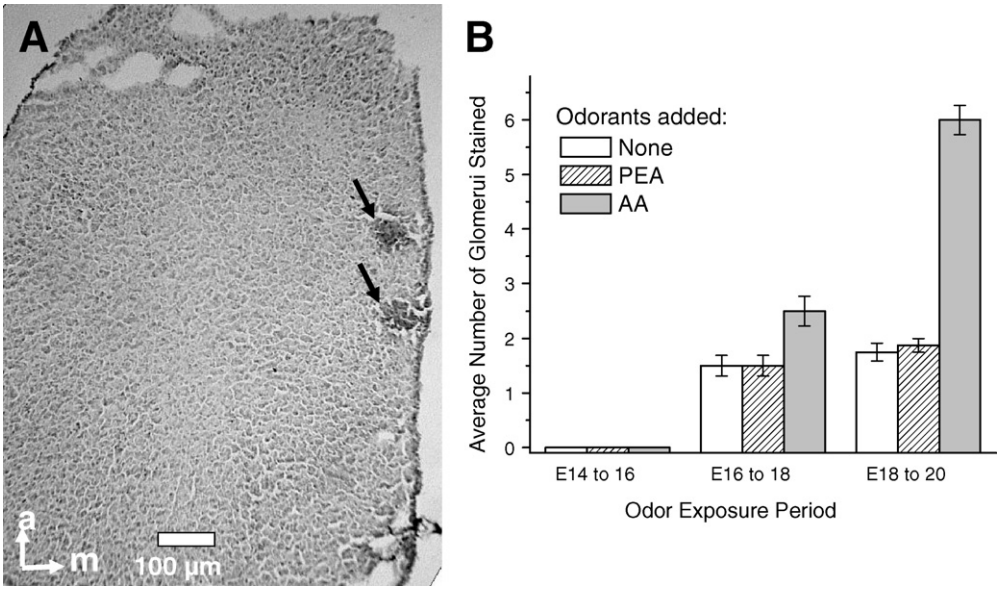

Fig. 2. cFos immunostaining of the embryonic chick olfactory bulb: embryonic chicks were exposed to odorants for two days, and their olfactory bulbs were dissected, fixed in 4% paraformaldehyde, preserved in 30% sucrose, cut into 40 μm sections, and mounted on gelatin-subbed slides. Sections were treated with anti-cFos antibodies, and antibody binding was visualized using ABC peroxidase agents and DAB as described in the text. (A) This figure shows a sample frontal section taken from an E20 chick exposed to amyl acetate at E18. For clarity, section was slightly overstained to show all the tissue; two stained glomeruli (black arrows) are visibly darker. White arrows indicate anterior (a) and medial (m) directions; scale bar: 100 μm. (B) To determine glomerular activation patterns, chicks were exposed to odorants at E14, E16, and E18, and the brains were fixed and sectioned and stained 2 days later. The number of stained glomeruli was determined by looking at all sections taken from each individual brain. The chart shows an average number of stained glomeruli (±S.E.M.) counted from eight separate brains per treatment. At E16 (odor added on E14), no glomeruli are visibly active. At E18, brains exposed to amyl acetate (AA) start to show differences from those that were exposed to phenylethyl alcohol (PEA) or not exposed to odor (control). At E20, there are marked differences in glomerular staining between AA and PEA/controls. Behavioral studies support this timeline: odor imprinting is effective from E18 to E21.

where axons are initially guided by cell adhesion molecules and subsequently, by the olfactory receptors themselves [119]. Some studies support the role of OSN activity-dependent connections in establishing olfactory bulb circuitry [129], while others suggest that axons can target the appropriate glomeruli in the absence of OSN function [62]. It is also known that the OSNs are trophically dependent on the olfactory bulb for development and survival [112]. In the chicken, studies suggest that olfactory receptor expression is initiated in the OSNs independently from the axonal innervation of the olfactory bulb; in quail chick chimeras, OSNs from chicken epithelium expressed their normal olfactory receptor repertoire when transplanted into a quail embryo [59]. It appeared that the olfactory receptors were the primary factors involved in axonal targeting at earlier stages (E5 to E8); subsequent synaptogenesis and refinement of connectivity through E15 is likely under the influence of the olfactory bulb [59].

The olfactory bulb of the chicken appears to develop in synchrony with the OSNs. Anatomical studies show that the chick olfactory bulb is visible at around E7, and the connection between olfactory sensory neurons and the main olfactory bulb occurs between E8 and 10 [2]. It is not clear whether the olfactory bulbs begin to function at this point. Therefore, to determine the timeline of functional development of the chicken olfactory system *in ovo*, we attempted to measure olfactory bulb activity in chick embryos. Exposure to odorants *in ovo* [17,117] or *in utero* (for mammals [49]) alters post-parturition behavior. We incubated chicken eggs and exposed them to different odorants by either externally (soaked filter paper taped onto the eggshell near the air cavity) or internally (injecting odorants into the airspace; see [117]) for 2-day periods starting at E14, E 16, or E18. We used two odorants: amyl acetate (AA, which is detectable by chicks [106]) and phenylethyl alcohol (PEA, which does not appear to be detectable by chicks). At the end of each period (E16, E18, and E20), some embryos were sacrificed, and the cFos staining pattern in the olfactory bulb was assessed using immunocytochemical techniques. Briefly, chick brains were dissected and fixed immediately in 4% paraformaldehyde for 24 h, transferred to 30% sucrose, sectioned into 40 μm horizontal sections and mounted on gelatin-subbed slides. Antibody blocking and visualization were done using the Vectastain Elite ABC Kit and the Diaminobenzidine (DAB) Kit (Vector Labs, Santa Cruz, CA) following the manufacturer's instructions; the tissue was incubated in the primary antibody (anti-cFos, 1:500, from Santa Cruz Biotechnology, Santa Cruz, CA) for at least 4 days. Positive cFos staining would be indicative of neuronal activity [43]. In addition, other eggs were allowed to hatch, and 24–48-h-old chicks were tested for odor preference in a Y-maze.

Our results showed that at E16, the glomeruli are still undeveloped: no cFos staining was evident in any of the embryos. By E18, there was evidence of differential staining between AA-exposed embryos versus PEA-exposed or control (no odor exposed) embryos (Fig. 2), suggesting that the periphero-central circuitry is established during this time window. These patterns become more pronounced by E20, with a clear difference between AA-exposed versus PEA-exposed bulbs (and no difference between control and PEA-exposed bulbs). While these studies are still in progress, the results support the notion that the chicken olfactory system is fully functional *in ovo*, and the critical period for the development of function of the olfactory system occurs at about E18. Indeed, behavioral assays from these chicks support this timeline: embryos imprinted with AA at E18 show preference to AA versus PEA or no odor when tested in a Y-maze (data not shown). Other studies on odor imprinting behavior in chickens [117] have shown a similar developmental timeline.

6. The nose: "a window to the brain"

The processes involved in the development of the olfactory system are most likely utilized post-parturition. It is well known

that olfactory neurons regenerate throughout the adult lifespan [31]. The generation of mature olfactory neurons from an adult stem cell population involves a series of mitotic divisions, followed by maturation steps that result in structurally and functionally mature OSNs (for review see [19]). Briefly, non-neuronal globose basal cells adjacent to the basal lamina of the olfactory epithelium undergo mitotic divisions to produce intermediate neuronal precursors. These precursors undergo subsequent maturation steps, resulting in immature neurons. These grow a new axon that targets the appropriate glomerulus in the olfactory bulb [23], as well as grow appropriate dendritic and ciliary structures to form a mature OSN. Thus in addition to OSNs, the olfactory epithelium has several other cell types [42,76]: immature neurons that lack cilia; stem or basal cells that represent a population of progenitor cells capable of proliferation and differentiation into neurons [18,20]. The maturation of olfactory neurons is influenced by growth factors that are secreted by other cells of the olfactory epithelium [28,31] or trophically by the olfactory bulb [112]. Basal cell division is triggered by dying olfactory neurons that release trophic factors such as the leukemia inhibitory factor (LIF) [6,34]. Thus, physical assault on the olfactory epithelium or on the olfactory nerve that results in OSN death subsequently results in upregulated mitosis and neurogenesis in the epithelium [113]. Subsequent stages of neurogenesis are influenced by a series of growth factors: transforming growth factor-α (TGF-α), interleukin-6 and -β, basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), insulin growth factor I (IGF-I), and LIF have been shown to affect olfactory cell differentiation and neurogenesis in the olfactory epithelium (see [19]). As a result, the olfactory epithelium recovers function following injury; this ability has been shown in birds as well [54,89]. Since the olfactory system has multiple cell types, growth factors, and maturation stages, it may serve as a useful model for the study of factors employed by the body to generate and/or regenerate neurons.

The regenerative ability of OSNs enables the olfactory system to propagate *in vitro* and continuously generate neurons from non-neuronal precursors. To culture olfactory neurons, olfactory tissue is typically dissected from the epithelium, separated into isolated cells or into pieces of tissue ("explants") using mechanical and/or enzymatic dissociation, and allowed to grow on culture dishes in humidified incubators at 38 °C and 5% CO_2 [104]. When in culture, olfactory neurons that grow and proliferate attain a visually distinct bipolar/spherical shape with two processes extending from opposite ends [41,104]. In addition, the globose basal cells can survive in culture and eventually divide to produce neuron precursors [20]. A number of molecular markers that have been used to identify OSNs and their precursors *in vivo* have also been shown to be expressed by olfactory cells *in vitro*, including the olfactory marker protein (OMP, expressed exclusively by mature olfactory neurons *in vivo* and *in vitro.* [67]), neural cell adhesion molecule (NCAM), β tubulin III (neuron-specific tubulin or NST), neuron-specific enolase, mircotubule associated proteins, and growth associated protein 43 (for review see [19]). Glial-like cells have been identified with markers such as S100β, glial fibrillary acidic protein (GFAP), vimentin (which

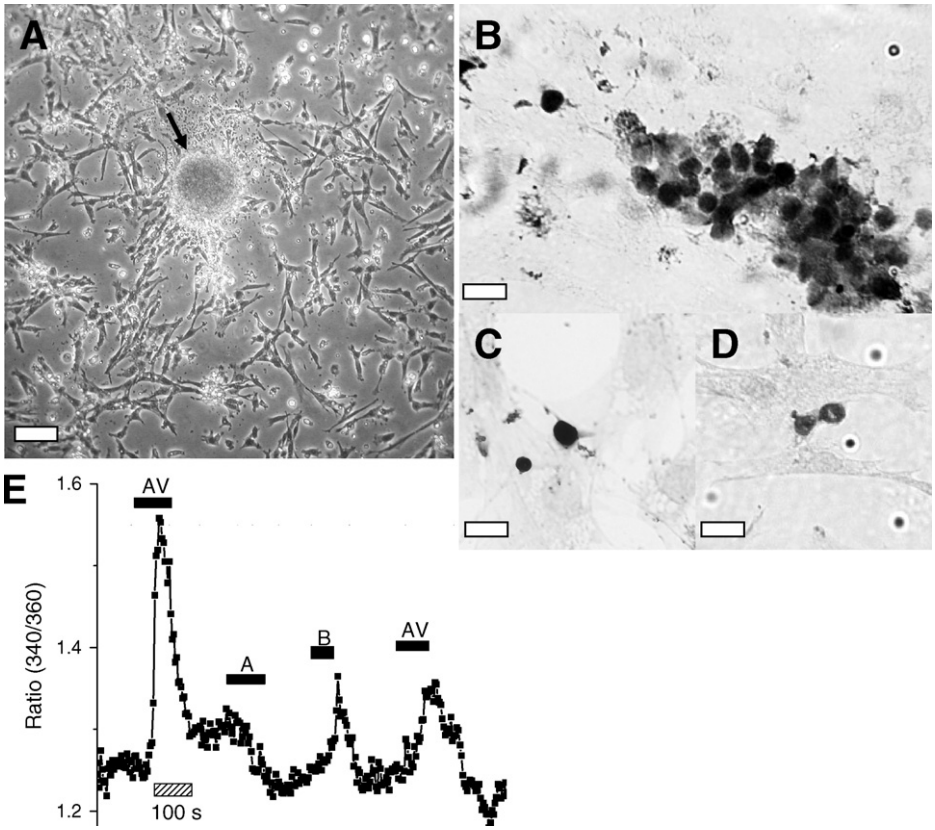

Fig. 3. Cells derived from the newborn chick olfactory epithelium grow readily *in vitro*. (A) Phase contrast photomicrograph of an explant culture after 4 days *in vitro*: a variety of cell types grew outwards from the explant (arrow); neurons appeared phase-bright. Scale bars: (A) 100 μm; (B–D) 20 μm. (B) Cells were stained for marker molecules using similar techniques described in Fig. 2. These cells stained positively for neuron-specific tubulin, a molecule expressed by OSNs *in vivo*. (C) An OMP-positive neuron had bipolar processes typical of OSNs *in vitro*. (D) Neurons also stained positively for G_{olf}, a molecule associated with cAMP-mediated odor signal transduction in OSNs. (E) Cultured avian olfactory neurons respond to odorant stimulation with changes in $[Ca^{2+}]_i$. Cells were grown for 5 days in vitro, visually identified by their characteristic morphology and tested with calcium imaging techniques as in Fig. 1. This specific cell responded to odorants with increases in $[Ca^{2+}]_i$, demonstrating that these cells *in vitro* possessed functional properties as well.

sometimes stains olfactory neurons), and fibronectin [92]. Many of these molecules are used in other parts of the brain. Studies using calcium imaging have shown that cultured olfactory cells respond to odorants with changes in $[Ca^{2+}]_i$ as a result of *in vitro* differentiation and maturation [41,124]; these odorant-elicited changes in $[Ca^{2+}]_i$ that are similar to those found in OSNs *in vivo* [41], suggesting the possible use of *in vitro* systems for the study of cell signal transduction cascades in the nervous system [103].

Preliminary studies in our laboratory show that the chick olfactory epithelium can be grown in culture using methods described previously [41]. Cultured chick olfactory cells (CCOCs) attained morphology typical of cultured olfactory neurons [41] and expressed marker molecules characteristic of neurons (such as NST and OMP [19], Fig. 3B and C) or molecules associated with olfactory function (Fig. 3D). The CCOCs are also odorant-sensitive: individual cells grown *in vitro* for at least 5 days responded to odorants with increases in $[Ca^{2+}]_i$ (Fig. 3E). Further experiments characterizing chick OSN and CCOC odorant sensitivity are currently in progress. These studies suggest the possible use of *in vitro* systems for study of neural molecular marker expression and neuronal cell signaling pathways. Such approaches have proved viable in studies on mammalian species [103], including humans [41,45].

Lastly, there is strong evidence for continuous neurogenesis in the vertebrate olfactory bulb throughout the adult lifespan [9]. In mice and rats, neuronal stem cells migrate from the subventricular zone through the rostral migratory stream and into the bulb [32], where they form local interneurons and granule cells [35]. Odor exposure increases neurogenesis in this system, leading to improved odor memory [102]. Since neural reorganization occurs in the adult bird brain, it is possible to gain insight into the details and mechanisms involved in this process using the olfactory system as a model (for review, see [37]).

Conflicts of interest

None.

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