LOCALIZATION OF THE OLFACTOR Y CYCLIC NUCLEOTIDE-GATED CHANNEL SUBUNIT 1 IN NORMAL, EMBRY ONIC AND REGENERATING OLFACTOR Y EPITHELIUM

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Abstract—The spatial and temporal expression of subunit 1 of the olfactory cyclic nucleotide-gated channel was investigated using affinity-purified anti-fusion protein antibodies. Immunoreactivity was most prominent in the ciliary layer of the olfactory epithelium, but high protein expression was also seen along the entire length of olfactory receptor neuronal axons to the level of the epithelial surface and not in axons (adenylyl cyclase type III). Following unilateral lesions of the olfactory bulb, olfactory cyclic nucleotide-gated channel subunit 1 immunoreactivity was present early and throughout developing olfactory receptor neurons; adenylyl cyclase type III immunoreactivity, in contrast, was detectable only later, and again present only in the ciliary layer.

These results support the hypothesis that this subunit of the olfactory cyclic nucleotide-gated channel may be involved in olfactory axon guidance, in addition to its well-described role in olfactory signal transduction. © 1999 IBRO. Published by Elsevier Science Ltd.

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Cyclic nucleotide-gated (cNG) channels mediate the electrophysiological responses in vision and in olfaction by binding cyclic GMP and cyclic AMP, respectively (for reviews, see Refs 21, 22 and 44). In olfaction, signal transduction is initiated when odorants interact with odorant receptors, 31,37 members of a large gene family of seven transmembrane-domain receptors. 11 Stimulated receptors may couple via an identified G-protein, Goolf 20 to adenylyl cyclase (AC) type III. 3 These signaling components are highly enriched in olfactory cilia, as has been shown by light and electron microscopy (for reviews, see Refs 27 and 30). In addition, odorants stimulate the production of inositol 1,4,5-trisphosphate (IP3). 8,38 Although the function of the odorant-induced IP3 signal is uncertain, 10 the location of IP3 receptors on the ciliary surface membranes 13 suggests that IP3 may mobilize calcium to modulate ciliary enzymatic activities, such as AC or phosphodiesterases. 39

Cyclic AMP then activates a cNG channel. 15,14 To date, several different subunits of this channel have been identified in vertebrates, with the added complexity that some exist in tissue-specific splice forms. Native retinal rod and olfactory cNG channels are thought to be heteromultimers composed of at least two types of subunit. 6,25 In ectopic expression systems, many of these subunits cross-assemble, which provides a large functional diversity. Subunit composition can affect several properties, such as calcium permeability, ligand selectivity and cyclic nucleotide sensitivity. 16 The cNG subunit first identified in the olfactory system was referred to as the olfactory cyclic nucleotide-gated channel (OcNC) subunit 1. 14,21 A second subunit cloned from the olfactory system (designated OcNC2 or Oc3) was shown to increase the cyclic nucleotide sensitivity of the channel. 5,25

Besides having a role in electrophysiological signaling to odors, cNG channels may have multiple functions, 23,45 including a developmental role. In an invertebrate, C. elegans, mutation of the tax-2 gene, which encodes a cNG channel, causes abnormalities in axonal outgrowth in a subclass of sensory neurons. 32 In addition, cNG channels appear to be present on growth cones of embryonic vertebrate neurons. 7 Initial studies on mice null for OcNC1 failed to recognize any defect in olfactory neuronal axon guidance, 10 but more recent work has shown that absence of the channel in OcNC1 knock-out mice leads to an altered biochemistry and morphology in the olfactory bulb. 4 OcNC1 in the peripheral olfactory system has been evaluated during embryogenesis by detection of mRNA, 26 but protein expression during embryogenesis has not yet been examined. In the present study, OcNC1 expression was determined spatially and temporally using affinity-purified anti-OcNC1 antibodies. The temporal profile of OcNC1 expression was determined during embryogenesis and during regeneration following olfactory bulbectomy (OBx).
EXPERIMENTAL PROCEDURES

Animal selection
Studies utilized adult male or fetal Sprague–Dawley rats (Harlan, Indianapolis, IN). All procedures described below were performed in accordance with Federal and NIH animal use guidelines, using institution-approved animal protocols.

Generation of anti-olfactory cyclic nucleotide-gated channel subunit 1 antibody
Anti-rat OcNC1 polyclonal antiserum was generated against a 318-base pair sequence, encoding amino acids 559–664 of OcNC1, by amplification polymerase chain reaction with oligonucleotide linker primers from a pBluescript (Stratagene) construct containing the entire OcNC1 coding sequence14 (gift from Dr Randy Reed, Johns Hopkins University School of Medicine). This product was subcloned into the pTrcHisA Expression Vector (Invitrogen), transformed into competent Escherichia coli bacteria and expression of fusion protein was induced by isopropylthio-β-galactoside. Fusion protein was purified using a nickel column and injected into rabbits for antibody synthesis according to previously established protocols.15 Antibody was affinity purified using a fusion protein affinity column, as described previously.40 This affinity-purified anti-OcNC1 antibody was characterized previously by western blot and by immunohistochemistry, including pre-absorption and pre-immune serum controls.

Anti-adenyl cyclase III antibody
Rabbit polyclonal anti-AC III antibody was a gift from Dr Randall Reed and co-workers (Johns Hopkins University School of Medicine) and has been characterized previously regarding its specificity in olfactory tissues.31

Embryo preparation
Timed-pregnant rats were killed with a non-survival dose of Xylaklet (25 mg/ml ketamine hydrochloride, 2.5% Xylazine (Rompun; A. J. Buck) in ethanol and 0.9% saline), after which embryos were removed and washed in phosphate-buffered saline (PBS). Rat embryos were dated defining the day of conception as embryonic day 0 (E0).

Olfactory bulbectomy
Adult Sprague–Dawley rats were anesthetized intraperitoneally with 0.8 ml Xylaklet and fixed in a stereotactic apparatus for surgery. The right olfactory bulb was exposed via a partial dorsal craniotomy and was ablated by suction. Care was taken to avoid damage to the contralateral (left) olfactory bulb. The ablation cavity was filled with Gelfoam (Upjohn Co.) to prevent invasion of the frontal cortex into this cavity, which could provide an alternative target for regenerating axons. Olfactory turbinates were carefully removed and postfixed in 4% PFA or Bouin’s fixative. Depolymerized paraformaldehyde (PFA) in PBS or Bouin’s fixative (Sigma) for 2 h at 4°C. Embryos were washed in PBS and immersion fixed for 6 h in either PFA or Bouin’s fixative. Two methods of fixation were used to permit comparison of immunoreactivity under different fixation conditions. As there was no difference in amount or pattern of immunoreactivity, Bouin’s fixative was used, as it produced better morphology. Tissue was cryoprotected in 20% sucrose in PBS overnight at 4°C, before being transferred to plastic molds and embedded in OCT medium (Tissue-Tek) on dry ice. Cryostat sections (12 μm) were cut, applied to Fisher Super-frost-Plus™ glass slides and dried overnight. Sections were blocked for 1 h in PBS containing 1% normal goat serum and then incubated overnight at 4°C in the same buffer containing either affinity-purified anti-OcNC1 antibody (1:250) or anti-AC III antibody (1:500). Control sections were incubated using pre-immune serum or antibody pre-absorbed with a 10-fold weight excess of antigen prior to primary antibody incubation. Staining was visualized using Vectastain Elite Kits (Vector Labs), and developed using diaminobenzidine (Sigma) and hydrogen peroxide in 50 mM Tris (pH 7.4). Slides were mounted with Aquapolymount (Polysciences) cytological mountant and coverslipped.

Electron microscopy: tissue preparation and immunocytochemistry
Sprague–Dawley rats were anesthetized with Co2 and the required tissues were used for freeze substitution without further chemical fixation. E18 and E21 rat fetuses were removed from dams asphyxiated with CO2. Nasal septa were rapidly frozen by dropping them on a liquid nitrogen-cooled copper block, slam freezing (Gentleman Jim Quick-Freeze System, Energy Beam Sciences, Agawam, MA36) and freeze substituted in dry acetone/0.1% uranyl acetate. Freeze substitution, infiltration and low-temperature embedding were carried out in a CS Auto Cryo-Substitution System (LEO-Reichert Instruments, Vienna, Austria). Infiltrated specimens were embedded in Lowicryl K11M while the temperature rose slowly from −60°C to ambient.26 Immunolocalization by electron microscopy was carried out as described previously.26,28 In brief, 10 mM Tris-buffered saline (pH 8.0) containing 500 mM NaCl and 0.1% acetylated BSA (Aurion, Electron Microscopy Sciences, Fort Washington, PA29) was used for blocking, incubations, dilutions and most washings. Sections were immersed in the blocking solution for 3–4 h at room temperature and incubated with primary antibodies at appropriate dilutions (see figure legends) overnight at 4°C. Antibody binding was visualized with goat anti-rabbit immunoglobulin G–10 nm gold [OD 520 (optical density) – 0.1; Aurion], to which sections were exposed for 4 h. Triton buffer was supplemented with 0.1% Tween-20 for the jet washings (1 min) after gold incubation. Thereafter, the grids were jet washed with distilled water for 1 min. Gold conjugates were diluted in the same buffer in which they were supplied, but supplemented with 0.1% acetylated BSA. Following labeling, the sections, 150–200 nm thick and mounted on 300-nm nickel electron microscope grids, were stained with 0.5% methanolic uranyl acetate, carbon coated on one side and examined at 120 kV in a Jeol 1010 CX electron microscope.

RESULTS

Western blot of anti-olfactory cyclic nucleotide-gated channel subunit 1 antibody
Western blots were performed to determine the specificity of the rabbit polyclonal antibody generated against the fusion protein representing amino acids 559–664 of the OcNC1 protein.14 Anti-OcNC1 antiserum was affinity purified using rinsed three times in SM-PBS and finally rinsed in PBS containing 0.1% Tween-20. Bands were visualized using Vectastain Elite Kits (Vector Labs). Controls were performed using blots incubated with pre-immune serum, and with antibody pre-absorbed with a 10-fold weight excess of antigen prior to primary antibody incubation.
a fusion protein column. Western blots of rat cilia preparations were probed using this anti-OcNC1 antibody (Fig. 1). Immunoreactive bands were observed at mol. wts of 78,000 and 140,000–150,000. Both bands were absent in pre-immune serum and when antibody was preabsorbed with fusion protein. The broadness of the higher mol. wt band suggested that post-translational modifications of the OcNC1 protein occur, such as glycosylation. This anti-OcNC1 antibody does not cross-react with either the OcNC2 or rod beta subunit, as these proteins migrate at different positions (data not shown).

Localization of olfactory cyclic nucleotide-gated channel subunit 1 in olfactory epithelium: light microscopy

Immunohistochemistry was performed on adult olfactory tissues to determine the regional distribution of OcNC1 protein. Immunoreactivity was visualized most strongly in the ciliary layer of the olfactory epithelium (Fig. 2A). This layer contains both the olfactory sensory cilia projecting from the dendritic knobs of the olfactory receptor neurons and microvilli emanating from neighboring sustentacular cells. Immunoreactivity was essentially lost after OBx, consistent with labeling of olfactory cilia (see Fig. 5E). Little immunoreactivity was detected in the deeper two-thirds of the olfactory epithelium, which contains the cell bodies of the
olfactory receptor neurons (ORNs). Examination of many sections failed to demonstrate staining in the deepest layer of the epithelium, the region containing basal cells. A surprisingly high level of immunoreactivity was seen in the axons of ORNs, shown in coronal (Fig. 2A) and sagittal sections (Fig. 2B). This immunoreactivity extended over the entire length of the axons (Fig. 2B). OcNC1 protein was also expressed in the olfactory nerve layer and glomeruli.
Lengths, but consist of distinctly thicker proximal portions.

**Localisation of olfactory cyclic nucleotide-gated channel subunit 1 to olfactory cilia by immunoelectron microscopy**

Ultrastructural analysis confirmed the ciliary localisation of OcNC1 immunoreactivity in the rat (Fig. 3A, B) and mouse (Fig. 13 in Ref. 30). For these studies, otherwise unfixed rapidly frozen olfactory epithelium was subjected to free substitution, resin (Lowicryl K11M) infiltration, low-temperature embedding and postfixation detection using goat anti-rabbit immunoglobulin G–10 nm gold as a secondary probe. Olfactory cilia are not homogeneous along their lengths, but consist of distinctly thicker proximal portions 2–3 μm long and approximately 0.3 μm in diameter, and much longer, 50–60-μm-long distal segments. Functional differences have been suggested for these two ciliary morphological regions, based on enzymatic cytochemical and immunoelectron microscopic localization studies.\(^2,3,0\) Although associated with proximal cilium compartments (Fig. 3A), the majority of staining is associated with the transverse-running, thinner distal cilium compartments. Large portions of immunogold grains are seen to cluster in these regions (Fig. 3B). Labeling was essentially absent from ORN dendritic knobs and dendrites; sustentacular cell microvilli also did not label (Fig. 3A, B).

Omitting primary antibody resulted in an absence of labeling (data not shown). Antibodies were also pre-absorbed with an excess of peptide overnight at 4°C, which resulted in a marked reduction of immunoreactivity (Fig. 3C). Nasal respiratory epithelia served as tissue control, and cilia and microvilli of this tissue did not bind antibody (Fig. 3D). Antibodies whose distribution had been characterized previously, those to Go, Gs and AC type III, served as positive controls. Finally, ultrastructurally, immunoreactivity was only seen in unfixed tissues; even mild fixation with PFA alone abolished immunoreactivity (data not shown, but see Ref. 29).

**Expression of olfactory cyclic nucleotide-gated channel subunit 1 during development of the olfactory epithelium**

The localization of expression of OcNC1 to olfactory axons in the adult suggested that OcNC1 might be involved in other aspects of ORN function. Rat embryos from E12 to postnatal day 1 (P1) were immunostained for OcNC1 and AC III to determine the pattern of protein expression during development (Fig. 4). AC III was chosen as a comparative marker, as it participates immediately upstream from OcNC1 in the odorant signaling cascade.

No immunoreactivity was detected for either OcNC1 or AC III at or prior to E13 (Fig. 4A, B). OcNC1 immunoreactivity was detected at E14, in cells located basally in the developing olfactory epithelium (Fig. 4C, arrow). Staining is seen in basally located cells (arrowheads) and in a small group of cells located in the submucosal region (asterisk). No staining was detected for AC III (Fig. 4D). By E15, a number of cells expressed OcNC1 (Fig. 4E, arrow). At E16, a subset of cells in the epithelium stained relatively more intensely for OcNC1 (arrows), while a higher proportion of cells stained at a lower level (Fig. 4G). At E18, immunoreactivity was increased (Fig. 4I) and more uniform (arrowheads). By E19, OcNC1 protein expression was observed in a uniform layer of ORNs situated in the middle two-thirds of the epithelium (Fig. 4K). AC III expression could be detected at E19, but occurred only at the level of the epithelial surface and never extended beyond the ciliary layer of the epithelial surface. This was the case throughout development and in adults (Fig. 4L; also see Ref. 28). By P1, ORNs stained uniformly for OcNC1 (Fig. 4M) and the cilial/epithelial surface layer stained for AC III (Fig. 4N). Thus, spatial expression of OcNC1 extended significantly beyond that of AC III, and occurred throughout the cells and also in submucosal axonal regions. This was true throughout development from the earliest time of label detection, E14.

Ultrastructurally, distinct OcNC1 immunoreactivity at the level of the cilia was confirmed for E18 and E21 embryos (Fig. 5A and B, respectively). Proximal segments of olfactory cilia labeled abundantly when distal segments had not yet formed (Fig. 5A). However, once the latter began to form, these segments labeled more abundantly than the proximal parts of the cilia (Fig. 5B). Axonal labeling could not be demonstrated ultrastructurally, as submucosal regions are severely damaged in unfixed, rapidly frozen specimens.\(^29\)

Expression of olfactory cyclic nucleotide-gated channel subunit 1 and adenylyl cyclase III in regenerating olfactory epithelium

To determine whether a differential spatial expression of OcNC1 and AC III also occurred during regeneration, immunohistochemistry was performed following unilateral OBx, which hyperinduces replacement of dying ORNs with newly generated neurons.\(^18\) Unilateral bullectomies were performed on young adult male rats.\(^40\) In control sham-operated animals, the patterns of protein expression of OcNC1 and AC III overlapped. Ciliary staining was intense for both proteins. In contrast, AC III was essentially absent from cell bodies and axons (also see Refs 3 and 21), while OcNC1 antiserum also labeled axons (Fig. 6A, B; also see Fig. 2).

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**Notes and References**

1. The localization of expression of OcNC1 to olfactory axons in the adult suggested that OcNC1 might be involved in other aspects of ORN function.

2. Functional differences have been suggested for these two ciliary morphological regions, based on enzymatic cytochemical and immunoelectron microscopic localization studies.

3. Although associated with proximal cilium compartments (Fig. 3A), the majority of staining is associated with the transverse-running, thinner distal cilium compartments. Large portions of immunogold grains are seen to cluster in these regions (Fig. 3B). Labeling was essentially absent from ORN dendritic knobs and dendrites; sustentacular cell microvilli also did not label (Fig. 3A, B).

4. Omitting primary antibody resulted in an absence of labeling (data not shown). Antibodies were also pre-absorbed with an excess of peptide overnight at 4°C, which resulted in a marked reduction of immunoreactivity (Fig. 3C).

5. Nasal respiratory epithelia served as tissue control, and cilia and microvilli of this tissue did not bind antibody (Fig. 3D).

6. Antibodies whose distribution had been characterized previously, those to Go, Gs and AC type III, served as positive controls.

7. Finally, ultrastructurally, immunoreactivity was only seen in unfixed tissues; even mild fixation with PFA alone abolished immunoreactivity (data not shown, but see Ref. 29).

8. The localization of expression of OcNC1 to olfactory axons in the adult suggested that OcNC1 might be involved in other aspects of ORN function.

9. Rat embryos from E12 to postnatal day 1 (P1) were immunostained for OcNC1 and AC III to determine the pattern of protein expression during development (Fig. 4). AC III was chosen as a comparative marker, as it participates immediately upstream from OcNC1 in the odorant signaling cascade.

10. No immunoreactivity was detected for either OcNC1 or AC III at or prior to E13 (Fig. 4A, B). OcNC1 immunoreactivity was detected at E14, in cells located basally in the developing olfactory epithelium (Fig. 4C, arrow).

11. Staining is seen in basally located cells (arrowheads) and in a small group of cells located in the submucosal region (asterisk). No staining was detected for AC III (Fig. 4D).

12. By E15, a number of cells expressed OcNC1 (Fig. 4E, arrow). At E16, a subset of cells in the epithelium stained relatively more intensely for OcNC1 (arrows), while a higher proportion of cells stained at a lower level (Fig. 4G).

13. At E18, immunoreactivity was increased (Fig. 4I) and more uniform (arrowheads).

14. By E19, OcNC1 protein expression was observed in a uniform layer of ORNs situated in the middle two-thirds of the epithelium (Fig. 4K).

15. AC III expression could be detected at E19, but occurred only at the level of the epithelial surface and never extended beyond the ciliary layer of the epithelial surface. This was the case throughout development and in adults (Fig. 4L; also see Ref. 28).

16. By P1, ORNs stained uniformly for OcNC1 (Fig. 4M) and the cilial/epithelial surface layer stained for AC III (Fig. 4N).

17. Thus, spatial expression of OcNC1 extended significantly beyond that of AC III, and occurred throughout the cells and also in submucosal axonal regions. This was true throughout development from the earliest time of label detection, E14.

18. Ultrastructurally, distinct OcNC1 immunoreactivity at the level of the cilia was confirmed for E18 and E21 embryos (Fig. 5A and B, respectively). Proximal segments of olfactory cilia labeled abundantly when distal segments had not yet formed (Fig. 5A).

19. However, once the latter began to form, these segments labeled more abundantly than the proximal parts of the cilia (Fig. 5B). Axonal labeling could not be demonstrated ultrastructurally, as submucosal regions are severely damaged in unfixed, rapidly frozen specimens.

20. To determine whether a differential spatial expression of OcNC1 and AC III also occurred during regeneration, immunohistochemistry was performed following unilateral OBx, which hyperinduces replacement of dying ORNs with newly generated neurons.

21. Unilateral bullectomies were performed on young adult male rats. In control sham-operated animals, the patterns of protein expression of OcNC1 and AC III overlapped. Ciliary staining was intense for both proteins.

22. In contrast, AC III was essentially absent from cell bodies and axons (also see Refs 3 and 21), while OcNC1 antiserum also labeled axons (Fig. 6A, B; also see Fig. 2).
Within three days post-lesion, immunoreactivity was significantly decreased for both proteins, although some labeling was retained at the ciliary level and, for OcNC1, also at the level of the axons (Fig. 6C, D). At one week post-lesion, immunoreactivity to OcNC1 was apparent throughout new neurons (Fig. 6E); weak immunoreactivity for AC III may be present in the ciliary layer (Fig. 6D). Two weeks following bullectomy, OcNC1 immunoreactivity was intense in cell bodies of ORNs, axons and in the ciliary layer (Fig. 6G). Interestingly, some OcNC1 immunoreactivity also seems to be present in precursor cells close to the basal lamina of the epithelium. However, examination of many fields failed to demonstrate consistent immunoreactivity across the basal layer.

In contrast to the staining pattern for OcNC1, ciliary layer immunoreactivity for AC III was barely detectable (Fig. 6H).
Three weeks after OBx, immunoreactivity to OcNC1 was similar to that of the normal epithelium, with the most intense immunoreactivity in ciliary and axonal layers (Fig. 6I). Although AC III expression did not display the intensity seen in unlesioned animals, immunoreactivity was present three weeks post-OBx and, as in unlesioned animals, predominantly in the ciliary layer (Fig. 6J). Thus, the spatial distribution of protein expression of OcNC1 and AC III during regeneration post-OBx was similar to that seen during fetal development. OcNC1 expression early on extending to the axons, while AC III was restricted only to the cilia.

**DISCUSSION**

The findings of this study reinforce the hypothesis that olfactory cNG channels have complex or multiple roles. OcNC1 expression, as detected by immunostaining, was found in axons, and occurred early during development and regeneration in ORNs, positioning OcNC1 to play a role in the development or axonal targeting of these neurons. Expression of OcNC1 was determined immunohistochemically at the light level in normal, fetal and regenerating rat olfactory epithelium, and at the electron microscopic level in late fetal and adult rat olfactory epithelium. In all cases, OcNC1 was rather uniformly distributed in the chemosensory ciliary layer of the olfactory epithelium and at the light level also in the axons. In these axons, staining extended their entire length, into the glomeruli of the olfactory bulb. In contrast, AC III immunoreactivity was essentially restricted to cilia during fetal development and regeneration, and in normal adult epithelium. Immuno-electron microscopic localization, performed to ensure that immunoreactivity is associated with sensory cilia and not sustentacular microvillar processes, revealed that OcNC1 is predominantly expressed in the distal, thin extensions of the sensory cilia, although some expression is also seen in the proximal segments of the cilia.

**Signal transduction**

The long and thin distal modified regions of the cilia that immunoreacted with the antibodies are the same regions of the cilia that labeled more abundantly than other regions of the cells with antibodies to Go (reviewed in Ref. 30) and, in the case of labeled cells, were also shown to contain ample odorant receptor. Therefore, the present study completes a series of studies that show that, at least for the AC-cNG channel signaling cascade, all proteins needed to transduce an olfactory signal, from odor receptor to current-generating channels, are positioned correctly with respect to the external odorous environment and the receiving cellular structures to transduce the odor message into an electrical signal. The only other ion channel thus far ultrastructurally examined and localized in olfactory epithelia is an amiloride-sensitive sodium channel. However, in contrast to the OcNC1 of olfactory cilia, this channel is found predominantly in microvilli of olfactory sustentacular cells.

The function of the cNG channel in odor transduction is well established. This channel produces an outwardly rectifying non-selective cationic conductance that can be gated by cyclic AMP and, unexpectedly, shows a slightly
higher affinity for cyclic GMP. The biological relevance of this channel’s sensitivity to cyclic GMP is unclear. Nitric oxide synthase, which generates nitric oxide to stimulate soluble guanylyl cyclase, is highly and transiently expressed in ORNs during development and regeneration. Cyclic GMP thus formed may gate the olfactory cNG channel to drive activity in an odorant-independent manner. Nitric oxide has been shown to stimulate OcNC2 directly. In addition, whole-cell calcium imaging suggested that more than 70% of the fractional conductance of the olfactory cNG channel is mediated by calcium with an extracellular calcium concentration of 3 mM. This implies that the olfactory cNG channel functions as a calcium channel in the presence of physiological levels of extracellular calcium. The existence of at least one other α subunit of the olfactory cNG channel, OcNC2, further distinguishes the olfactory channels. This subunit confers higher affinity for cyclic AMP; therefore, sensitivity to cyclic nucleotides can be altered, depending on cellular need. The olfactory cNG channel also differs from other ligand-gated channels in that it does not

Fig. 6. Immunolocalization of OcNC1 and AC III post-unilateral OBx. Sections were prepared from control animals (A, B), three days post-OBx (C, D), one week (E, F), two weeks (G, H) and three weeks (I, J) post-OBx. In non-lesioned animals, ciliary layer staining was prominent for OcNC1 (A) and AC III (B). Immunostaining had disappeared for both proteins by three days after lesioning (C, D). OcNC1 immunoreactivity recurred with neurogenesis by one week (E), whereas AC III immunoreactivity was not visible until two weeks post-lesion (H). Three weeks following OBx, the labeling patterns for both proteins resemble those of controls (I, J). Scale bar = 50 μm. Ax, axon bundles; CL, cilia layer; ORN, olfactory receptor neuronal cell body layer; NI, normal; 3d, three days; wk, week post-OBx.
desensitize. Thus, it can continue to conduct current and admit calcium as long as cyclic nucleotide levels are sufficiently elevated.\textsuperscript{16}

**Development**

The similarity of the spatiotemporal expression of OcNC1, with respect to cilial and axonal expression, during embryogenesis and regeneration may indicate that OcNC1 mediates other signaling functions aside from odorant detection. Several studies have investigated the onset of expression of transduction proteins during olfactory epithelial development. Using total RNA prepared from embryonic heads which was subjected to reverse transcription–polymerase chain reaction, Margalit and Lancet\textsuperscript{26} found that onset of RNA expression for G\textsubscript{olf} (E16) lagged somewhat behind that of AC (E15), and that messages for odorant receptors and OcNC1 were first seen at E19. However, other in situ hybridization\textsuperscript{42,43} and immunocytochemical studies\textsuperscript{28} showed that odor receptors, OcNC1, AC III and olfactory marker protein are all expressed earlier than G\textsubscript{olf}. These differences may reflect variation between techniques and differences generated by analysing different tissue samples, as some proteins, such as OCNC1, are also expressed in the brain.\textsuperscript{7}

The early expression of OcNC1 protein during ORN development may provide a molecular basis for the early electrophysiological responses seen by Gesteland et al.\textsuperscript{17} In the rat, electro-olfactograms could be recorded as early as E14 and single units by approximately E16. Individual ORNs could respond to many odorants non-selectively, and cells appeared to become more selective just prior to birth. These and our data suggest that cNGs may be responsible for current generation at times when some other components, notably G\textsubscript{olf} and part of the odor receptors, of the signaling cascade are not yet present.\textsuperscript{5,28,43} As not all signaling components are yet correctly positioned to perceive odorants in developing ORNs, the normal odorant-mediated pathway for depolarization cannot occur. Therefore, an alternative pathway to achieve depolarization may exist in developing olfactory neurons. In particular, nitric oxide synthase, which is only present at high levels in ORNs during development and regeneration,\textsuperscript{40} may generate cyclic GMP to open the cNG channels in the absence of odorants. Alternatively, nitric oxide may affect channel opening directly.\textsuperscript{5} The transformation of responsivity during development would further imply that the control of transcription of olfactory signal transduction components is not straightforward or mediated en bloc by the same transcription factors, but instead occurs in a sequential manner. Finally, OcNC1 immunoreactivity seems to be transiently present in precursor cells close to the basal lamina of the epithelium during fetal development (e.g., E14; Fig. 4C) and following lesioning (e.g., Fig. 5G), suggesting that OcNC1 expression may even occur prior to differentiation.

**Axons**

Although OcNC1 immunoreactivity is most intense in the ciliary layer, significant signal was also seen in ORN axons, also associated with glomeruli in the olfactory bulb in normal adults (Fig. 2). This suggests a role for OcNC1 in transduction beyond the initial events of odorant detection, such as axon guidance or targeting. The fact that axonal protein expression of OcNC1 in ORNs occurs early during both embryogenesis and regeneration may mean that OcNC1 plays a role in the development or maturation of ORNs.

Sullivan et al.\textsuperscript{43} interpreted the early expression of OcNC1 to represent migrating luteinizing hormone-releasing hormone (LHRH) neurons and/or olfactory axons. Those OcNC1-positive cells seen below the basal lamina of the olfactory epithelium may indeed represent LHRH neurons. However, most immunostaining appeared to be present uniformly in the axon bundles, suggesting that the majority of axons expressed OcNC1, as opposed to the minority one might expect if OcNC1 were only associated with migrating LHRH-expressing neurons.\textsuperscript{43}

The embryonic expression in the axons of OcNC1 may mean that this channel also functions, aside from its well-described role in olfactory signaling, to define neuroconnections and that it is important for target recognition.\textsuperscript{43} A similar role has been proposed for odor receptors, which are also expressed early on in ORN axons.\textsuperscript{19,43} AC III is not likely to play a role in this. Current and previous data\textsuperscript{19,28} showed that AC III is never expressed at the level of the axons, but at the epithelial surface only. This is true as soon as AC III begins to appear. Like AC III, G\textsubscript{olf} is also an unlikely candidate for playing a role in axon pathfinding, as it begins to become expressed somewhat later than both cNG channels and AC III.\textsuperscript{5,28,43} At the level of the axons, OcNC1 may respond to cyclic nucleotides to modulate calcium concentrations\textsuperscript{16} to support axonal maintenance, as well as outgrowth or target innervation.\textsuperscript{7}

**CONCLUSIONS**

We have characterized an antibody to OcNC1, which was used for immunolocalization of OcNC1 in normal adult, regenerating and embryonic olfactory epithelium. The expression of OcNC1 protein occurs early in olfactory development and during regeneration after lesion of the olfactory bulb. Besides its expression in ORN cilia, OcNC1 immunoreactivity is visualized in axons. The spatiotemporal pattern of expression of OcNC1 suggests that it could be involved in maintenance or development of axonal projections, and that it may function in activity-driven developmental events and maintenance of ORNs, in addition to its role in olfactory signaling.

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