THE ROYAL SOCIETY

*doi* 10.1098/rstb.2000.0670

# Immunohistochemical evidence for the $Na^+/Ca^{2+}$ exchanger in squid olfactory neurons

**Mary T. Lucero*, Wei Huang and Tu Dang**

*Department of Physiology, School of Medicine, University of Utah, Room 155, 410 Chipeta Way, Salt Lake City, UT 84108-1297, USA*

The olfactory organs from the squid *Lolliguncula brevis* are composed of a pseudostratified epithelium containing five morphological subtypes of chemosensory neurons and ciliated support cells. Physiological recordings have been made from two of the subtypes and only the type 4 neuron has been studied in detail. Odour-stimulated increases in intracellular calcium and rapid activation of an electrogenic $Na^+/Ca^{2+}$ exchanger current in type 4 neurons suggest that the exchanger proteins are localized very close to the transduction machinery. Electrophysiological studies have shown that olfactory signal transduction takes place in the apical ciliary regions of olfactory neurons. Using polyclonal antiserum against squid $Na^+/Ca^{2+}$ proteins, we observed specific staining in the ciliary region of cells that resemble type 2, 3, 4 and 5 neurons. Staining was also observed in axon bundles, and in muscle tissue. Collectively, these data support the model that $Na^+/Ca^{2+}$ exchanger proteins are localized to transduction machinery in cilia of type 4 neurons and suggest that the other olfactory subtypes also use $Ca^{2+}$ during chemosensory responses.

**Keywords:** cephalopod; chemosensory; olfaction

## 1. INTRODUCTION

Squid and other coleoid cephalopods are highly evolved invertebrates that use visual, tactile, proprioceptive and chemosensory receptors to collect information about their complex and ever-changing aquatic environment. Our work has focused on the squid olfactory organs, paired sensory structures located posteriorly and slightly ventrally to each eye. Behavioural studies in our own and other laboratories have shown that certain aquatic odorants such as amino acids are attractive to cephalopods, while quaternary ammonium ions, dopamine and squid ink are aversive and elicit escape jetting responses (Gilly & Lucero 1992). Aquatic chemical signals also appear to play a role in cephalopod mate selection (Boal & Golden 1999). Thus, the squid olfactory system provides many of the same chemosensory functions as in other animals: prey or food detection, predator and toxic substance avoidance, and mate selection.

Our recent studies have focused on the mechanisms involved in transducing the chemical odorant signal into an electrical signal that is transmitted to the brain. Squid possess five types of olfactory sensory neurons (OSNs) and electrical recordings have been made from two morphologically distinct subtypes, the type 2 and type 4 (as described by Emery (1975)). We found that both are electrically excitable (Lucero *et al.* 1992). We investigated the voltage-gated Na channels in the type 4 OSNs and found tetrodotoxin (TTX)-sensitive, rapidly inactivating $Na^+$ currents, which produce regenerative action potentials as well as persistent TTX-sensitive $Na^+$ currents that may be important in setting the tonic level of activity (Chen & Lucero 1999). At least three types of K channels are also present in the type 4 OSNs: a delayed rectifier K channel blocked by tetraethylammonium, a rapidly inactivating K channel blocked by 4-aminopyridine, and a $Ca^{2+}$-activated K channel blocked by charybdotoxin (Lucero & Chen 1997). In addition to voltage-gated channels, squid OSNs produce either depolarizing or hyperpolarizing receptor potentials when exposed to specific odours. The behaviourally aversive odours, including squid ink, L-dopa, dopamine and betaine, elicit hyperpolarizing receptor potentials while an attractive odour, L-glutamate, activates a depolarizing receptor potential.

Interestingly, both dopamine and glutamate, which activate opposing effector channels, increase intracellular $Ca^{2+}$ $[Ca^{2+}]_i$ (Piper & Lucero 1999). $Ca^{2+}$ can act as both an electrical and a chemical signal, and therefore its cellular concentrations are tightly controlled. $Ca^{2+}$ pumps on mitochondria and endoplasmic reticulum, as well as $Na^+/Ca^{2+}$ exchangers (NCXs) present in plasma membranes, play important roles in maintaining low $[Ca^{2+}]_i$. In vertebrates, NCXs have been localized to the cilia and dendritic knobs of OSNs where they rapidly expel $Ca^{2+}$ following odour-mediated increases (Noe *et al.* 1997). In perforated-patch recordings of squid type 4 OSNs, we found that an electrogenic NCX is activated during glutamate-induced odour responses (Danaceau & Lucero 2000*a*). In type 4 OSNs, glutamate application activates an influx of $Ca^{2+}$ that is rapidly extruded by the NCX. The standard stoichiometry of three $Na^+$ in for each $Ca^{2+}$ out results in a net inward exchanger current which amplifies the glutamate-induced receptor current.

Our observations of an NCX current in squid type 4 ORNs led us to question whether the exchanger proteins

*Author for correspondence (mary.lucero@m.cc.utah.edu).

 

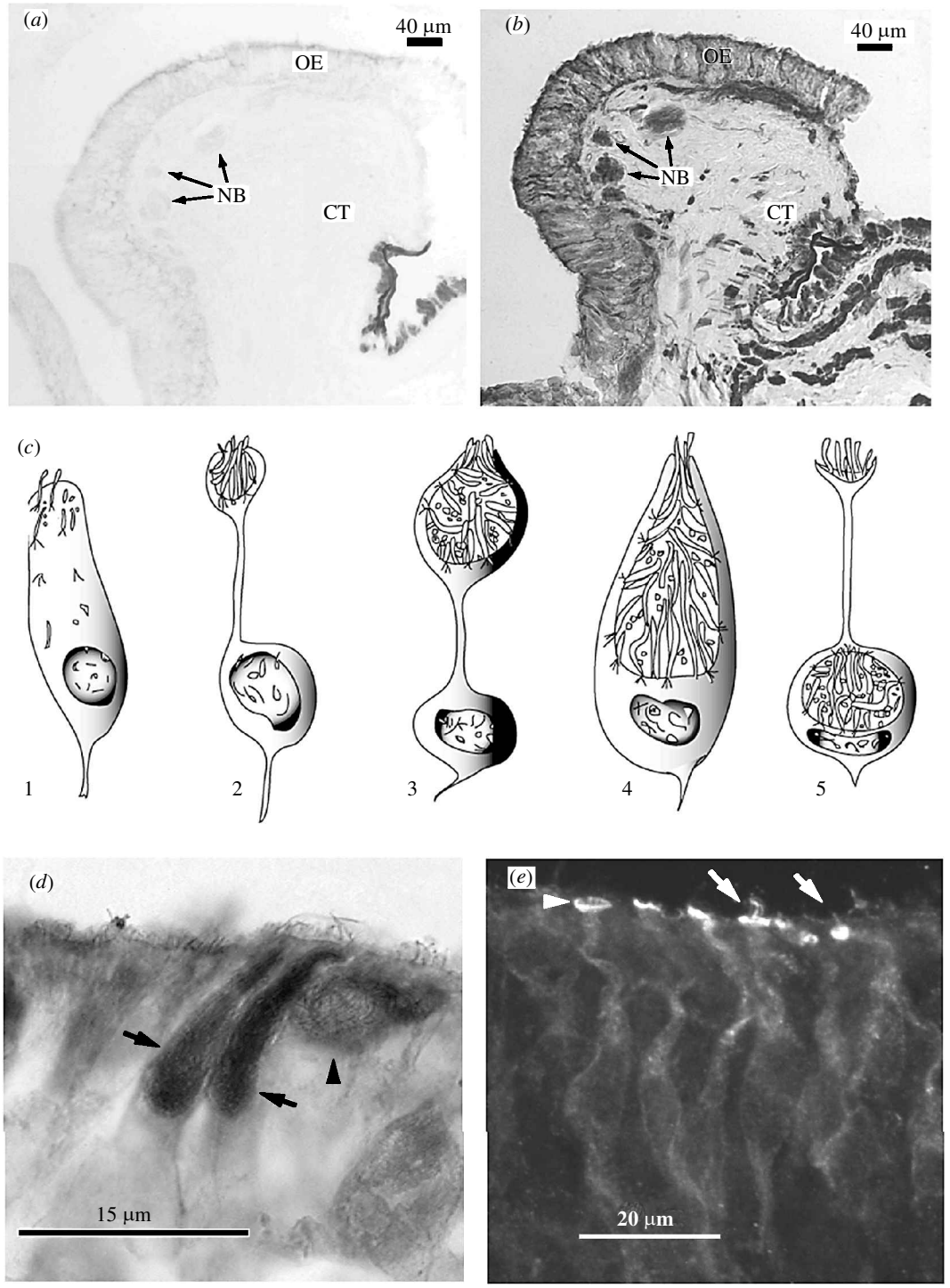


Figure 1. Immunohistochemical localization of NCX protein in squid OE. (*a*) Negative control of squid olfactory epithelium with zero primary antiserum, 1:200 goat anti-rabbit secondary antibody, treated with DAB for 60 s. (*b*) Anti-NCX staining at 1:10 000 dilution and 60 s DAB from a section on the same slide. (*c*) Line-drawing of the five subtypes of squid OSNs adapted from Emery (1975). Numbers indicate cell type. (*d*) Anti-NCX staining (1:10 000; DAB 80 s) of two type 4 OSNs (arrows). The ciliary pocket of a type 3 neuron is indicated by the arrowhead. (*e*) Anti-NCX staining (1:10 000; fluorescein–avidin 1:100, 1 h) of type 2 or type 5 OSNs. Arrows indicate the cilia, arrowhead indicates a ciliary region. With permission, Emery (1975), *Tissue and Cell*.

are concentrated in the cilia and apical dendritic processes of squid OSNs, and whether the other OSN cell types, which have only been characterized morphologically, contain NCX proteins. The presence of NCX in ciliary regions of the other squid OSN types would support the idea that these cells also use $Ca^{2+}$ increases in cilia during odour signal transduction. We found that the polyclonal antiserum generated against a peptide fragment spanning the intracellular loop from cloned squid neuronal NCX, NCX-SQ1 (He *et al.* 1998), produced intense staining on the ciliary and dendritic regions of cells that resemble type 2, 3, 4 and 5 OSNs.

Specific staining was also observed in olfactory nerve bundles (NBs) and in muscle surrounding the olfactory organ. We were unable to unequivocally identify type 1 cells and therefore cannot say whether or not the antibody recognized NCX in these cells.

## 2. MATERIAL AND METHODS

Juvenile squid, *Lolliguncula brevis* (4–5 cm mantle length) were obtained from the National Resource Center for Cephalopods (University of Texas Medical Branch at Galveston, TX). Animals were anaesthetized with 50 mM $MgCl_2$ artificial seawater and killed by decapitation. Olfactory organs from nine animals were dissected and pinned into a dish containing ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with added sucrose to bring the osmolality to 780 mOsm $kg^{-1}$. Following a 1 h fixation, olfactory organs were rinsed three times with 15% sucrose and cryoprotected overnight in 30% sucrose. Squid olfactory organs ranged from 550 to 750 µm in length, and 46 to 63 serial 12 µm-thick cryostat sections were cut from each. Sections were collected on glass slides, dried, and treated with 0.3% hydrogen peroxide in 0.3% Triton X-100, 0.1 M PBS for 10 min, and rinsed, and then with 10% normal goat serum for 30 min, and avidin and biotin blocked for 15 min each (Blocking Kit; Vector Laboratories, Burlingame, CA, USA), and stained overnight with 1:2500 to 1:20 000 dilutions of rabbit NCX-SQ1 antiserum (anti-NCX; a generous gift from Dr K. Philipson, University of California at Los Angeles, CA, USA). The primary antiserum was diluted in a solution containing 2.5 mg bovine serum albumin per 500 µl 0.3% Triton X-100, 0.1 M PBS. Biotinylated goat anti-rabbit secondary antibody (1:200 dilution) was applied for 30 min. Immunoreactivity was visualized using either a peroxidase-based stain or an avidin-based fluorescein. For the former, a Vectastain[1] Elite ABC-Peroxidase kit (1:50 dilution; Vector Laboratories) and 3,3′-diaminobenzidine (DAB) was applied for 60–80 s. In the fluorescent labelling, a 1:100 dilution of Fluorescein Avidin DCS (Vector Laboratories) was applied for 30–60 min and washed for 10 min. Low-power digital photographs were obtained using an Olympus DP-10 Color digital camera system and montages were assembled using Photoshop[1], v. 5.5. High-magnification images were obtained using either light microscopy for DAB-stained tissue or confocal laser scanning microscopy (Zeiss LSM510, Axioskop II) for fluorescein staining. The same 63× oil Plan-Apochromat lens with a 1.4 numerical aperture was used for both light and confocal microscopy. Negative controls included omission of primary or secondary antibodies.

## 3. RESULTS

Figure 1*a* shows a low-magnification view of a negative control obtained by omission of anti-NCX but inclusion of secondary antibody. The olfactory epithelium (OE) had very light, non-specific staining that darkened slightly near the apical border. Dark staining appeared along a ridge of non-sensory tissue posterior to the OE. This staining pattern was similar in all controls that included secondary antibody. In negative controls that omitted both primary and secondary antibodies, there was no staining (data not shown). Figure 1*b* shows a section taken 12 µm from the control and stained with 1:10 000 anti-NCX. Dark staining appeared in cells of the OE as well as in NBs and muscle tissue (data not shown). The connective tissue (CT) underlying the OE was unstained.

Figure 1*c* shows a drawing of the five sensory cell types. It is based on a similar drawing by Emery (1975) and on our own observations. The type 1 cells show few distinct characteristics and are thus difficult to identify at the light microscopy level. The type 2 cells are similar in shape to vertebrate OSNs and show a ciliated apical dendritic swelling, a long thin dendrite and oval soma. Type 3 cells have a larger apical cup filled with cilia, a thin dendritic neck and a smaller basal soma. Type 4 cells, also named pyriform receptors for their pear shape, contain a large distinctive cone-shaped apical pocket of cilia, which connects directly to the basal soma. Type 5 cells have a large round ciliary pocket located near the basement membrane, a long thin dendritic neck and a ciliated apical plate.

Serial sections of 12 squid olfactory organs were stained and analysed. We found that different cell types appeared in different regions of the OE, with overlapping boundaries. Thus, multiple cell types could be visualized in a single section, but the distribution of cell types varied according to where the section was taken in the epithelium. Figure 1*d* shows a higher-magnification light microscopy image of anti-NCX immunoreactivity in a section containing two type 4 neurons and the apical ciliary pocket typical of a type 3 neuron. DAB staining is seen in the ciliary region as indicated by the horizontal arrows. Staining is also seen in the ciliary region of a putative type 3 neuron as indicated by the arrowhead. The dendritic neck and soma of the type 3 neuron were not present in this plane of section. Figure 1*e* shows a single projection of seven confocal laser-scanned fluorescent images. NCX immunoreactivity is seen in what appear to be type 2 neurons based on their morphology and apical location in the epithelium; however, the apical process is more flattened (arrowhead), similar to type 5 neurons. The brightest staining was obtained in the apical ciliated regions with lighter diffuse staining outlining the dendritic neck and soma. The arrows point to the cilia extending from the apical pocket. Specific staining was also seen in the apical regions of more typical type 2 neurons as well as the apical plate and ciliated region of type 5 neurons (data not shown).

## 4. DISCUSSION

The high level of dilution (1:10 000) and the low background staining in the CT (figure 1*a*,*b*) suggest that the squid anti-NCX antibody is recognizing specific epitopes in squid OE. Specificity of the antiserum for the NCX protein was previously demonstrated by Western blot analysis both of native NCX protein from squid stellate ganglia and of the membrane protein fraction of NCX-SQ1 cRNA-injected *Xenopus* oocytes (He *et al.* 1998). In the present study, we found anti-NCX immunoreactivity in the ciliary regions of squid OSNs as well as in OSN axon bundles and in muscle tissue (data not shown). The dark staining in OSN axons suggests that these small unmyelinated axons require exchanger activity to maintain low $[Ca^{2+}]_i$. Immunoreactivity in the muscle, although not previously reported, was not surprising because NCX plays a major role in extruding $Ca^{2+}$

following contraction in many muscle types. The localization of the staining to the ciliary pocket of type 4 neurons correlates well with our previous electrophysiological studies on these cells. We applied the odorant L-glutamate to specific regions of type 4 neurons and found that apical stimulation near the entrance to the ciliary pocket activated the largest receptor currents (Danaceau & Lucero 2000*b*). These data suggested that as with OSNs from other species, the odour transduction machinery is localized to the cilia. The present work extends our previous studies to the other OSN cell types that have not been characterized physiologically. The confocal images showed neurons with somas similar to type 2 neurons but with apical pockets that were more flattened and similar to type 5 neurons. Further study will be necessary to determine whether these cells represent an additional cell type or a developmental intermediate. The intense staining in the ciliary regions of putative type 2, 3 and 5 neurons indicates that it will be interesting to look for odour-induced changes in $[Ca^{2+}]_i$ in these cells.

We thank Dr L. Stensaas for his help and advice, J. D. Lucero for technical assistance, and Dr K. D. Philipson for providing the NCX-SQ1 antiserum. We thank the National Resource Center for Cephalopods for providing squid. We also thank Dr S. Collin and Dr J. Marshall for organizing an excellent meeting. This work was supported by National Institutes of Health (NIH) National Institute on Deafness and Other Communication Disorders grant no. DC02587 to M.T.L. and NIH National Institute of Neurological Disorders and Stroke grant no. NS07938.

## REFERENCES

Boal, J. G. & Golden, D. K. 1999 Distance chemoreception in the common cuttlefish, *Sepia officinalis* (Mollusca: Cephalopoda). *J. Exp. Mar. Biol. Ecol.* **235**, 307–317.

Chen, N. & Lucero, M. T. 1999 Transient and persistent tetrodotoxin-sensitive sodium currents in squid olfactory receptor neurons. *J. Comp. Physiol.* A **184**, 63–72.

Danaceau, J. P. & Lucero, M. T. 2000*a* Electrogenic $Na^+/Ca^{2+}$ exchange: a novel amplification step in squid olfactory transduction. *J. Gen. Physiol.* **115**, 759–768.

Danaceau, J. P. & Lucero, M. T. 2000*b* Mixture interactions of glutamate and betaine in single squid olfactory neurons. *J. Comp. Physiol.* A **186**, 57–67.

Emery, D. G. 1975 The histology and fine structure of the olfactory organ of the squid *Lolliguncula brevis* Blainville. *Tissue Cell* **7**, 357–367.

Gilly, W. F. & Lucero, M. T. 1992 Behavioral responses to chemical stimulation of the olfactory organ in the squid *Loligo opalescens*. *J. Exp. Biol.* **162**, 209–229.

He, Z., Tong, Q., Quednau, B. D., Philipson, K. D. & Hilgemann, D. W. 1998 Cloning, expression, and characterization of the squid $Na^+$–$Ca^{2+}$ exchanger (NCX-SQ1). *J. Gen. Physiol.* **111**, 857–873.

Lucero, M. T. & Chen, N. S. 1997 Characterization of voltage- and $Ca^{2+}$-activated $K^+$ channels in squid olfactory receptor neurons. *J. Exp. Biol.* **200**, 1571–1586.

Lucero, M. T., Horrigan, F. T. & Gilly, W. F. 1992 Electrical responses to chemical stimulation of squid olfactory receptor cells. *J. Exp. Biol.* **162**, 231–249.

Noe, J., Tareilus, E., Boekhoff, I. & Breer, H. 1997 Sodium/calcium exchanger in rat olfactory neurons. *Neurochem. Int.* **30**, 523–531.

Piper, D. R. & Lucero, M. T. 1999 Calcium signalling in squid olfactory receptor neurons. *Biol. Signals Recept.* **8**, 329–337.