Sensory sea slugs
Towards decoding the molecular toolkit required for a mollusk to smell

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Mollusks are a large and diverse group of aquatic and terrestrial animals that rely heavily on chemical communication. Aplysia is an excellent model in which to investigate and develop breakthrough principles into the molecular aspects of chemoreception in mollusks. We recently identified a large family of rhodopsin-like G-protein coupled receptors expressed in the chemosensory rhinophore of Aplysia that may be key components of sensory detection. Here, we summarize these findings and provide further insight into the molecular olfactory toolkit used by Aplysia, by taking advantage of our knowledge of their attraction pheromones. Our characterization of rhinophore genes upregulated following pheromone stimulation helps explain the dynamics of olfactory gene expression following chemical stimulation.

Chemical signals are known to convey a remarkable amount of information. To understand how this occurs, research has been devoted towards identifying the compounds that are involved, how they are produced, and how olfactory organs are able to ‘decode’ information from chemical signals. Although well studied in the broad behavioral and physiological context, there is relatively little knowledge of how olfaction works at the molecular level in a large diversity of aquatic invertebrate animals, from corals and sponges to octopuses and oysters. Incredibly, despite mollusks representing the second most abundant living phyla and the most diverse,1 it is only now that we are starting to elucidate the molecular toolkit used by mollusks to enable them to smell.

The seminal olfactory discoveries obtained at the molecular and cellular level from the mouse (representing chordates), Drosophila (arthropods) and Caenorhabditis (nematodes) provides compelling evidence for the necessity to study scientifically tractable animals, prior to exploration into those of commercial relevance. For example, the identification of olfactory receptors in Drosophila in 1999,2 was pivotal to the research that led to the multitude of olfactory-related entomological discoveries within the insect group, including the moth, honeybee and mosquito. We need to interrogate the molluscan olfactory system in a similar fashion.

The first stage of olfaction is handled by the sensory neurons in the olfactory epithelium where they express genes that encode products devoted to binding environmental odorants and transferring this information intracellular. In mammals, detection of environmental chemicals results from binding of molecules to one or more of a large family of seven-transmembrane domain rhodopsin and metabotropic G-protein coupled receptors (GPCRs) that are expressed almost exclusively on the surface of sensory neurons within the main olfactory epithelium and vomeronasal organ.3 Meanwhile, Benton et al. 2009,4 were first to discover that variant ionotropic receptors were chemosensory in insects and appear to enhance processing speed. Insect olfactory systems respond to a smaller range of odorants than terrestrial vertebrates, and their genomes in turn encode a smaller family of receptors.5
We use Aplysia as our model system as it is almost unique amongst mollusks in that it has been highly characterized at multiple levels and therefore fulfills many of the requirements necessary to investigate the molecular basis of olfaction. First, various aspects of the structure and function of olfactory organs, life history, such as larval development and laboratory rearing, have been well described. Second, the availability of multiple expressed sequence tag transcriptome libraries, including central neuron transcripts, as well as the Aplysia genome, enables detailed genetic analyses. Finally and perhaps most important, they have no acoustic sense and little to no ability to recognize objects by vision, instead, their world is chemically driven and many key physiological and behavioral events must be mediated by secreted water-soluble chemicals, leading to aggregation, habitat selection, defense and courtship and mating. For example, induction of larval metamorphosis requires a chemical cue from algal sources. Also, mate attraction and subsequent mating is stimulated by the release of conspecific water-borne sexual pheromones. Experimental attraction assays followed by molecular and biochemical analysis were important to demonstrate that Aplysia had developed a potent solution to attracting a mate by releasing attraction pheromones, consisting of four small proteins that were subsequently named attractin, enticin, temptin and seductin (Fig. 1).

At the anatomical level, odorant detection is achieved by a pair of rhinophore, specialized anterior sensory organs on the dorsal surface of the head that act as a finely tuned nose. These also allow animals to locate and discriminate food, conspecific pheromones and predators. From the rhinophore sensory epithelium we identified genes encoding proteins that may directly or indirectly be involved in Aplysia olfaction, including, a large multi-gene receptor family encoding rhodopsin-like GPCRs (>90), identified by analyzing the unfinished Aplysia genome database and using algorithms that identify genes encoding multi-transmembrane proteins. Phylogenetic analysis revealed they consist of three distinct monophyletic protein subfamilies and expression analysis using laser capture microdissection-reverse transcription-polymerase chain reaction and sequencing revealed expression of at least some of these genes within the sensory neurons located within the rhinophore groove. These gene families encode novel candidate chemoreceptors that show only distant identity with known vertebrate, insect and nematode chemoreceptors. Based on molecular genetic and expression analyses, Aplysia G proteins, an IP$_3$ receptor, and a phospholipase C, are expressed and localize to the olfactory sensory epithelium of the
This suggests that the receptors identified may be G-protein coupled, similar to that of vertebrates. However, Aplysia also appear to have their own variant ionotropic receptors, similar to that found in insects, some of which are found in rhinophore. (V. Croset, S. Cummins, R. Benton, in preparation) Further understanding of these will significantly expand our understanding of how these animals detect water-soluble chemicals.

Our knowledge of the attraction pheromones and site of pheromone detection provides an excellent opportunity to gain insight into the dynamics of olfactory gene expression. Towards achieving this, we performed a suppressive subtraction hybridization, to identify those genes upregulated following pheromone exposure (method summarized in Fig. 2A). In total, 40 clones were sequenced and identification of unigenes achieved by blastn or tblastn of the NCBI database, resulting in 27 significant (cut-off of $10^{-6}$ E-value) matches. These included complementary DNAs (cDNAs) encoding an isoform of $\alpha$-tubulin and actin, the neuropeptide schistosomin, an Aplysia temptin-like peptide, an inhibition of apoptosis protein and several cDNAs encoding products involved in translation, including ribosomal protein L18a (Table 1). The identified temptin-like gene shows similarity with the previously described temptin pheromone only within the conserved calcium-binding EGF-like domain. The cytoskeletal $\alpha$-tubulin is known to be present not only in the cell bodies but also in the distal neurites of cultured Aplysia californica sensory neurons, where serotonin can induce its specific translation. Thirteen unigenes remain unidentified. Of those, two cDNAs contain a 303 base pair open reading frame that encode a 91 amino acid precursor predicted to be

| Gene name                      | BP  | Search | Species              | NCBI identifier | Probability | Genbank   |
|-------------------------------|-----|--------|----------------------|-----------------|-------------|-----------|
| Adac-schistosomin-like        | 286 | blastn | Aplysia californica  | AY833132.1      | 1.00E-93    | HM191485  |
| Adac-$\alpha$-tubulin         | 600 | blastn | Aplysia californica  | AF481055.1      | 4.00E-45    | HM191486  |
| Adac-temptin-like             | 783 | tblastn| Aplysia brasiliana   | AYS82745.1      | 9.00E-30    | HM191487  |
| Adac-apoptosis inhibitor protein | 575 | tblastn| Gallus gallus        | XM_471413.2     | 3.00E-25    | HM191488  |
| Adac-actin                    | 576 | blastn | Aplysia californica  | X52868.1        | 4.00E-45    | HM191489  |
| Adac-ribosomal               | 465 | blastn | Aplysia californica  | E514513.1       | 7.00E-32    | HM191484  |
| Adac-thioredoxin-like         | 763 | blastn | Mus musculus         | NM_025299.3     | 3.00E-32    | HM191490  |

Figure 2. Identification of Aplysia rhinophore genes upregulated following exposure to attraction pheromones. (A) Summary of experimental procedure for gene identification. (B) Nucleotide and predicted amino acid sequences of a novel gene identified (clones 16 and 31), encoding a predicted secreted peptide. An arrow denotes the predicted signal sequence cleavage site, a dibasic cleavage site is underlined ($K_R$) and cysteines are highlighted in grey. A cartoon schematic representation is shown below. Met, Methionine; Signal, secretion signal sequence. (C) Comparative amino acid alignment of Aplysia dactylomela (Genbank: HM191483) and Aplysia kurodai (Genbank: EY420369) novel gene product. Amino acids that are identical are shaded in black.
post-translationally cleaved and secreted extracellular (Fig. 2B). A similar gene has also been identified from the *Aplysia kurodai* neural transcriptome 

(Fig. 2C), suggesting a neuronal origin and conservation of function. All of the genes identified from this subtraction may be involved in pheromone detection, processing or modulation.

The identification of these, along with the candidate chemoreceptors, will enable their use as tools to further investigate the dynamics of olfactory gene expression and provide a critical bridge between olfactory genes, circuits and behaviour in the broad evolutionary context. As a gastropod mollusk, *Aplysia* does share many biological characteristics with economically important aquaculture mollusks, including abalone, as well as pest mollusks such as Pomacea, Cernuella, Theba (all crop pests), and Biomphalaria (intermediate parasite host). Indeed, further understanding of the genetic basis of smell in these animals using olfactory principles derived from the *Aplysia* work may provide opportunities to develop agents that could enhance or disrupt chemical signaling.

**Methods**

*Aplysia dactylomela* (12 animals collected from Caloundra, Queensland) were divided into three groups containing four animals each and kept in individual cages containing 2 L filtered sea water. Treatments included: Group (1) Animals were injected with egg-laying hormone (40 pg/ml). Sixty minutes later, all egg cords were transferred to 40 ml fresh filtered sea water and gently shaken for 30 min. Egg eluate (containing pheromones) was then taken for; Group (2) Animals were exposed to 10 ml of filtered sea water. At 15 min, rhinophore were removed from the base, combined and immediately frozen on dry ice. These comprised pheromone-stimulated rhinophore (PSR). Group (3) Animals were exposed to 10 ml of filtered sea water. At 15 min, rhinophore were removed from the base, combined and immediately frozen on dry ice. These comprised pheromone-stimulated rhinophore (PSR).

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