

Structural and functional analysis of *Aplysia* attractins, a family of water-borne protein pheromones with interspecific attractiveness

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Mate attraction in *Aplysia* involves a long-distance water-borne signal (the protein pheromone attractin), which is released during egg laying. *Aplysia californica* attractin attracts species that produce closely related attractins, such as *Aplysia brasiliana*, whose geographic distribution does not overlap that of *A. californica*. This finding suggests that other mollusks release attractin-related pheromones to form and maintain breeding aggregations. We describe four additional members of the attractin family: *A. brasiliana*, *Aplysia fasciata*, *Aplysia depilans* (which aggregates with *A. fasciata* aggregations), and *Aplysia vaccaria* (which aggregates with *A. californica* aggregations). On the basis of their sequence similarity with *A. californica* attractin, the attractin proteins fall into two groups: *A. californica*, *A. brasiliana*, and *A. fasciata* (91–95% identity), and *A. depilans* and *A. vaccaria* (41–43% identity). The sequence similarity within the attractin family, the conserved six cysteines, and the compact fold of the NMR solution structure of *A. californica* attractin suggest a common fold for this pheromone family containing two antiparallel helices. The second helix contains the IEECKTS sequence conserved in *Aplysia* attractins. Mutating surface-exposed charged residues within this heptapeptide sequence abolishes attractin activity, suggesting that the second helix is an essential part of the receptor-binding interface.

Pheromones are thought to play an important role in coordinating reproductive behavior in many aquatic species, but only two water-borne peptide pheromones have been chemically and behaviorally characterized in invertebrates in detail: attractin from the marine opisthobranch gastropod mollusk *Aplysia californica* (1–5) and nereithione from the marine polychaete worm *Nereis* (6, 7). *A. californica* is a simultaneous hermaphrodite that does not normally fertilize its own eggs. Field studies have shown that *A. californica* are solitary animals most of the year, but during the reproductive season, they move into breeding aggregations (“brothels”), mate, and lay eggs. The aggregations usually occur where egg cordons are laid, often deposited one on top of another. Egg cordons are attractive in T-maze assays, and attraction is due to chemical, rather than visual, cues (8).

After ovulation, eggs travel to the fertilization chamber, which is surrounded by the albumen gland (refs. 9 and 10, and Fig. 4 and *Supporting Text*, which are published as supporting information on the PNAS web site). This gland packages eggs into a long string-like cordon, which has a high surface-to-volume ratio. Egg cordons are considered to be a source of both water-borne and contact pheromones, which attract animals to the area and induce them to mate and lay eggs (8, 11). Of the pheromones produced by the albumen gland, only attractin has been identified.

Attractin, a 58-residue protein with three intramolecular disulfide bonds (2, 3), was isolated from *A. californica*, a Pacific

Coast species, but was bioassayed with *Aplysia brasiliana*, a species found in the Gulf of Mexico, for several reasons: *A. californica* locomote slowly or not at all during T-maze attraction assays and tend to crawl out of T-mazes before being exposed to chemical test stimuli; *A. brasiliana* is genetically closely related to *A. californica* despite their different geographic locations; and *A. brasiliana* are rapid swimmers that often reach test stimuli in T-maze assays within 10–15 seconds. *A. brasiliana* attractin differs from *A. californica* attractin at only three positions (this study). *A. californica* attractin is attractive to *A. brasiliana*, reduces the latency to mating, and increases the time spent mating; the peptide also stimulates hermaphroditic mating, reduces the latency to hermaphroditic mating, and increases the duration of the activity (2, 5). These effects may result from attractin stimulating both animals to mate as males. These findings are consistent with field observations in which multiple *Aplysia* species are often found in the same egg-laying and mating aggregations, for example, *A. californica* and *Aplysia vaccaria* from the Pacific Coast (12, 13), which occasionally mate with each other (S. LePage, Marine Research and Educational Products, Carlsbad, CA, personal communication); and *Aplysia fasciata* and *Aplysia depilans* from the Mediterranean Sea, in which egg-laying and mating aggregations have been repeatedly observed (14), although interspecific mating has not. T-maze attraction assays have shown that attractin acts as part of a bouquet of water-borne odors: (i) egg cordons are attractive in the absence of *Aplysia*; and (ii) attractin is attractive only when *Aplysia* are part of the stimulus (5); the animal does not need to be a conspecific.

In previous work (3, 4), we used mass spectroscopy and NMR to determine the three disulfide bonds of recombinant *A. californica* attractin (C4–C41, C13–C33, and C20–C26). The NMR solution structure of *Aplysia* attractin has a compact folded structure with two antiparallel helices (4). *Aplysia* attractin has a different helix packing compared with the peptide pheromones from the ciliated protozoan *Euplotes* (15, 16). However, the second helix of attractin, which contains a highly conserved sequence motif, IEECKTS, is structurally similar to the third helix of the *Euplotes raikovi* pheromones (Er), which is involved

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Abbreviations: ASW, artificial seawater; Er, *Euplotes raikovi* pheromone.

Data deposition: The attractin sequences have been deposited in the Protein Information Resource database, <http://pir.georgetown.edu> [accession nos. A59061 (*A. californica*), B59060 (*A. brasiliana*), A59447 (*A. fasciata*), A59446 (*A. depilans*), and A59424 (*A. vaccaria*)].

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Fig. 1. Comparison of amino acid sequences of *A. californica*, *A. brasiliana*, *A. fasciata*, *A. vaccaria*, and *A. depilans* attractin. Identities are shaded black. Asterisks indicate amino acids (Glu-31, Glu-32, and Lys-34) substituted in one *A. californica* attractin triple mutant; plus signs indicate amino acids (Asp-5, Asp-22, and Glu-39) substituted in a second *A. californica* attractin triple mutant.

in receptor recognition in the ciliated protozoan. In this study, we tested the hypothesis that the charged surface-exposed residues of this motif are essential for attraction by bioassaying mutant proteins in T-maze attraction assays.

We hypothesized that each species secretes a unique attractin. In this study, therefore, we characterized attractins from four *Aplysia* species and have identified the residues that are important for attraction activity. We conclude that, in contrast to the species-specific, water-borne peptide pheromonal attractants in amphibians (17–20), the attractins are an unusual family of protein pheromones in invertebrates that are not species specific.

Materials and Methods

Animals. Animals were obtained from the following locations: *A. californica* and *A. vaccaria* (Pacific Coast of California; Marine Research and Educational Products, Escondido, CA); *A. fasciata* and *A. depilans* (Mediterranean coast of Israel); *A. brasiliana* (Gulf of Mexico, South Padre Island, TX).

Characterization of Attractin-Related Proteins. Albumen glands were removed from *A. brasiliana*, *A. fasciata*, *A. vaccaria*, and *A. depilans*, extracted, and purified by RP-HPLC, and attractin was characterized as described for *A. californica* attractin (2). Attractin and related fragments were subjected to N-terminal sequence analysis. In addition, we sequenced fragments obtained upon digestion with endoproteinase Glu-C (21), cyanogen bromide, or endoproteinase Lys-C. *A. vaccaria* attractin residues 49–52 and *A. depilans* attractin residues 25, 49, and 50 were determined by 3'-RACE (see *Supporting Text*).

Protein Expression and Site-Directed Mutagenesis. *A. californica* attractin was expressed and purified as described (3, 5). By using *A. californica* attractin cDNA (1), two attractin mutants were generated and expressed in a baculovirus expression system. The first triple mutant contained mutations in the three charged residues in the sequence IIEECKTS that are strictly conserved in all five *Aplysia* species: attractin E31Q, E32Q, K34Q. The second triple mutant contained mutations in three other charged residues that are either strictly conserved or contain conservative substitutions: attractin D5A, D22A, E39A (see *Supporting Text*).

Egg Cordon Elutions. To demonstrate that egg laying would trigger attractin secretion, egg laying was induced in *A. brasiliana* by injecting *A. californica* atrial gland extract (9), which contains egg-laying hormone-related peptides (22–24), into the hemocoel; egg laying in *A. vaccaria* was induced by injecting *A. vaccaria* atrial gland extract. One hour after injection and at 30-min intervals thereafter, egg cordons were removed, briefly rinsed in fresh artificial seawater (ASW), and transferred to fresh ASW for elution. Eluates were filtered (0.45 μ m) and purified on C18 Sep-Pak Vac cartridges and rinsed with 0.1% heptafluorobutyric acid, and samples were eluted sequentially with 10% and 50% acetonitrile/0.1% heptafluorobutyric acid, and lyophilized. The 50% eluate fractions were resuspended in 0.1% heptafluorobutyric acid and fractionated by analytical Vydac C18 RP-HPLC (2).

orobutyric acid and fractionated by analytical Vydac C18 RP-HPLC (2).

Pheromonal Attraction of *Aplysia*. The T-maze and conduction of T-maze assays using *A. brasiliana* have been described (2, 5, 8) (see *Supporting Text*).

Results

Purification and Characterization of Attractin-Related Proteins and the Structural Basis for Their Attraction. The final purification of attractin from four species is shown in Fig. 5 (which is published as supporting information on the PNAS web site). Edman sequence analyses revealed a single distinct attractin-related protein in albumen gland extracts of *A. brasiliana*, *A. fasciata*, *A. vaccaria*, and *A. depilans*. Additional sequence information was obtained by sequencing endoproteinase Glu-C, endoproteinase Lys-C, and cyanogen bromide peptides and by 3'-rapid amplification of cDNA ends (Table 1, which is published as supporting information on the PNAS web site). The complete sequences of the attractin proteins are shown in Fig. 1. The six cysteines, three charged residues (Asp-5, Asp/Glu-22, and Glu-39), and the sequence Ile-30-Glu-31-Glu-32-Cys-33-Lys-34-Thr-35-Ser-36 (IIEECKTS) was conserved in all five *Aplysia* attractins. *A. brasiliana*, *A. fasciata*, *A. vaccaria*, and *A. depilans* share 95%, 91%, 43%, and 41% sequence identity, respectively, with *A. californica* attractin. The attractin proteins fall into two groups according to their sequence similarity with *A. californica*: *A. californica*, *A. brasiliana*, and *A. fasciata* (91–95% identity), and *A. vaccaria* and *A. depilans* (41–43% identity). The attractins we have characterized are representatives of three subgenera that comprise 32 of the 35 known *Aplysia* species (Table 2, which is published as supporting information on the PNAS web site) and \approx 15% of all *Aplysia* species (Fig. 1).

Three of the attractin-related proteins are posttranslationally modified in the N-terminal region (Asn-8, *A. brasiliana*, *A. fasciata*; Asn-25, *A. depilans*) and two in the C-terminal region (Ser-49, Ser-50, Thr-51, Thr-52, *A. vaccaria*; Ser-49, Ser-50, *A. depilans*). N-glycosylation at Asn-8 of *A. californica* attractin is not required for attraction (2, 3, 5). Because *A. californica* attractin is progressively degraded from the C terminus but not from the N terminus, and only the N-terminal 41–47 aa are required for attraction activity (2), posttranslational modifications in the C-terminal region of *A. vaccaria* and *A. depilans* attractin may serve to prolong the half-life and biological activity of the proteins or to serve some other unknown function, but this was not examined.

MS Characterization of *A. vaccaria* and *A. depilans* Attractins. Mass spectra of reduced and alkylated *A. vaccaria* and *A. depilans* attractin did not have peaks corresponding to the predicted masses of the unmodified, but alkylated, attractins. Instead, significantly higher masses are observed. As shown in Fig. 6 (which is published as supporting information on the PNAS web site), *A. vaccaria* spectra had a high-mass peak grouping, similar to that seen in spectra of *A. californica* attractin (2), with a major

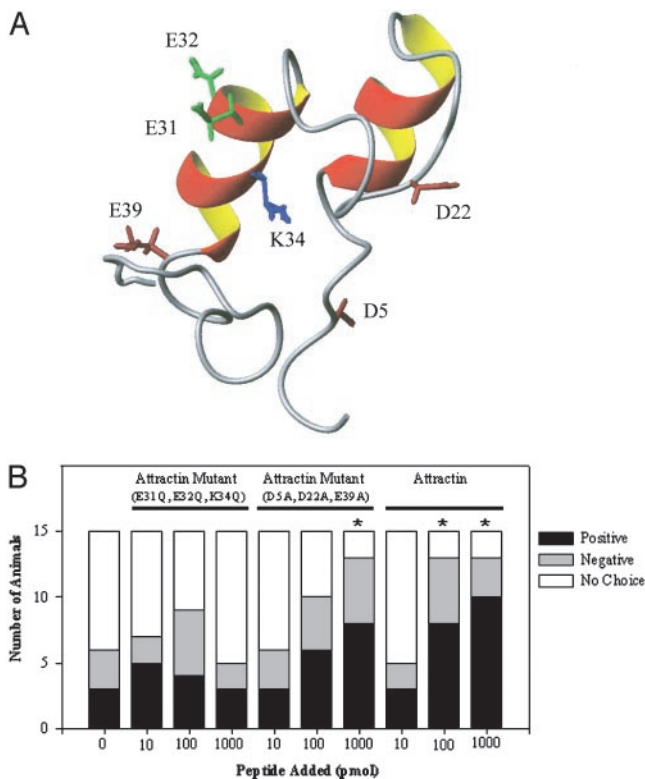


Fig. 2. NMR structure of *A. californica* attractin and biological activity of attractin mutants in T-maze attraction assays. (A) NMR structure showing the side chains of the residues mutated in these studies. (B) The number of *A. brasiliiana* attracted to a nonlaying conspecific (0 pmol) was not significantly increased when 10, 100, or 1,000 pmol of the attractin triple mutant with three substituted charged amino acids (Gln for Glu-31, Glu-32, and Lys-34) was placed in the adjacent ASW. In contrast, the number of *A. brasiliiana* attracted to a nonlaying conspecific (0 pmol) was significantly increased (*, $P < 0.001$) when 1,000 pmol of a second attractin triple mutant with three other substituted charged amino acids (Ala for Asp-5, Asp-22, and Glu-39) was placed in the adjacent ASW. In control experiments, when 100 pmol of recombinant *A. californica* attractin was tested, 8 of 15 animals (53%) were attracted to attractin (*, $P < 0.001$); when 1,000 pmol was tested, 10 of 15 animals (67%) were attracted to attractin (*, $P < 0.001$). This bar graph is based on 150 single-arm experiments, 15 per stimulus. In each experiment, animals chose between a stimulus in one arm and no stimulus in the other.

peak at m/z 7962, followed by five lower-intensity peaks spaced 163 ± 4 Da apart, suggesting a glycosylated protein with variable numbers of hexose units. *A. depilans* spectra also suggest significant posttranslational modification.

Residues Important for Attraction Activity. Conservation of Asp-5, Asp/Glu-22, Glu-31, Glu-32, Lys-34, and Glu-39 in five *Aplysia* attractins suggested that some of these charged residues might be important for attraction activity. To test this hypothesis, three charged amino acids within the IIECKTS region were substituted in *A. californica* attractin (Gln for Glu-31, Glu-32, and Lys-34; Figs. 1 and 2A). This triple mutant attractin was inactive in T-maze assays (Fig. 2B), indicating that Glu-31, Glu-32, and perhaps Lys-34 are important for attraction activity. Three other charged amino acids were substituted in a second triple mutant attractin (Ala for Asp-5, Asp-22, and Glu-39; Figs. 1 and 2A). In contrast, this triple mutant attractin retained activity (Fig. 2B). These data suggest that at least one, and possibly all, of the charged residues in the IIECKTS region are important for attraction activity. The effects of mutating uncharged residues (Ile-30, Cys-33, Thr-35, Ser-36) in the IIECKTS region were not examined.

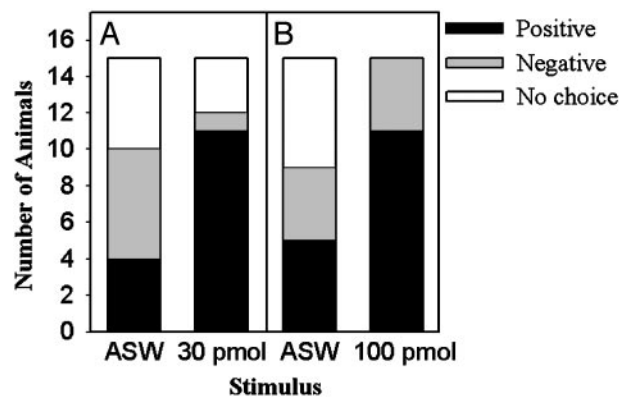


Fig. 3. *A. vaccaria* attractin attracts *A. brasiliiana*. (A) The number of *A. brasiliiana* attracted to a nonlaying conspecific (artificial seawater; ASW) increased when 30 pmol *A. vaccaria* attractin was added to the adjacent seawater; fewer animals went to the opposite arm (negative) of the T-maze, and fewer failed to make a choice (no choice). (B) Same as A, except that 100 pmol of *A. vaccaria* attractin was added.

***A. vaccaria* Attractin Attracts *A. brasiliiana*.** In control assays [nonlaying conspecific (*A. brasiliiana*), no added pheromone], four animals entered the arm of the T-maze containing the stimulus animal and remained, six went to the opposite arm, and five did not make a choice (Fig. 3A). These control assays established chance levels of attraction at four animals.

The response pattern changed when 30 pmol of *A. vaccaria* attractin was placed in the ASW adjacent to the stimulus animal: 11 of 15 animals (73%) entered the arm of the T-maze containing *A. vaccaria* attractin, one went to the opposite arm, and three did not make a choice. The response pattern differed significantly from that to a nonlaying conspecific alone [$\chi^2(2) = 17.22$; $P < 0.001$]. Due to the limited seasonal availability and lifespan of *A. brasiliiana*, assays testing 30 and 100 pmol of *A. vaccaria* attractin were performed in different years, and a second set of control assays was performed: five animals entered the arm of the T-maze containing the stimulus animal and remained, four went to the opposite arm, and six did not make a choice (Fig. 3B); these control assays established chance levels of attraction at five animals. When 100 pmol of *A. vaccaria* attractin was tested, 11 of 15 animals (73%) entered the arm of the T-maze containing *A. vaccaria* attractin, and four went to the opposite arm. The response pattern differed significantly from that to a nonlaying conspecific alone [$\chi^2(2) = 13.20$; $0.001 < P < 0.005$]. The response pattern to 10 pmol of *A. vaccaria* attractin was not significantly different from that to a nonlaying conspecific ($n = 15$; data not shown). In summary, despite the sequence dissimilarity of *A. brasiliiana* and *A. vaccaria* attractin, *A. vaccaria* attractin was attractive to *A. brasiliiana*.

We examined whether egg laying would trigger attractin secretion in *A. brasiliiana* and *A. vaccaria*. RP-HPLC purification of *A. brasiliiana* and *A. vaccaria* egg cordons eluates is shown in Fig. 7, which is published as supporting information on the PNAS web site. Peaks were pooled based on absorbance, then lyophilized, and analyzed by N-terminal sequence analysis. The partial sequences of the peaks indicated in Fig. 7A and B corresponded to the five N-terminal residues of *A. brasiliiana* and *A. vaccaria* attractin, respectively, demonstrating that they are released during egg laying in these species.

Discussion

Based on sequence comparison and an NMR structure of *A. californica* attractin (3, 4), we previously proposed that a conserved region of the protein is responsible for interaction with cellular receptors. The results shown here offer further evidence

that the heptapeptide sequence, IIECKTS, conserved in attractins from five different *Aplysia* species, is indeed important for biological activity. Altering the three charged amino acids in this sequence, which forms the second helix of the 3D structure, effectively abolished the activity of attractin in T-maze assays. In contrast, mutating three conserved charged residues at other areas of the protein reduced but did not destroy attractin activity. All attractins characterized to date have six conserved cysteines and three acidic residues corresponding to Asp-5, Glu-31, and Glu-32 of *A. californica*. These three residues are solvent exposed in the 3D NMR solution structure (ref. 4 and Fig. 2A). The 3D structure allowed us to distinguish residues that are conserved for structural reasons (e.g., cysteines, the core residue Ile-30) from those whose surface exposure could reflect a role in receptor recognition (Asp-5, Ser-11, Met-15, Asp/Glu-22, Glu-31, Glu-32, Glu-39) (4). The triple mutant *A. californica* attractin E31Q, E32Q, K34Q (Fig. 2A) lacks activity in T-maze assays, suggesting that Glu-31, Glu-32, and Lys-34 may be involved in receptor binding and pheromonal attraction and may account for the interspecific attraction activity of attractin.

We further showed that even attractin from a genetically distant *Aplysia* species, *A. vaccaria*, is attractive to *A. brasiliana*. The responses to 30 and 100 pmol of *A. vaccaria* attractin (Fig. 3A and B) may have been maximal because they were comparable to the maximal response obtained for *A. californica* attractin (Fig. 2B). In these T-maze assays, it should be noted that, because attractin is delivered as a pulse and the seawater is stationary during the assay, the attractin concentration gradually changes with time, and the concentration detected by *A. brasiliana* depends on when they reach the stimulus (range: \approx 10 seconds to 20 min). In the present study, the attractiveness of *A. vaccaria* attractin to *A. brasiliana* suggests that, despite only \approx 40% sequence conservation in some cases, *Aplysia* attractins share a common 3D structure and receptor-binding site that is probably in the conserved C-terminal helical domain. We therefore conclude that the attractins are part of a signaling/chemotaxis mechanism that is common to many marine organisms.

The signaling system may be even more fundamental. As noted in previous work (3), attractins have similar sequence and structural properties to those of the well-studied pheromones of the ciliated protozoan *Euplotes*. In particular, the second helix of attractin, containing the IIECKTS motif, is quite similar to the third helix of *Er-11* (4). This third helix of the *Euplotes* pheromones is involved in receptor recognition (25, 26). Although *Euplotes* pheromone sequences differ significantly, sharing only six cysteines and an N-terminal aspartic acid, all have the same compact “pyramid” 3D structure of three α -helices (25–29). The

pheromone *Er-1* can bind to the mammalian receptor for interleukin-2 (30), raising the possibility that water-borne pheromones may be ancestors of cytokines in higher organisms.

In most organisms, sex pheromones attract potential mates (e.g., ref. 18). If mate attraction were the sole function of attractin, one might expect that the pheromone would attract only conspecifics. However, our data demonstrate that attractin is a relatively promiscuous signal. This finding suggests that the 3D structure of attractin (4) has been conserved during evolution. Other additional functions, for example the reduced latency to mating and the stimulation of hermaphroditic mating (5), may prevent alteration of the attractin structure. There may be other behavioral functions attributable to attractin.

The benefits derived from the aggregation of multiple *Aplysia* species may exceed those derived solely by accessing potential mates. One benefit may be defense from predators. Consistent with this hypothesis, other communal animals that are thought to herd for defense may form aggregates composed of multiple species (31–34). Secretion of a pheromone that attracts individuals of a different species may still be useful in attracting a potential mate, if the individual that is attracted subsequently lays eggs and releases an attractin signal that attracts a conspecific. The concentration of attractin released from the egg cordons of multiple individuals should be higher than that from a cordon laid by a single individual. Because attractin is partially degraded within 30 min of eluting from egg cordons (2), a higher concentration of active attractin may be sustained over longer distances, thereby increasing the possibility that additional individuals will be recruited to breeding aggregations. Some of the new recruits may be appropriate partners for mating.

This work adds further support to the hypothesis that the attraction mechanism is similar to a signaling mechanism conserved in unicellular organisms. Attractins may also be regulators of other functions, as indicated by their ability to reduce the latency to mating and stimulate hermaphroditic mating. Thus, attractins are proving a multifaceted system to study signaling in marine invertebrates.

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- Fan, X., Wu, B., Nagle, G. T. & Painter S. D. (1997) *Mol. Brain Res.* **48**, 167–170.
- Painter, S. D., Clough, B., Garden, R. W., Sweedler, J. V. & Nagle, G. T. (1998) *Biol. Bull.* **194**, 120–131.
- Schein, C. H., Nagle, G. T., Page, J. S., Sweedler, J. V., Xu, Y., Painter, S. D. & Braun, W. (2001) *Biophys. J.* **81**, 463–472.
- Garimella, R., Xu, Y., Schein, C. H., Rajarathnam, K., Nagle, G. T., Painter, S. D. & Braun, W. (2003) *Biochemistry* **42**, 9970–9979.
- Painter, S. D., Clough, B., Black, S. & Nagle, G. T. (2003) *Biol. Bull.* **205**, 16–25.
- Zeeck, E., Müller, C. T., Beckmann, M., Hardege, J. D., Papke, U., Sinnwell, V., Schroeder, F. C. & Francke, W. (1998) *Chemoecology* **8**, 33–38.
- Ram, J. L., Müller, C. T., Beckmann, M. & Hardege, J. D. (1999) *FASEB J.* **13**, 945–952.
- Painter, S. D., Chong, M. G., Wong, M. A., Gray, A., Cormier, J. G. & Nagle, G. T. (1991) *Biol. Bull.* **181**, 81–94.
- Coggeshall, R. E. (1972) *Tissue Cell* **4**, 105–127.
- Painter, S. D., Kalman, V. K., Nagle, G. T., Zuckerman, R. A. & Blankenship, J. E. (1985) *J. Morphol.* **186**, 167–194.
- Begnoche, V. L., Moore, S. K., Blum, N., van Gils, C. & Mayeri, E. (1996) *J. Neurophysiol.* **75**, 2161–2166.
- Kupfermann, I. & Carew, T. (1974) *Behav. Biol.* **12**, 317–337.
- Pennings, S. C. (1991) *J. Exp. Mar. Biol. Ecol.* **149**, 249–266.
- Achituv, Y. & Susswein, A. J. (1985) *J. Exp. Mar. Biol. Ecol.* **85**, 113–122.
- Luginbuhl, P., Ottiger, M., Mronza, S. & Wüthrich, K. (1994) *Protein Sci.* **3**, 1537–1546.
- Zahn, R., Damberger, F., Ortenzi, C., Luporini, P. & Wüthrich, K. (2001) *J. Mol. Biol.* **313**, 923–931.
- Kikuyama, S., Toyoda, F., Ohmiya, Y., Matsuda, K., Tanaka, S. & Hayashi, H. (1995) *Science* **267**, 1643–1645.
- Kikuyama, S., Yamamoto, K., Iwata, T. & Toyoda, F. (2002) *Comp. Biochem. Physiol.* **132**, 69–74.
- Wabnitz, P. A., Bowie, J. H., Tyler, M. J., Wallace, J. C. & Smith, B. P. (1999) *Nature* **401**, 444–445.
- Yamamoto, K., Kawai, Y., Hayashi, T., Ohe, Y., Hayashi, H., Toyoda, F., Kawahara, G., Iwata, T. & Kikuyama, S. (2000) *FEBS Lett.* **472**, 267–270.
- Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. & Wingfield, P. T. (1997) *Current Protocols in Protein Science* (Wiley, New York).
- Nagle, G. T., Painter, S. D., Blankenship, J. E., Dixon, J. D. & Kurosky, A. (1986) *J. Biol. Chem.* **261**, 7853–7859.
- Nagle, G. T., Painter, S. D., Blankenship, J. E. & Kurosky, A. (1988) *J. Biol. Chem.* **263**, 9223–9237.
- Rothman, B. S., Hawke, D. H., Brown, R. O., Lee, T. D., Dehghan, A. A., Shively, J. E. & Mayeri, E. (1986) *J. Biol. Chem.* **261**, 1616–1623.

