

Water taste: the importance of osmotic sensing in the oral cavity

Timothy A. Gilbertson, Arian F. Baquero and Kristina J. Spray-Watson

ABSTRACT

The sense of taste plays critical roles in nutrient identification and toxin avoidance. The ability to respond to hypoosmotic stimuli in mammalian taste receptor cells may reflect the importance of osmotic sensing by the gustatory system. Transduction for hypoosmotic stimuli involves water influx through aquaporins followed by activation of volume-regulated anion channels. The ability of these transduction elements to be regulated by natriuretic hormones at the mRNA and protein level in other transporting epithelia suggest that the gustatory system may respond to extrinsic signals related to the restoration of salt and water balance. Plasticity in the peripheral gustatory system is consistent with the activity in the taste system being reflective of underlying nutritional status. Clearly, more research is needed to determine the link between nutrition, taste and the control of food and water intake.

Key words | aquaporins, osmotic sensing, receptor cell, taste, water

Timothy A. Gilbertson (corresponding author)
Arian F. Baquero
Kristina J. Spray-Watson
Department of Biology, Utah State University,
5305 Old Main Hill,
Logan UT 84322-5305,
USA
Tel.: +1 (435) 797-7314
Fax: +1 (435) 797-1575
E-mail: tag@biology.usu.edu

INTRODUCTION AND BACKGROUND

The initial events in ingestion involve taking in solids and liquids into the oral cavity where they interact with a variety of potential sensors including chemoreceptors, mechanoreceptors and thermal receptors to provide information about the stimulus. Though the flavor of food includes both taste and olfaction, including retronasal olfaction, the selective acceptance or rejection of ingested material is often driven by its taste properties. In simplest terms, the taste system provides two main functions related to ingestive behavior. It must recognize those nutrients needed for survival and reject those toxic compounds which could potentially harm an individual (Herness & Gilbertson 1999).

Our classification of the four or five major types of taste stimuli is indicative of these dual functions of the taste system. The nutrient recognition function is reflective of our ability to sense carbohydrates (sweet taste), minerals (salty taste) and protein (umami taste) – all essential nutrients in the diet. Recently, there has been proposed a mechanism for the recognition of the essential fatty acids which may

contribute to the chemosensory properties of fats in the oral cavity (Gilbertson *et al.* 1997; Gilbertson & Kim 2002; Mattes 2002; Fukuwatari *et al.* 2003; Hiraoka *et al.* 2003) and which may also reflect the ability of the taste system to recognize those compounds the organism must have in order to survive. Conversely, bitter taste reflects our inherent ability to recognize those compounds which are potentially toxic. Plant alkaloids, insect and animal venoms are exquisitely bitter tasting and when ingested drive their rejection from the oral cavity. Coincidentally, the majority of pharmaceutical compounds are perceived as bitter, perhaps owing to their ability to modulate normal physiological processes in the body, an undesirable outcome in an otherwise healthy individual.

Accordingly, the vast majority of taste receptor research has centered on the elucidation of mechanisms underlying the transduction of the major classes of taste stimuli. In recent years, the identification and characterization of various types of mammalian taste receptors has enhanced

our understanding of the molecular and cellular processes involved in taste perception. For example, the cloning of the T1R and T2R families of taste receptors by Zuker (Zhao *et al.* 2003) and colleagues has shed light into the receptors that underlie sweet (T1R2+T1R3), bitter (T2R) and, umami (T1R1 + T1R3) tastes (for review, see Gilbertson *et al.* 2000; Smith & Margolskee 2001; Gilbertson & Boughter 2003).

The basic pathway for taste transduction is initiated with the interaction of the tastant with ion channels or receptors on the apical membranes of taste receptor cells. Though the downstream pathways have yet to be fully elucidated, in general, this interaction produces a depolarization (receptor potential) in the receptor cell, generation of action potentials during chemostimulation, opening of voltage-gated Ca^{2+} channels and eventual release of neurotransmitters onto gustatory afferents. A variety of signal transduction schemes have been implicated in transduction of the various tastants (Gilbertson *et al.* 2000; Smith and Margolskee 2001; Gilbertson & Boughter 2003). Though the molecular underpinnings of taste are beginning to be elucidated, there remains disagreement over the way information is coded by peripheral taste receptors. Namely, recent molecular work demonstrates that there is receptor segregation in taste cells (Zhang *et al.* 2003; Mueller *et al.* 2005) consistent with a labeled line for taste information (Hellekant *et al.* 1998), while recent cellular and imaging studies (Gilbertson *et al.* 2001; Caicedo *et al.* 2002) argue for more broadly tuned receptors, suggestive of a population coding strategy in taste. More research will be needed to clarify conflicts between the molecular and physiological data.

Largely overlooked in the majority of basic research on taste is how water (i.e. hypoosmotic stimuli) is recognized by the peripheral gustatory system. Clearly, water would fit the very definition of a required nutrient and the body has elaborate regulatory systems devoted to the maintenance of osmotic balance in the body. Further, it has been known for over 40 years that water is a *bona fide* taste stimulus – early research demonstrated that water (i.e. lack of solutes) was a powerful stimulus generating activity in a number of afferent nerves, including gustatory afferents, emanating from the oral cavity. Afferent nerve recordings from the cat and rat show water induced activity in chorda tympani and laryngeal nerves (Lindemann 1996). The cellular mechanism

contributing to this response until recently had been unexplored in any significant detail.

Recently, while performing experiments using an intact lingual epithelial preparation (Modified Ussing chamber Gilbertson and Zhang, 1998a; Gilbertson *et al.* 2001) which permitted rinsing of the apical surface of the taste buds with distilled water, we found that water stimulation following isotonic saline elicited robust, transient depolarizations from roughly 60% of taste receptor cells. One possible explanation for this response could be the removal of the Na^+ ions alleviates Na^+ self-inhibition of the apical epithelial Na^+ channels (ENaC) involved in sodium salt transduction (Gilbertson & Zhang 1998b). Alternatively, the influx of water itself into taste cells could be activating other signaling pathways leading to depolarization of the cell. To investigate this possibility we examined the effects of hypoosmotic stimulation on electrophysiological responses in isolated mammalian taste buds. In both rat (Gilbertson 2002) and mouse (Spray *et al.* 2005) hypoosmotic stimuli elicit significant increases in whole cell conductance consistent with activation of the taste cells during non-isosmotic stimulation. Studies in rat demonstrated that water influx during hypoosmotic stimulation caused cell swelling and subsequent activation of volume-regulated anion (Cl^-) channels (Gilbertson 2002). Given our data showing the expression of several aquaporins in taste receptor cells (Kim *et al.* 1999; Spray *et al.* 2005), including the apically expressed AQP5, it seems likely that rapid water influx into taste cells under these conditions is mediated through these water channels. A model for “water” transduction in taste cells is shown in Figure 1.

These data along with results showing the ability of hyperosmotic stimuli to alter activity in the peripheral taste system (Lyall *et al.* 1999) are consistent with the ability of the taste receptor cells to act as osmotic sensors. One might speculate that oral osmotic sensors in the taste system may play a critical role in helping direct water (and mineral) intake in order to help the body restore osmotic balance and dovetails well with the nutrient recognition role of the gustatory system. While the kidneys play a key role in retaining water under dehydrated conditions, intake of water is required for restoration of salt and water balance. In this way, the peripheral osmotic sensing provided by

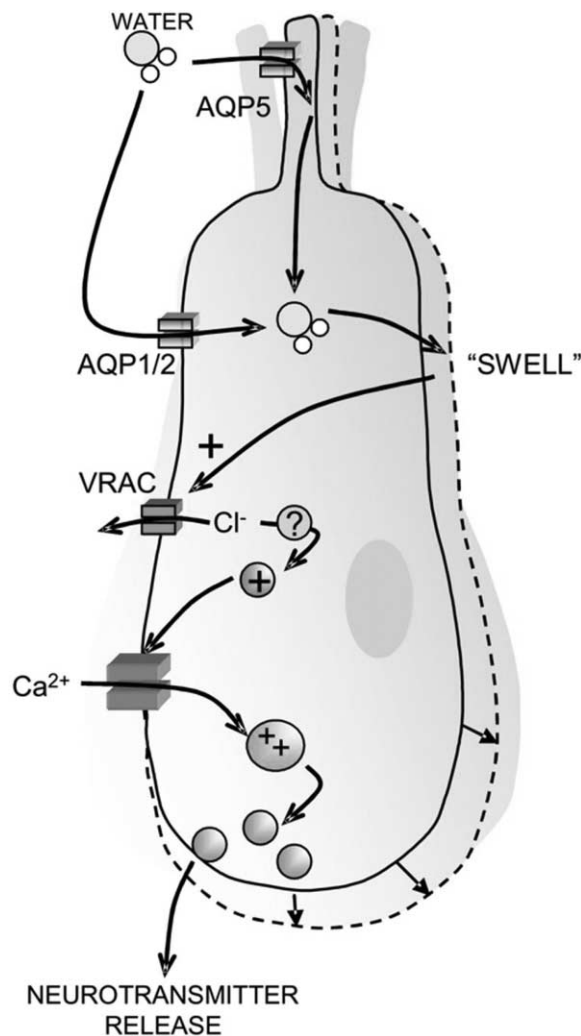


Figure 1 | Putative transduction pathway for hypoosmotic stimuli (water) in mammalian taste receptor cells. Water influx through aquaporins (AQP) on the apical membrane (AQP5) or basolateral membrane (AQP1, AQP2) leads to cell swelling and activation of volume-regulated anion channels (VRAC). Data showing hypoosmotic stimuli depolarizes taste cells suggests that opening VRACs leads to depolarization and eventual opening of voltage-sensitive Ca²⁺ channels. The rise in intracellular Ca²⁺ triggers release of neurotransmitter onto gustatory afferents.

taste receptor cells may be critical for helping promote water intake.

GENERAL ISSUES

Three obvious issues arise when one considers the state of our understanding of the peripheral taste system's role in nutrient recognition. Issues concerning the function of identified receptors in native taste receptor cells including

taste cell specificity, transduction mechanisms of non-conventional taste stimuli (including water) and the ability of the peripheral taste system to respond to nutritional needs remain to be investigated with the same fervor given to receptor identification for the five main classes of taste stimuli. As alluded to above, in recent years, much of the emphasis in peripheral taste system research has focused on the identification of receptors and ion channels involved in the initial recognition of the major classes of taste stimuli. As a result, there have been tremendous strides made in taste receptor identification and the initial events in taste transduction (*see above*). While the understanding of taste receptor structure/function has progressed, the downstream signaling pathways activated by tastants *in vivo* have not yet been elucidated. This is due, in part, to the reliance on heterologous expression systems to understand receptor specificity and function which does not necessarily accurately recreate the internal milieu of the native taste receptor cells. The role of these receptors (e.g. T1R and T2R families) in terms of their cellular function at the level of the receptor cell *in vivo* remains to be determined. This may, in turn, help to rectify some of the aforementioned arguments concerning whether taste receptors are specific, namely the presence of a labeled line pathway from a taste-specific receptor to the central nervous system (Hellekant *et al.* 1998; Zhang *et al.* 2003; Mueller *et al.* 2005) or display multiple chemical sensitivity (Gilbertson *et al.* 2001; Caicedo *et al.* 2002).

Though taste receptor cells have been shown to respond to a variety of chemical compounds which fall outside the usual classes of salty, sour, sweet, bitter and umami, little emphasis has been placed on these non-conventional taste stimuli. Further, the chemical environment of the taste stimulus (temperature, viscosity, tonicity, etc.) has been largely ignored in peripheral physiological studies which emphasize responses to pure tastants rather than to attempt to recreate the "normal" stimulus environment during ingestion of liquid and solid food. Non-conventional taste stimuli including astringents (Schiffman *et al.* 1992), menthol (Lundy & Contreras 1993; Green & Schullery 2003), water (Gilbertson 2002), fats (Gilbertson *et al.* 1997) and capsaicin (Park *et al.* 2003), to name but a few, have all been shown to alter activity in gustatory afferents. Clearly, it is rare when one ingests a pure macronutrient or individual

tastant and an understanding of the effect of the chemical composition of ingested nutrients, including the role of saliva (Matsuo 2000), is warranted. For example, normal taste stimuli may range from the very hypoosmotic like water (low millimolar concentration) to incredibly hyperosmotic (several molar concentration), yet few studies have looked at the ability of the taste system to respond to varying tonicity of solutions.

Inherent in the assumption of studies which look at the responses of taste receptor cells is that the physiological state of the cell is identical across time. This reflects a notion, fairly commonplace in the taste field, that the taste cell merely detects sapid molecules and does little to reflect the underlying physiological state of the organism or contribute in any significant way to the control of food intake over the short or long term. However, numerous studies have shown that taste receptor cells possess an impressive variety of receptors specific for neuropeptide and steroid hormones that are important for the control of food intake and maintaining nutrient balance. These studies have demonstrated the presence of receptors for, or gustatory responses to, such factors as cholecystokinin (CCK), substance P and calcitonin gene related peptide (CGRP), (Herness *et al.* 2002; Simon *et al.* 2003), met-enkephalin (Yoshie *et al.* 1993), leptin (Kawai *et al.* 2000; Shigemura *et al.* 2004), aldosterone (Herness 1992; Lin *et al.* 1999; Burks *et al.* 2005), vasopressin (Gilbertson *et al.* 1993), atrial natriuretic peptide and oxytocin (Gilbertson 1998). Clearly, the expression of this rich array of receptors would be predicted to alter responses of the peripheral taste system under those physiological conditions that precipitate their release. There is a decided lack of comprehensive studies aimed at elucidating the connection between nutritional (hormonal) status, taste and food/water intake.

BARRIERS TO PROGRESS

The aforementioned issues surrounding peripheral taste research in general apply to the investigations surrounding the gustatory response to water and its involvement in the restoration of salt and water balance in the body. Thus, attempts to determine the importance of the taste system's ability to respond to water or hypoosmotic stimuli (Gilbert-

son 2002) and hyperosmotic stimuli (Lyall *et al.* 1999) must by necessity span from the transduction molecules (aquaporins; volume-regulated anion channel) through the afferent nerve, central nervous system and, ultimately, behavior. Such multilevel analyses often yield results which are not intuitively consistent from the molecule to behavior.

To discover the underlying transduction cascades often depends upon the use of transgenic models, including knockouts of suspected key proteins, or, more recently, application of small interfering RNA molecules (siRNA), (Schutze 2004; Holen & Mobbs 2004). These approaches when successful yield important clues as to the importance of specific molecules, however, negative data may be attributable to a variety of reasons including technical concerns, compensatory gene expression and influence of genetic background (*cf.* Leiter 2002; Hall 2004). Taken together, the approach used, techniques employed and the model organism selected may all profoundly affect the outcome and general applicability of the resulting dataset.

RESEARCH GAPS

In terms of attempting to understand the gustatory cue for water, to elucidate its underlying transduction pathway and to determine its role, if any, in the restoration of salt and water balance in the organism, there is significant work to be done. None of these three points have been definitively addressed to date. The ability to answer the remaining questions involving water taste and its importance to the organism has implications beyond the peripheral taste system. In particular, determining the transduction pathway for nutrients like water and the plasticity of this signaling pathway according to nutritional need will greatly expand our understanding of the link between nutritional status and the ability to sense nutrients which, in turn, may lead to compensatory changes in nutrient intake. This link between chemosensation, nutrition and food/water intake is largely unknown at present.

Recommendations for research

Given the discussion above, it is not surprising that several

areas should be actively investigated relating to water perception.

- A basic understanding of the important transduction elements is required to begin to explore issues related to the role of this pathway in water intake and in the restoration of salt and water balance in the body. Identification of several aquaporins (Kim *et al.* 1999; Spray *et al.* 2005) and classes of volume-regulated anion channels in taste cells (Rao *et al.* 2003) has been an important first step in this area. Further identification of specific molecules and signaling elements in the water pathway using genetic, molecular, cellular and behavioral approaches is warranted.
- Identification of regulatory pathways within taste cells which may modulate expression of water transduction elements should begin to be explored. For example, numerous regulatory pathways involved in other transporting epithelia (kidney, lung, colon) which regulate salt and water movement have been identified (for review see Gormley *et al.* 2003). Their identification and function in the taste system would suggest they may play similar roles there. Recently, for example, our laboratory (Burks *et al.* 2005) identified a number of early aldosterone regulated gene products which are expressed in mammalian taste buds including serum- and glucocorticoid-regulated kinase (sgk-1), corticosteroid hormone inducible factor (CHIF), neural developmentally down regulated gene product 4 (NEDD4-2) and Kirsten ras. This is consistent with the idea that these regulatory pathways may influence taste performance directly, in addition to their roles in maintaining salt and water balance in other organs (Tong *et al.* 2004).
- A third area for recommended future research involves determining how these transduction and regulatory elements are altered in response to changes in salt and water balance at the organism level. That is, are there predictable changes in expression or function of these proteins which correspond with nutritional needs? Under conditions of water deprivation, for example, one might hypothesize that there are compensatory changes in expression of aquaporins which will alter taste sensitivity and eventually lead to increases in water intake necessary to restore salt and water balance.

Further, investigations of the performance of the peripheral taste system in pathophysiological conditions which alter the salt and water balance should yield additional insights into the relationship between taste and nutritional status.

CONCLUSIONS

One of the important roles of the taste system is in nutrient recognition. This has been clearly actively pursued in research attempting to identify the gustatory pathways underlying the recognition of carbohydrate (sweet), protein (umami), minerals (salty) and, to a lesser extent, fat. Water, which has been known for decades to activate the peripheral gustatory system, has received comparatively little attention. Recent research exploring the effects of non-isotonic solutions in the taste system has begun to fill this void. Further research is necessary, if we are to begin to understand the role that the recognition of water plays in the gustatory system and, in the broader context, in the restoration of salt and water balance in the body.

REFERENCES

- Burks, C. A., Hansen, D. R. & Gilbertson, T. A. 2005 Quantitative PCR analysis of the aldosterone-regulated salt transduction pathway in taste cells. *Chem. Senses* **30**, A21.
- Caicedo, A., Kim, K. N. & Roper, S. D. 2002 Individual mouse taste cells respond to multiple chemical stimuli. *J. Physiol.* **544**, 501–509.
- Fukuwatari, T., Shibata, K., Iguchi, K., Saeki, T., Iwata, A., Tani, K., Sugimoto, E. & Fushiki, T. 2003 Role of gustation in the recognition of oleate and triolein in anosmic rats. *Physiol. Behav.* **78**, 579–583.
- Gilbertson, T. A. 1998 Role of the taste system in ingestive behavior. Studies in NaCl and fatty acid transduction. *Ann. N.Y. Acad. Sci.* **855**, 860–867.
- Gilbertson, T. A. 2002 Hypoosmotic stimuli activate a chloride conductance in rat taste cells. *Chem. Senses* **27**, 383–394.
- Gilbertson, T. A. & Boughter, J. D., Jr 2003 Taste transduction: Appetizing times in gustation. *NeuroReport* **14**, 905–911.
- Gilbertson, T. A., Boughter, J. D., Jr, Zhang, H. & Smith, D. V. 2001 Distribution of gustatory sensitivities in rat fungiform taste cells: Whole cell responses to apical chemical stimulation. *J. Neurosci.* **21**, 4931–4941.
- Gilbertson, T. A., Damak, S. & Margolskee, R. F. 2000 The molecular physiology of taste transduction. *Curr. Opin. Neurobiol.* **10**, 519–527.

- Gilbertson, T. A., Fontenot, D. T., Zhang, H., Liu, L. & Monroe, W. T. 1997 Fatty acid modulation of K⁺ channels in taste receptor cells: Gustatory cues for dietary fat. *Am. J. Physiol.* **272**, C1203–C1210.
- Gilbertson, T. A. & Kim, I. 2002 The chemoreception of fat. In: *Chemistry of Taste, Symposium series 825* (Given P. & Paredes D. eds), pp. 180–191. American Chemical Society Publishing, Clarendon Hills, IL.
- Gilbertson, T. A., Roper, S. D. & Kinnamon, S. C. 1993 Proton currents through amiloride-sensitive Na⁺ channels in isolated hamster taste cells: enhancement by vasopressin and cAMP. *Neuron*. **10**, 931–942.
- Gilbertson, T. A. & Zhang, H. 1998a Characterization of sodium transport in gustatory epithelia from the hamster and rat. *Chem. Senses* **23**, 285–293.
- Gilbertson, T. A. & Zhang, H. 1998b Self-inhibition in amiloride-sensitive sodium channels in taste receptors cells. *J. Gen. Physiol.* **111**, 667–677.
- Gormley, K., Dong, Y. & Sagnella, G. A. 2003 Regulation of the epithelial sodium channel by accessory proteins. *Biochem. J.* **371**, 1–14.
- Green, B. G. & Schullery, M. T. 2003 Stimulation of bitterness by capsaicin and menthol: differences between lingual areas innervated by the glossopharyngeal and chorda tympani nerves. *Chem Senses*. **28**, 45–55.
- Hall, J. 2004 Opinion: Unravelling the general properties of siRNAs: strength in numbers and lessons from the past. *Nat. Rev. Genet.* **5**, 552–557.
- Heltekant, G., Ninomiya, Y. & Danilova, V. 1998 Taste in chimpanzees. III: Labeled-line coding in sweet taste. *Physiol. Behav.* **65**, 191–200.
- Herness, M. S. 1992 Aldosterone increases the amiloride-sensitivity of the rat gustatory neural response to NaCl. *Comp. Biochem. Physiol.* **103**, 269–273.
- Herness, M. S. & Gilbertson, T. A. 1999 Cellular mechanisms of taste transduction. *Annu. Rev. Physiol.* **61**, 873–900.
- Herness, S., Zhao, F. L., Lu, S. G., Kaya, N. & Shen, T. 2002 Expression and physiological actions of cholecystokinin in rat taste receptor cells. *Neurosci.* **22**, 10018–10029.
- Hiraoka, T., Fukuwatari, T., Imaizumi, M. & Fushiki, T. 2003 Effects of oral stimulation with fats on the cephalic phase of pancreatic enzyme secretion in esophagostomized rats. *Physiol. Behav.* **79**, 713–717.
- Holen, T. & Mobbs, C. V. 2004 Lobotomy of genes: use of RNA interference in neuroscience. *Neurosci.* **126**, 1–7.
- Kawai, K., Sugimoto, K., Nakashima, K., Miura, H. & Ninomiya, Y. 2000 Leptin as a modulator of sweet taste sensitivities in mice. *Proc. Natl. Acad. Sci. U S A.* **97**, 11044–11049.
- Kim, I., Liu, L., Sears, N. L., Kim, J. H. & Gilbertson, T. A. 1999 Regionalized expression of aquaporins suggests a role for water channels in osmotic sensing by taste cells. *Soc. Neuroscience Abs.* **25**, 2184.
- Leiter, E. H. 2002 Mice with targeted gene disruptions or gene insertions for diabetes research: problems, pitfalls, and potential solutions. *Diabetologia.* **45**, 296–308.
- Lin, W., Finger, T. E., Rossier, B. C. & Kinnamon, S. C. 1999 Epithelial Na⁺ channel subunits in rat taste cells: localization and regulation by aldosterone. *J. Comp. Neurol.* **405**, 406–420.
- Lindemann, B. 1996 Taste reception. *Physiol. Rev.* **76**, 718–766.
- Lundy, R. F., Jr & Contreras, R. J. 1993 Taste prestimulation increases the chorda tympani nerve response to menthol. *Physiol. Behav.* **54**, 65–70.
- Lyall, V., Heck, G. L., DeSimone, J. A. & Feldman, G. M. 1999 Effects of osmolarity on taste receptor cell size and function. *Am. J. Physiol.* **277**, C800–C813.
- Matsuo, R. 2000 Role of saliva in the maintenance of taste sensitivity. *Crit. Rev. Oral Biol. Med.* **11**, 216–229.
- Mattes, R. D. 2002 Oral fat exposure increases the first phase triacylglycerol concentration due to release of stored lipid in humans. *J. Nutr.* **132**, 3656–3662.
- Mueller, K. L., Hoon, M. A., Erlenbach, I., Chandrashekar, J., Zuker, C. S. & Ryba, N. J. 2005 The receptors and coding logic for bitter taste. *Nature* **434**, 225–229.
- Park, K., Brown, P. D., Kim, Y. B. & Kim, J. S. 2003 Capsaicin modulates K⁺ currents from dissociated rat taste receptor cells. *Brain Res.* **962**, 135–143.
- Rao, S., Hansen, D. R. & Gilbertson, T. A. 2003 Chloride channels in taste cells: Multiple subtypes and functions. *J. Gen. Physiol.* **122**, 29a.
- Schiffman, S. S., Suggs, M. S. & Simon, S. A. 1992 Astringent compounds suppress taste responses in gerbil. *Brain Res.* **595**, 1–11.
- Shigemura, N., Ohta, R., Kusakabe, Y., Miura, H., Hino, A., Koyano, K., Nakashima, K. & Ninomiya, Y. 2004 Leptin modulates behavioral responses to sweet substances by influencing peripheral taste structures. *Endocrinology* **145**, 839–847.
- Schutze, N. 2004 siRNA technology. *Mol. Cell. Endocrinol.* **213**, 115–119.
- Simon, S. A., Liu, L. & Erickson, R. P. 2003 Neuropeptides modulate rat chorda tympani responses. *Am. J. Physiol.* **284**, R1494–R1505.
- Smith, D. V. & Margolskee, R. F. 2001 Making sense of taste. *Sci. Am.* **284**, 32–39.
- Spray, K. J., Baquero, A. & Gilbertson, T. A. 2005 The cellular and molecular basis for water taste in mice. *Chem. Senses* **30**, A52.
- Tong, Q., Booth, R. E., Worrell, R. T. & Stockand, J. D. 2004 Regulation of Na⁺ transport by aldosterone: signaling convergence and cross talk between the PI3-K and MAPK1/2 cascades. *Am. J. Physiol.* **286**, F1232–F1238.
- Yoshie, S., Wakasugi, C., Kanazawa, H. & Fujita, T. 1993 Met-enkephalin-Arg6-Gly7-Leu8-like immunoreactivity in mammalian taste buds. *Arch. Histol. Cytol.* **56**, 495–500.
- Zhang, Y., Hoon, M. A., Chandrashekar, J., Mueller, K. L., Cook, B., Wu, D., Zuker, C. S. & Ryba, N. J. 2003 Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* **112**, 293–301.
- Zhao, G. Q., Zhang, Y., Hoon, M. A., Chandrashekar, J., Erlenbach, I., Ryba, N. J. & Zuker, C. S. 2003 The receptors for mammalian sweet and umami taste. *Cell* **115**, 255–266.