Heightened Avidity for Trisodium Pyrophosphate in Mice Lacking Tas1r3

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Abstract

Laboratory rats and mice prefer some concentrations of tri- and tetrasodium pyrophosphate (Na₃HP₂O₇ and Na₄P₂O₇) to water, but how they detect pyrophosphates is unknown. Here, we assessed whether T1R3 is involved. We found that relative to wild-type littermate controls, Tas1r3 knockout mice had stronger preferences for 5.6–56 mM Na₃HP₂O₇ in 2-bottle choice tests, and they licked more 17.8–56 mM Na₃HP₂O₇ in brief-access tests. We hypothesize that pyrophosphate taste in the intact mouse involves 2 receptors: T1R3 to produce a hedonically negative signal and an unknown G protein-coupled receptor to produce a hedonically positive signal; in Tas1r3 knockout mice, the hedonically negative signal produced by T1R3 is absent, leading to a heightened avidity for pyrophosphate.

Key words: intake, palatability, preference, taste, T1R3

Introduction

Pyrophosphates—compounds with a P₂O₇ anion—have several commercial applications including as food additives. They chelate minerals, prevent discoloration, support emulsion stability, and improve the texture of processed meat, perhaps by increasing water retention of proteins (e.g., Wu et al. 1990; Agnihotri and Pal 1997; Xiong and Kupski 1999; International Food Additives Council 2013). A major use of pyrophosphates is as palatability enhancers of domestic cat food (Brunner 2001; Shao and Stammer 2005; Brand et al. 2013), but why cats find them attractive is unknown. To investigate potential mechanisms, we recently observed the behavioral and electrophysiological responses of laboratory rats and mice to several pyrophosphate salts and found that they moderately prefer some concentrations of tri- and tetrasodium pyrophosphate (Na₃HP₂O₇ and Na₄P₂O₇) to water (McCaughey et al. 2007). Behavioral tests and recordings of neural activity in the nucleus of the solitary tract showed that the preference for these pyrophosphates was not due simply to their saltiness; moreover, Na₃HP₂O₇ mixed with amiloride had a taste profile that was distinct from those of sweet, salty, sour, bitter, and umami stimuli, suggesting that the pyrophosphate moiety has a unique taste (McCaughey et al. 2007). However, little is known about the molecular gustatory transduction mechanisms for pyrophosphates or the mechanisms that underlie their palatability.

Mice with a deactivating mutation or targeted knockout of Itrp3 or Calhm1 do not show a preference for Na₃HP₂O₇ (Tordoff and Ellis 2013; Tordoff, unpublished data). ITTPR3 and CALHM1 are required components of the G protein-coupled receptor (GPCR) transduction cascade, so this implies that pyrophosphate transduction involves a GPCR (see Huque et al. 1992; Hisatsune et al. 2007; Tordoff and Ellis 2013). T1R3 is a GPCR involved in the detection of sweet (e.g., Nelson et al. 2001; Damak et al. 2003; Zhao et al. 2003; Tordoff, Shao, et al. 2008), umami (e.g., Damak et al. 2003; Chaudhari et al. 2009; Li 2009), calcium–magnesium (Tordoff 2008; Tordoff, Reed, et al. 2008; Tordoff, Shao, et al. 2008; Tordoff et al. 2012), iron (Riera et al. 2009), and zinc (Riera et al. 2009). To investigate whether T1R3 also is necessary for the detection of pyrophosphates, we investigated the taste-related behavior of Tas1r3 knockout (KO) mice in response to Na₃HP₂O₇.

Methods

Mice and maintenance

Experiments followed the guidelines outlined in the National Research Council’s Guide for the Care and Use of Laboratory Animals, eighth edition. Protocols were approved by the Institutional Animal Care and Use Committee of the Monell Chemical Senses Center.
The mice were derived from an initial group of 3 breeding pairs of \textit{Tas1r3} heterozygote (Het) mice on a C57BL/6 (B6) background that were provided by Dr Robert Margolskee in 2007. A breeding nucleus was maintained in our colony by backcrossing and by mating heterozygotes brother-to-sister. The experiments described here used wild-type (\textit{Tas1r3} WT) and KO (\textit{Tas1r3} KO) homozygote mice resulting from heterozygote crosses. Care was taken to ensure that each experiment involved mice from several litters and that each litter contributed equal numbers of WT and KO mice (i.e., we used littermates as controls). During the third to fourth week after birth, a 1-mm tail tip snip was collected from each mouse, and this was processed and analyzed by a commercial service (Transnetyx, Inc.) to identify the mouse’s genotype.

All mice were maintained in a vivarium at 23°C on a 12:12 light:dark cycle with lights off at 7 PM. They were housed in plastic “tub” cages (26.5 cm × 17 cm × 12 cm) with stainless-steel grid lids and wood shavings scattered on the floor (a picture and additional husbandry conditions are available online; Tordoff and Bachmanov 2001). The mice ate pelleted AIN-76A diet and drank deionized water. They were housed in groups of the same sex until 7–10 days before tests began, at which time they were housed alone.

\section*{Two-bottle choice tests}

\subsection*{\textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7}, preference}

Three independent studies were conducted to assess preferences for \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7}. Study 1 involved 4 male and 3 female \textit{Tas1r3} WT mice and 5 male and 2 female \textit{Tas1r3} KO mice aged 14–19 weeks. Study 2 involved 7 male and 1 female \textit{Tas1r3} WT mice, 6 male and 2 female \textit{Tas1r3} Het mice, and 6 male and 2 female \textit{Tas1r3} KO mice aged 9–17 weeks. Study 3 involved 12 female WT and 12 female \textit{Tas1r3} KO mice aged 12–18 weeks. The mice in Study 1 had previously received brief-access tests with CaCl\textsubscript{2} solutions as part of another experiment. Those in Study 3 had previously received brief-access gustometer tests with “hedonically negative” \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} solutions (described below). The mice in Study 2 were naive.

All the mice received a series of 48-h tests with 2 graduated drinking tubes (allowing measurements to be made to the nearest 0.1 mL). During the first test, both drinking tubes contained deionized water. In subsequent tests, one drinking tube contained water and the other contained \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7}. Every 48 h, the concentration of \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} was increased in 0.25-log steps from 1 mM (Study 1 and Study 2) or 1.78 mM (Study 3) to 178 mM. The positions of the drinking tubes were switched every 24 h to control for any side preferences.

\subsection*{\textit{NaCl}, preference}

To examine whether the results we observed with \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} could be attributed to the sodium ion, we tested some of the mice that had participated in Study 1 and Study 2 with 5 concentrations of NaCl. There were 8 male and 7 female \textit{Tas1r3} WT mice and 7 male and 5 female \textit{Tas1r3} KO mice, aged 8–14 weeks. During the first test, both drinking tubes contained deionized water. In subsequent tests, one drinking tube contained water and the other contained NaCl. The concentration of NaCl was increased every 48 h (31.6, 100, 178, 316, and 512 mM). The positions of the drinking tubes were switched every 24 h to control for any side preferences.

\section*{Statistical analyses}

The change in liquid levels in a drinking tube was considered to be the mouse’s fluid intake (we did not correct for spillage, which is usually <0.5 mL/test; Tordoff and Bachmanov 2003). Solution preference scores were calculated based on the intake of taste solution divided by total liquid intake, expressed as a percentage.

Results were analyzed by mixed-design analyses of variance with factors of group (WT, Het, or KO) and concentration. In initial analyses, we also included sex as a factor, but, in agreement with previous work (Tordoff 2007), there were no sex differences in preference scores, so results from the 2 sexes were combined. Differences between the groups in consumption of specific concentrations of \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} were determined using post hoc Fisher’s least significant difference (LSD) tests. One-sample \( t \)-tests were used to determine whether mice in each genotype group preferred, were indifferent to, or avoided each \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} concentration (i.e., differed from 50\% preference).

Analyses of the 3 studies involving 2-bottle choice tests with \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} produced similar results (Supplementary Figure S1), so the data were combined for presentation and analysis here. The combined analysis involved 27 \textit{Tas1r3} WT and 27 \textit{Tas1r3} KO mice. Intakes related to the test with 1 mM \textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} and all data from \textit{Tas1r3} Het mice were excluded from these omnibus analyses because they were not components of all 3 studies.

\section*{Brief-access gustometer tests}

\subsection*{Procedure}

\textit{Na}\textsubscript{3}HP\textsubscript{2}O\textsubscript{7} acceptance was assessed in 2 experiments, one designed for “hedonically positive” and one designed for “hedonically negative” taste compounds. The different methods were required in order to titrate the motivation of the mice to respond to “good” and “bad” tasting solutions; unmotivated mice often fail to respond even to good-tasting solutions, and very thirsty mice drink at maximal rates to all but the worst-tasting solutions.

The procedures are described in detail elsewhere (Taruno et al. 2013). In order to train each mouse to sample taste solutions, it was first water-deprived for 22.5 h and then placed in a mouse gustometer (MS160; DiLog Instruments) and allowed uninterrupted access to water for 30 min from the time it first licked the drinking spout. It was then returned
to its home cage and given water for 1 h. On the following 3 days, this procedure was repeated except the shutter allowing access to water was closed 5 s after each time the mouse began to lick, and it was reopened after a 7.5-s interval.

The experiment with hedonically positive concentrations of Na₃HP₂O₄ involved 10 male and 8 female Tαs₁r₃ WT mice and 7 male and 11 female Tαs₁r₃ KO mice. Each mouse received 3 test sessions. Prior to a session, the mouse received free access to food and water for 24 h. It then received 1 g of food and 2 mL of water, and the test session began 24 h later. The test session consisted of 19 repeated series of 5-s exposures to the following, presented in a quasi-random order: 0, 17.8, 31.6, 56, and 75 mM Na₃HP₂O₄. By “quasi-random order,” we mean that a concentration appeared once and only once in every series of 5 tests. By “0 mM Na₃HP₂O₄,” we mean deionized water, which was also used as the solvent for the Na₃HP₂O₄ solutions. There was no requirement for mice to respond to a taste solution. Typically, they licked during the first 3–5 series of presentations and then stopped behaving, presumably because they were no longer thirsty. After the session, the mice received a recovery day with free access to food and water for 24 h. Their water was then removed for 22.5 h in preparation for the next session.

The experiment with hedonically negative concentrations of Na₃HP₂O₄ involved 12 female Tαs₁r₃ WT and 12 female Tαs₁r₃ KO mice. These mice received 3 test sessions consisting of 10 repeated series of access to the following in quasi-random order: 0, 75, 150, 350, and 750 mM Na₃HP₂O₄. Additional 5-s “washout” trials with water were interposed between each test trial. Thus, a mouse received 5-s access to a taste solution followed by 7.5 s with the shutter closed, then 5-s access to water followed by 7.5 s with the shutter closed, followed by the next taste solution for 5 s, and so on. The mouse was water deprived for 22.5 h before each test (i.e., unlike mice for the hedonically positive tests, it received food but not 2 mL of water).

**Statistical analysis**

Results from the 2 experiments were analyzed separately. The mean number of licks in response to each Na₃HP₂O₄ concentration made by each mouse was obtained by averaging the results of identical exposures. These values for individual mice were then used in mixed-design analyses of variance with factors of group (WT or KO) and Na₃HP₂O₄ concentration. Significant differences between pairs of means were assessed using post hoc LSD tests. Two female Tαs₁r₃ KO mice in the hedonically positive experiment failed to train—they did not reliably lick in the gustometer even for water—so data from these mice were excluded from analyses.

**Results**

**Two-bottle choice tests**

Na₃HP₂O₄

Relative to Tαs₁r₃ WT mice, Tαs₁r₃ KO mice drank significantly more 5.62, 10, 17.8, 31.6, and 56.2 mM Na₃HP₂O₄, and had significantly higher preferences for these concentrations (genotype × concentration interactions, intake, $F(9,468) = 4.26, P < 0.0001$; preference, $F(9,468) = 6.07, P < 0.001$; [Figure 1]). Water intakes were reciprocal to Na₃HP₂O₄ intakes, $F(9,468) = 3.29, P = 0.0006$, so that total fluid intakes did not differ between the WT and KO groups. There was also no difference between the groups in body weights ($Tαs₁r₃ WT = 22 ± 0.4 g, Tαs₁r₃ KO = 21 ± 0.5 g$).

Relative to indifference (50%), the Tαs₁r₃ WT mice preferred 10, 17.8, and 31.6 mM Na₃HP₂O₄; the lowest concentration they avoided was 56.2 mM Na₃HP₂O₄. The Tαs₁r₃ KO mice preferred 5.62, 10, 17.8, and 31.6 mM Na₃HP₂O₄, and the lowest concentration they avoided was 100 mM Na₃HP₂O₄.

**NaCl**

There were no differences between the Tαs₁r₃ WT and KO groups in response to any concentration of NaCl ([Figure 2]). There were also no differences in body weight ($Tαs₁r₃ WT = 26 ± 1 g, Tαs₁r₃ KO = 25 ± 1 g$).

**Brief-access tests**

Na₃HP₂O₄—positive hedonic method

Using the method designed to test preferred taste solutions, there was a significant interaction between genotype and Na₃HP₂O₄ concentration, $F(4,128) = 4.36, P = 0.0024$. The Tαs₁r₃ WT and KO mice had similar lick rates for water, but the Tαs₁r₃ KO mice licked significantly more frequently for 17.8, 31.6, and 56 mM Na₃HP₂O₄ ([Figure 3A]). The Tαs₁r₃ WT mice licked more 75 mM Na₃HP₂O₄ than water but not more of the lower Na₃HP₂O₄ concentrations than water. In contrast, the KO mice licked more 17.8, 31.6, 56, and 75 mM Na₃HP₂O₄ than water ($Ps < 0.0002$), and they also licked more of the 31.6 and 56 mM Na₃HP₂O₄ than 17.8 mM Na₃HP₂O₄.

Na₃HP₂O₄—negative hedonic method

Using the method designed to test disliked taste solutions, the Tαs₁r₃ WT and Tαs₁r₃ KO mice did not differ in their licking rates to any concentration of Na₃HP₂O₄ ([Figure 3B]). There was no interaction of genotype with Na₃HP₂O₄ concentration. High Na₃HP₂O₄ concentrations reduced licking rates of both groups relative to their licking rates for water or low concentrations, $F(4,84) = 70.7, P < 0.0001$.

**Discussion**

The main finding of this series of experiments is that Tαs₁r₃ KO mice have a stronger avidity for Na₃HP₂O₄ than do Tαs₁r₃ WT controls, both in long-term choice and brief-access acceptance tests. We expected that if Tαs₁r₃ was involved in the detection of pyrophosphate taste, then genetic knockout of the
receptor would abolish the preference for moderate concentrations of Na$_3$HP$_2$O$_7$ observed in WT and B6 mice (here and McCaughey et al. 2007), in much the same way as does knock-out of Itpr3 (Tordoff and Ellis 2013) or Calhm1 (unpublished results). Thus, our finding that Tas1r3 KO mice had stronger preferences for low-to-moderate concentrations of Na$_3$HP$_2$O$_7$ than did Tas1r3 WT controls was surprising, leading us to replicate the finding twice (Supplementary material) and to seek supporting evidence from brief-access taste tests.

Pyrophosphate preference scores of KO mice could potentially be higher than those of WT mice if the scores of the WT mice were aberrantly low. However, the preference scores of Tas1r3 WT mice observed here are similar to those
reported for inbred B6 mice (McCaughhey et al. 2007), which is not unexpected because the Tartr3 KO mouse line is carried on a B6 background. The B6 strain and our Tartr3 WT controls had relatively modest peak preferences for moderate concentrations of Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} (~60–72% for 10–56 mM in McCaughhey et al. 2007 or 10–32 mM, here). Tartr3 KO mice had stronger peak preferences that included lower preferred concentrations (~75–80% for 5.6–32 mM).

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\text{Na}_2\text{H}_2\text{P}_2\text{O}_7 \quad \text{probably has a salty taste component to rats (McCaughhey et al. 2007), and the most preferred concentrations of Na}_2\text{H}_2\text{P}_2\text{O}_7 (10–56 \text{mM}) provide sodium in the most preferred concentration range (30–168 \text{mM}; peak sodium preference is usually ~150 \text{mM; Bare 1949; Denton 1984; but see Tordoff et al. 2007). Thus, a concern was that the abnormal response of Tartr3 KO mice to sodium pyrophosphate may be due to altered saltiness perception. However, Tartr3 WT and KO mice had indistinguishable NaCl intakes and preferences (consistent with Nelson et al. 2001; Damak et al. 2003). Thus, a difference in response to saltiness cannot explain the enhanced pyrophosphate preference shown by Tartr3 KO mice. Instead, the effect of Tartr3 KO to increase Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} preference must involve alterations in response to the taste of the pyrophosphate moiety.}

The responses of mice given 2-bottle choice tests can be influenced by postingestive actions of the taste solutions and by learning. These problems are minimized with brief-access tests. However, brief-access tests are not without their own problems, including a lack of sensitivity caused by unmotivated animals failing to respond to bad-tasting solutions or over-motivated animals responding maximally to good-tasting ones (see Glendinning et al. 2002). To circumvent these floor and ceiling effects, we used methods tailored to the concentration (and thus hedonic value) of Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} being tested. Reassuringly, the results we obtained paralleled those observed with 2-bottle choice tests: The Tartr3 KO mice licked more for moderate, hedonically positive Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} concentrations than did WT controls, but the 2 groups did not differ in response to high, hedonically negative Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} concentrations. The finding that both WT and KO mice licked more for at least one concentration of Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} than they did for water supports a role for immediate sensory factors (e.g., taste) in guiding preferences for pyrophosphates.

Knockout of taste-related genes tends to result in a loss of taste sensitivity, as indicated by smaller preferences for hedonically positive compounds or larger preferences for hedonically negative ones. Thus, our observation that Tartr3 KO mice have heightened avidity for some concentrations of Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} is unusual, although not without precedent. Some concentrations of Polycose are licked more avidly by Tartr3 KO mice than WT mice (Treusukosol et al. 2009 but not Zukerman et al. 2009). Also, moderate concentrations of calcium and magnesium salts are preferred to water by Tartr3 KO mice but avoided by WT mice (Tordoff, Shao, et al. 2008). There are examples of Tartr3 KO imparting a negatively hedonic valence to normally preferred taste solutions: High concentrations of the artificial sweeteners, saccharin, sucralose, acesulfame K, and SC45647 are preferred by WT mice but avoided by Tartr3 KO mice (Damak et al. 2003; Tordoff, Shao, et al. 2008). The most likely explanation for this is that bitter side tastes are unmasked when sweet taste is lost. For calcium and magnesium taste, the interpretation is that the action of a second receptor, perhaps CaSR, is unmasked when T1R3 is absent (Tordoff, Shao, et al. 2008; Tordoff et al. 2012). We argue by analogy that the increased avidity for Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} observed in Tartr3 KO mice is due to a different mechanism.

Figure 3 Licking rates of Tartr3 wild-type (WT) and knockout (KO) mice during tests giving 5-s access to various concentrations of Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}. Slightly different methods, including different levels of deprivation, were used to test mice with Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} concentrations that were positively hedonic (A; n = 18 WT and 16 KO) or negatively hedonic (B, n = 12 WT and 12 KO). Vertical bars are standard errors of the mean. *P < 0.05 relative to WT group.
to the action of \( \text{Na}_3\text{HP}_2\text{O}_7 \) on a second receptor, which is masked by T1R3 activation in WT mice.

Thus, we hypothesize that in the intact mouse, pyrophosphate acts on 2 receptors: T1R3 to produce a hedonically negative signal and an unknown receptor to produce a hedonically positive signal. With T1R3 eliminated in the KO mouse, the positive hedonic signal produced by the unknown receptor predominates. The unknown receptor could potentially be within the same taste bud or the same taste cell as T1R3 or even be a dimer with T1R3 that has different partners when T1R3 is absent. A modification of this hypothesis is that pyrophosphate acts both on the T1R3 receptor and directly on its intracellular transduction cascade. In homogenized catfish taste tissue, pyrophosphate inhibits inositol-1,4,5-trisphosphate kinase (Huque et al. 1992), which is required to terminate IP₃ signaling. Perhaps this action is facilitated in the mouse lacking T1R3 although this begs the question of how pyrophosphate gains access to the interior of a taste receptor cell.

How can T1R3 mediate responses with opposite hedonic valences, such as those to sweeteners and to pyrophosphates? We can only conclude that there are separate T1R3 populations. The existence of separate sweet- and umami-sensitive T1R3 populations is well established (e.g., Zhao et al. 2003), and we have argued elsewhere for an additional pool of T1R3-containing cells sensitive to the taste of calcium (Tordoff, Shao, et al. 2008). Pyrophosphate-sensitive T1R3-containing cells may be yet another distinct population. These are most likely Type II taste receptor cells because the behavioral responses to sweet, umami, calcium, and pyrophosphate are all influenced by knockout of \text{Itpr3} and \text{Calhm1} (Tordoff and Ellis 2013 and unpublished results), which are expressed specifically in Type II cells (Hisatsune et al. 2007; Taruno et al. 2013).

It has not escaped our attention that both cats (Li et al. 2005) and \text{Tas1r3} KO mice lack the ability to taste sweetness, and both respond avidly to pyrophosphates. This may, of course, be a coincidence, but it may also point to the loss of sweet taste somehow amplifying the attractiveness of pyrophosphate taste. Perhaps in the absence of active sweet signaling pathways in the cat or \text{Tas1r3} KO mouse, neural connectivity is “rewired” such that pyrophosphate-sensitive fibers activate circuitry associated with reward and pleasure that normally receives input from sugar-responsive taste cells. It will take additional studies to test these and other possibilities.

Our work helps elucidate how, but not why, rodents are attracted to pyrophosphates. Pyrophosphates are a rich source of phosphorus, which is a required nutrient and the target of a specific appetite (see Denton 1984; Czarnogorski et al. 2004; Ohnishi et al. 2007). Observations that domestic cats, which are obligate carnivores, have strong preferences for pyrophosphates (e.g., Brunner 2001; Shao and Stammer 2005; Brand et al. 2013) and that pyrophosphates are formed endogenously, raise the possibility that pyrophosphate taste may signal animal protein or metabolic energy. However, pyrophosphate concentrations in blood are in the low micromolar range (Ryan et al. 1979), which is 3 orders of magnitude below the behavioral thresholds for \( \text{Na}_3\text{HP}_2\text{O}_7 \) observed here. The pyrophosphate concentrations of plants are even lower (Vigorito and Sclafani 1987). Thus, why animals evolved a taste for pyrophosphates is a mystery.

**Supplementary material**

Supplementary material can be found at [http://www.chemse.oxfordjournals.org/](http://www.chemse.oxfordjournals.org/)

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