Published in final edited form as:

Physiol Behav. 2012 March 20; 105(5): 1214–1218. doi:10.1016/j.physbeh.2011.12.023.

Chorda tympani nerve modulates the rat's avoidance of calcium chloride

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Abstract

Calcium intake depends on orosensory factors, implying the presence of a mechanism for calcium detection in the mouth. To better understand how information about oral calcium is conveyed to the brain, we examined the effects of chorda tympani nerve transection on calcium chloride (CaCl₂) taste preferences and thresholds in male Wistar rats. The rats were given bilateral transections of the chorda tympani nerve (CTX) or control surgery. After recovery, they received 48-h two-bottle tests with an ascending concentration series of CaCl₂. Whereas control rats avoided CaCl₂ at concentrations of 0.1 mM and higher, rats with CTX were indifferent to CaCl₂ concentrations up to 10 mM. Rats with CTX had significantly higher preference scores for 0.316 and 3.16 mM CaCl₂ than did control rats. The results imply that the chorda tympani nerve is required for the normal avoidance of CaCl₂ solution.

Keywords

calcium; taste preference; mineral appetite; ingestive behavior; avoidance threshold; gustatory nerves

1. Introduction

Many physiological processes require constant levels of calcium; consequently, extracellular calcium concentrations are under precise control, at ~1 mM. It is therefore not surprising that calcium intake is also controlled, and that calcium-replete rats and mice generally avoid calcium solutions at the concentrations that are typically used in psychophysical experiments (i.e., 10 – 300 mM) [1,2]. Restraints on calcium intake prevent large volumes of concentrated calcium from overwhelming homeostatic controls.

The preponderance of evidence suggests that these restraints on calcium intake are exerted primarily by orosensory factors: Rats generally dislike concentrated calcium solutions; they make facial movements indicative of avoidance when forced to ingest it [3]. However, the restraints are lifted when extracellular calcium concentrations drop and a need for calcium arises. Relative to calcium-replete rats, calcium-deprived rats respond more frequently with an ingestive sequence of orofacial movements to oral calcium salt infusions [3]. They also

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have increased intakes of calcium salt solutions in brief-exposure tests, and consume significantly larger volumes of CaCl_2 solution when “sham-drinking” (i.e., when postingestive effects are minimized) [4,5].

Recent studies point to the involvement of T1R3 and CaSR as receptors in calcium detection in the oral cavity [6,7]; however, the exact mechanisms remain unknown [8,9]. In addition to a receptor unit, a sensing mechanism requires a neural pathway to provide the signal to the brain. Fibers of the chorda tympani (CT) nerve—a branch of the facial nerve, which is one of the three major nerves innervating taste papillae—respond by excitation to calcium salts applied to the tongue [10-12]. Moreover the response of the CT nerve to calcium salts depends on the animals’ physiological status and correlates with responses to calcium salt solutions in behavioral tests [13,14]. These electrophysiological data demonstrate that the CT nerve provides information about the concentration of calcium ions in the oral cavity. However, it is unknown whether the signal carried by the CT nerve plays a significant role in the regulation of calcium consumption.

CT nerve transection influences the ingestive responses to different taste stimuli in specific ways. It elevates NaCl detection thresholds in rats and mice, and blunts the rise in consumption of NaCl by sodium-deplete rats [15-18]. However, CT transection does not affect licking responses to sucrose or quinine solutions (when other gustatory nerves are left intact [19,20]). These behavioral effects are congruent with the relative contribution of the CT nerve to the responses elicited by these taste stimuli: the CT nerve response to NaCl is larger than those elicited in the glossopharyngeal and greater superficial petrosal nerves; conversely, the CT nerve responses elicited to sucrose and quinine are smaller than those elicited in the greater superficial petrosal nerve [21,22]. Thus, the relative contribution of each nerve depends on the taste stimulus being investigated.

To clarify the contribution of the CT nerve in the regulation of ingestive responses to CaCl_2 , we transected the CT nerve of rats and assessed their taste preferences for an ascending series of CaCl_2 solutions using 48-h, two-bottle tests. Preference test results were used to determine avoidance thresholds. Control rats were given sham CT nerve transection.

2. Methods

2.1. Subjects

Male Wistar rats were received from Charles River (Wilmington, MA) at the age of 49 - 52 days. The experiment was conducted in two batches involving a total of 49 rats. Each rat was housed individually in a stainless steel wire mesh hanging cage ($19.5 \times 17.5 \times 24$ cm). AIN-76A purified powdered diet (#100000, Dyets, Bethlehem, PA) and deionized (DI) water were provided *ad libitum*. The vivarium was maintained at 23 °C on a 12:12 h light/dark cycle with lights off at 7 pm. The rats were allowed 2 weeks to adapt to the facility, and testing began when they were 63 - 66 days old. The experiment protocol was approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee.

2.2. Pre-operative test

We gave the rats a pre-operative test to familiarize them with the test procedures, and to allow us to match the CT surgery and sham surgery groups for initial side preferences. The rats received a 48-h two-bottle choice test with deionized water provided in both bottles. We used 300-ml glass water bottles with neoprene stoppers and stainless steel drinking spouts. Bottles were placed on the front mesh wall of each rat’s cage with their spout tips positioned 1 - 2 cm above the cage floor and 2 - 4 cm apart. The position of the water bottle and “solution” bottle (which in this case was also water) was switched after 24 h. Measurements were made in the early light period (between 10:00 - 11:30 am). Bottles were weighed on a

top-loading electronic balance to the nearest 0.1 g every 24 h; fluid intake was calculated with the assumption that 1 g = 1 ml. Daily intakes from each bottle were calculated as an average of the two consecutive 24-h intakes. Intakes from both bottles were summed to obtain total fluid intakes. Solution preferences were calculated as the ratio of solution intake divided by total fluid intake, and were expressed as a percentage.

2.3. Chorda tympani transection surgery

All the rats were deeply anesthetized with 1% isoflurane. For the rats receiving CT nerve transection (CTX; n=25), the animal's head was tilted 80° away from the surgeon. The auditory meatus was temporarily widened with a No. 7 microforceps and a second microforceps was used to remove the tympanic membrane. Visualization of the CT was achieved by deflection of the malleus rostrally. To prevent regeneration of the transected nerve, its visible part was removed with microforceps along with the malleus and remaining ossicles. Removing the bones eliminated the path the nerve normally follows during development, resulting in a physical separation of the peripheral and central ends of the transected CT, which makes nerve regeneration unlikely in the 4-week time frame of this experiment. This procedure was conducted bilaterally.

Twenty-four rats served as surgical controls. In these rats, the auditory meatus was temporarily widened, as described earlier, and a small hole was made in the tympanic membrane with the tips of the microforceps. Care was taken to avoid contact with the underlying ossicles. This procedure controlled for any postoperative effects of anesthesia and consequences of inner ear exposure. Puncture of the tympanic membrane does not affect taste responses (rats: [23-26], hamsters: [27,28], mice: [29]).

After surgery, all rats were treated with 2.0 mg/kg, i.m. of the antibiotic, gentamicin, as a prophylactic against infection, and 1.0 mg/kg s.c. of buprenorphine as a postoperative analgesic.

2.4. Postoperative preference tests

All rats were allowed to recover from surgery for 5 - 6 days and were then tested with a series of 48-h, two-bottle choice tests between deionized water and CaCl₂ (Sigma-Aldrich, St. Louis, MO) dissolved in deionized water. After each 48-h test, the concentration of CaCl₂ was increased in 0.5-log steps (0.0, 0.1, 0.316, 1.0, 3.16, 10.0, 32.0, 100 mM; in ascending order).

2.5. Choice of the solutions to test

Our choice of taste solutions was primarily guided by the electrophysiological data on the CT nerve response to calcium salt solutions in rat. The CT nerve responds to application of low concentrations of CaCl₂ and calcium lactate to the tongue [14]. Thresholds of CT responses for both salts were found to be similar (0.3 mM); however, the magnitude of the response to calcium lactate was only one fifth of the response to CaCl₂. Thus CaCl₂ appeared to be more suitable for studying changes in behavior.

2.6. Histology

On Day 28 after surgery, rats were euthanized by CO₂ asphyxiation and their tongues were collected for histological verification of the surgery procedure. The CT innervates the fungiform papilla of the anterior tongue, and bilateral transection of the CT leads to degeneration of the no-longer innervated papillae and the taste buds within [30,31]. Therefore, a decrease in the percentage of fungiform papillae with taste pores is indicative of successful CT transection. To establish the effectiveness of CT transection in our study, we counted fungiform taste papilla and taste pores.

Tongues were stored in 4 % paraformaldehyde for at least 24 h. For counting taste papilla and taste pores, the anterior tongue was removed and soaked in distilled water for 0.5 - 1.0 h, immersed in 0.5 % w/v methylene blue for 5 min and then briefly rinsed with distilled water. The ventral surface of the tongue was removed, taking care to leave intact the pore-rich portion of the epithelium that curves under the front of the tongue. The remaining muscle layer connected to the lingual epithelium was removed by scraping with a # 10 scalpel blade. The epithelium was pressed between two slides in order to observe the fungiform papillae under light microscopy and a 2x image of each anterior tongue quarter was recorded. An experiment-blind observer counted the number of fungiform papillae containing taste pores and papillae devoid of taste pores for all groups; these data were used to calculate the percentage of fungiform papillae containing taste pores.

2.7. Data analysis

Solution intakes and preference scores were analyzed by two-way mixed-design ANOVAs with surgical group (CTX and Control) as the between-group factor and concentration as the within-group factor (with eight levels). Planned Student's *t*-tests were used to evaluate differences between individual means.

The avoidance thresholds for groups and individuals were predicted from the CaCl_2 preference scores using a three-parameter logistic function modified from Ritz and Striebig [32]. Threshold was defined as the concentration at half performance (preference score of 25%) that approximates the 50% level of correct responses, as used previously [33,34]. The preference scores for all tested concentrations except 0 mM within each treatment group were fit to a regression curve using the function: $f(x) = 50 / (1 + \exp(b(\log(x) - \log(c))))$. Within the function, (*x*) is the stimulus concentration, (*b*) is the slope, and (*c*) is the threshold concentration. The maximum performance was set to 50% preference as complete indifference and minimum performance was set to 0% preference as complete avoidance. Using this approach, three control rats had individual avoidance thresholds that were higher than 100 mM CaCl_2 and were thus excluded from analysis because we considered these values unreliable (i.e., these rats did not avoid all of the higher CaCl_2 concentrations tested and so the estimated thresholds were not indicative of actual behavior).

Statistical analyses were conducted using the Statistica software package [35] for ANOVA and using the R statistical language and environment [36] for evaluating avoidance thresholds. $P < 0.05$ was used as the level of statistical significance unless specified otherwise. All data are presented as means $\pm$ SEM.

3. Results

The results from one rat in the control group and one in the CTX group were excluded from the analysis because histology indicated they had unilateral lesions. The remaining rats in the CTX group had a significantly lower proportion of fungiform papillae with a taste pore than did the control rats (2.3 ± 0.4 % and 97.4 ± 0.3 %, respectively, $t_{45} = 174.2$, $p < 0.00001$), indicating successful surgery. This reduction in the proportion of fungiform taste buds with taste pores was similar to those observed in previous work [29,37].

The total volume of water consumed by the control and CTX rats on the first two days of postoperative testing (with access to two bottles of water) did not differ significantly (control = 49 ± 4 ml, CTX = 44 ± 3 ml), and was similar to preoperative total water intake (control = 44 ± 3 ml, CTX = 47 ± 4 ml). Surgery had no effect on total water intake (two-way ANOVA, group $\times$ test interaction $F_{1,44} = 1.5$, $p = 0.22$). The rats did not exhibit a preference towards specific bottles. Total fluid intakes increased in both groups over the course of the 16-day postsurgery test period ($F_{7,301} = 9.1$, $p < 0.00001$) but there was no

difference between the groups at any time (two-way ANOVA, group $\times$ test interaction $F_{7,301} = 1.6$, $p = 0.13$; Fig.1a).

The CTX and control groups differed in intake of 0.316 and 3.16 mM CaCl_2 but not other concentrations (group $\times$ concentration interaction, $F_{7,301} = 2.5$, $p = 0.015$; planned comparisons $t_{45} = 3.58$, $p < 0.001$ and $t_{45} = 2.34$, $p = 0.02$ for 0.316 and 3.16 mM CaCl_2 respectively). The CaCl_2 solution intake of both groups decreased as a function of solution concentration (Fig. 1b). The control group drank significantly less of 0.1 mM CaCl_2 solution and higher concentrations than they did of 0 mM CaCl_2 (i.e. DI water). In contrast, the CTX group drank less of only 10, 32 and 100 mM CaCl_2 than they did of 0 mM CaCl_2 (Fig.1b). Surgery ($F_{1,43} = 10.8$, $p = 0.002$) and solution concentration ($F_{7,301} = 54.2$, $p < 0.00001$) had a significant effect on CaCl_2 preference scores. Control rats were indifferent to 0 mM CaCl_2 and avoided 0.1 - 100 mM CaCl_2 (preference ratios, 41% - 7%; Fig. 1c). The CTX rats were indifferent to 0 - 3.16 mM CaCl_2 and avoided 10 - 100 mM CaCl_2 (preference ratios, 32% - 8%). Avoidance was determined as a significantly lower preference score in comparison with the preference score at concentration of 0 mM within the same group. The CTX rats had significantly higher preference scores in comparison to control rats for 0.316 and 3.16 mM CaCl_2 but not for other CaCl_2 concentrations (planned comparisons, $t_{45} = 4.16$, $p < 0.001$; $t_{45} = 3.52$, $p = 0.001$ for 0.316 and 3.16 mM CaCl_2 , respectively). The two groups did not differ in preference for 1 mM CaCl_2 but this appeared to be due to high solution intake by the control group and not to the response of the CTX group (Fig.1b).

The means of calculated individual CaCl_2 avoidance thresholds for the CTX group were 32 ± 6 mM compared with 14 ± 4 mM for the control group ($t_{42} = 2.46$, $p = 0.02$). This is consistent with a shift in the frequency distribution of avoidance thresholds in CTX rats towards higher CaCl_2 concentrations in comparison with the control rats (Figure 2).

4. Discussion

This is the first study to characterize the effect of gustatory nerve transection on the response of rats to a range of CaCl_2 solution concentrations. The results suggest that CT nerve section increases CaCl_2 avoidance thresholds and that a functioning CT nerve is required for the avoidance of CaCl_2 that is usually observed in intact rats.

Rats with CT nerve transection drank more CaCl_2 solution in the 0.316 - 3.16 mM concentration range than did rats with an intact nerve. The lower solution intake was not due to lower fluid intake in general; CT nerve transection elevated CaCl_2 preferences without influencing total fluid intakes. Intact rats demonstrated changes in behavioral responses to CaCl_2 at a concentration as low as 0.1 mM which is similar to the threshold value of 0.3 mM observed with electrophysiological recordings of the CT nerve [14]. It noteworthy that the range of 0.316 - 3.16 mM narrowly brackets extracellular calcium concentrations (~1 mM) and is within the limits of calcium concentrations found in natural drinking sources such as river water [38]; it is thus physiologically and ecologically relevant.

The avoidance threshold estimated from the intersection of the regression curve with the 25% preference ratio was more than twice as high for CTX rats than control rats (32 ± 6 mM vs. 14 ± 4 mM). The power of this regression analysis is that even if there are random fluctuations of individual means from the expected concentration-response curve (e.g., due to sampling error), the threshold calculations take into account the whole range of tested concentrations, which minimizes the impact of deviations at individual concentrations.

The fact that rats with CTX were indifferent to a range of concentrations that controls avoided suggests that the CT nerve conveys signals about the concentration of Ca^{2+} and/or Cl^- in the oral cavity. While we are aware that responses to CaCl_2 may be due to its anion as

well as to its cation component, and thus the CT nerve may convey information about both ions, there is considerable evidence that Ca^{2+} and not Cl^- is responsible for the development of the aversive response to CaCl_2 solutions. Rats prefer NH_4Cl and KCl (not to mention NaCl) over water at concentrations that CaCl_2 is avoided, and they exhibit similar aversive responses to different calcium salts, which indicates that Ca^{2+} and not Cl^- is the aversive component of CaCl_2 [39]. In both cases, the lack of avoidance indicates that the aversive component is not detected or that it is perceived differently.

Calcium taste is complex. Humans describe calcium as predominantly bitter accompanied by salty and metallic taste sensations [40,41]. CT transection does not affect the response to bitter compounds in rats [42]. Thus, the aversive response to CaCl_2 solution that is attenuated by CT nerve transection may depend on a non-bitter calcium-like characteristic of CaCl_2 . At higher concentrations—when the aversive response is evident in both groups—the bitter component of CaCl_2 may become prevalent and hence equally detectable by both intact and CTX rats. However, the avoidance of CaCl_2 at higher concentrations by CTX rats may also involve postingestive mechanisms and learning.

The finding that rats with intact nerves avoided high concentrations of calcium is consistent with earlier work; however, we did not observe preferences greater than indifference (i.e. “liking”) for low concentrations of calcium salts, as has been reported previously [39]. We suspect there are two reasons for this. First, calcium-liking appears most prominent in young, growing rats, whereas the rats used here were adults that had largely plateaued in growth. Second, and more critically, calcium-liking is rat strain-specific and it does not include the Wistar strain used here [2]. Thus, a preference for calcium relative to water would not be expected in the subjects of this experiment.

As well as conveying chemosensory signals in afferent fibers, the CT nerve carries efferent fibers that innervate the submandibular and sublingual salivary glands. Denervation of both of these glands leads to a significant decrease in saliva production. Thus the question arises of whether the effect of CT nerve section on behavior is caused by the disruption of its sensory fibers or its secretomotor ones. Reduced resting saliva production may compromise the ionic environment to which the receptors are adjusted, resulting in a change of sensitivity of receptor cells. Indeed surgical removal of submandibular and sublingual glands decreases total salivary sodium and potassium content [43]. However nothing is known about changes in the concentration of the active (ionized) form of these ions and there are no data on changes of calcium content. With respect to NaCl , St John and colleagues found that CT nerve section produces a significantly greater effect on taste responses than does partial desalivation [25], and there is little reason to suspect that the same would not hold for other compounds. The CT nerve provides only part of the stimulation of the submandibular and sublingual glands; there is also modulation by the sympathetic nerves [44], which remain intact after CT surgery. And lastly, the effect of reduced saliva production in response to food ingestion should have minimal influence at the moment of tasting in our experiment because calcium was provided in solution (already dissolved and thus accessible to taste receptors). In summary, it appears doubtful that decreased salivary production is the cause of the effects observed in our experiment, although this possibility cannot be ruled out.

5. Conclusions

Our observation that the behavioral response to CaCl_2 is perturbed by CT transection is consistent with previous work showing that the preference of calcium-deficient animals for CaCl_2 is present during brief-access and sham-feeding tests, which minimize or eliminate postingestive influences [4]. Together, these findings provide compelling evidence that the consumption of calcium depends on orosensory factors. Our results suggest that the

avoidance of CaCl_2 concentrations up to ~10 mM depends solely on the CT nerve; the input from other gustatory nerves does not come into play until this concentration is reached. Given that freshwater calcium levels rarely exceed 5 mM, it would appear that the CT nerve conveys the signal that is necessary for the avoidance of ecologically relevant concentrations of calcium.

Acknowledgments

This work was supported by The National Institute of Deafness and Other Communication Disorders Grant DC-10149. CT nerve transection was performed at the Monell phenotyping core laboratory, which is supported, in part, by funding from the NIH-NIDCD Core Grant (1P30DC011735-01). Matthew Rosazza and Tiffany Weiss provided expert technical assistance. We thank Dr. Alexander Bachmanov for his comments on the manuscript.

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Highlights

- We examined the effects of chorda tympani nerve transection on CaCl_2 taste preferences.
- Chorda tympani nerve transection raised the preference scores for CaCl_2 .
- Chorda tympani nerve is required for the normal avoidance of CaCl_2

Figure 1.

Total fluid intake (a), CaCl_2 intakes (b) and preference scores (c) in rats with bilateral CT transection (CTX; $n = 24$; ○) or sham surgery rats (control; $n = 23$; ●). Symbols represent means $\pm$ SEM. CTX vs. Control comparisons: * $p < 0.05$; Control comparisons relative to water (0 mM) $^\dagger p < 0.05$; CTX comparisons relative to water $^\# p < 0.05$.

Figure 2. Frequency distribution of avoidance thresholds in rats with sham surgery (control) (a) or rats with bilateral chorda tympani nerve transection (CTX) (b).