State-dependence of olfactory perception as a function of taste cortical inactivation.

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Methods

Subjects. We used female Long-Evans rats (Charles River Laboratories) weighing between 250-300g at the time of surgery as subjects (see Supplementary Table 1). All rats were individually housed and kept on a 12/12 hr light/dark cycle. Experimental sessions occurred between 1:00 P.M. and 6:00 P.M, with rats that had been acclimated to the behavioral room for at least 1 hr. All methods complied with IACUC guidelines[1].

Nasal epithelial deciliation. We anesthetized rats *via* intraperitoneal (ip) injections of a ketamine/xylazine/acepromazine cocktail (100mg/kg, 5mg/kg, and 1mg/kg, respectively). We placed anesthetized rats on their flanks, and inserted fifteen millimeters of an Eppendorf cannulae (20 µl, 0.3 mm diameter) into each nostril in turn, so that 40-50 µl of a 0.125% Triton-X 100 solution (Sigma, diluted in 0.9% Saline) could be first infused into the exposed nostril over the course of five min, and then withdrawn. Following the infusion, we placed rats on their stomachs for 5 minutes to allow any residual infusate to drain from the nares, and repeated the process on the other side[2, 3]. Control rats received vehicle infusions. Rats rested for no less than 20-23 hours following nasal infusions.

Topical lingual anesthesia. We briefly anesthetized rats with Isoflurane, and applied Anbesol™, a topical anesthetic with an active ingredient of 20% benzocaine, directly to the dorsal surface of the tongue with a sterile cotton swab (control animals were swabbed with 0.9% Saline). Rats regained consciousness and began walking normally in less than one minute, after which STFP training commenced.

Cortical cannulation. Rats were anesthetized as above, and given hourly update doses of the cocktail (20% of initial dose) as needed to maintain deep anesthesia. We placed the anesthetized rat in a stereotaxic frame, incised and

retracted the scalp, leveled the skull, implanted five support screws, and lowered either electrode bundles or guide cannulae (23 gauge, 15mm in length) bilaterally into taste cortex (AP: 1.4mm, ML ± 5.0mm, DV -4.5mm from dura[4]), and then anchored with dental acrylic. In a subset of rats, we implanted at DV -4.0mm, and lowered the infusion cannula 0.5-1 mm into the brain (see Figure 2A). Another subset received implants into somatosensory cortex dorsal to taste cortex. Stainless steel stylets inserted into the guide cannulae ensured patency. Rats in which one cannula missed its target were excluded from analysis.

Electrophysiology. We recorded single-neuron activity from taste cortex during taste sampling sessions[5], assaying responses to a taste array of 0.2M citric acid, 0.001M quinine and 0.1M saline before and after rats received lingual swabs of topical anesthesia (20 trials per taste), and again 20 minutes later (another 20 trials per taste to assess recovery). A parallel processor amplified (1000-2000), filtered (300-800 HZ) and digitized the signals. A waveform template, augmented with offline cluster software (Plexon) allowed us to isolate single neurons of a 3:1 signal-to-noise ratio.

Chow. 1.0 g of ground cinnamon (McCormick), 2.0 g Hershey's Cocoa, 2.0 g of ground marjoram (McCormick) or 0.4 g of ground cumin (Badia) to 100 g were added to powdered laboratory rat chow (Lab Diet Formula 5P00 Prolab RMH 3000).

STFP[6-8].

Adaptation. Rats learned to eat plain powdered chow from ramekins (1 hr in a clean chamber), until food consumption within two consecutive sessions was at least 2 g. Next, demonstrator-observer pairs interacted in a test cage without bedding for 30 min; these adaptation interactions minimized later aggressive behavior. All rats maintained > 85% free-feeding weight.

Training interaction. On training day, demonstrators ate one randomly flavored chow for 1 hour. Only demonstrators ingesting ≥2g of powdered chow proceeded to the interaction. Observer and demonstrator pairs interacted for 30 min (15 min for lingual anesthesia experiments), monitored through Plexiglas cage tops. Following

the interaction, subject rats received 7g of unflavored chow in their home cages. For a subset of training sessions, a mesh screen separated subject from demonstrator, preventing visual and gustatory interaction.

Preference test. 23 hours after the training interaction, subject rats received 1 hour *ad lib* access to a pair of ramekins in a clean chamber. One cup contained the "demonstrated" food, while the other contained chow mixed with a novel spice; cocoa was paired with cinnamon and marjoram with thyme (demonstrated spice counterbalanced within pairs, but full counterbalancing had the same effect, data not shown). Cup positions varied randomly; the depth of the feeding cups limited chow spillage. The effect size was nearly identical for all 4 tastes (Fig. S7).

Remote memory test. For 5 days following the Test 1 preference test, subject rats received pellet rat chow. On the 6^{th} day, food was removed. On the 7^{th} day, rats were tested for preference retention. Cup position was reversed from the first test.

N**aïve preferences.** Rats with cannulae implanted into taste cortex were tested for their preferences for cinnamon or cocoa flavored foods. Rats received either muscimol or saline infusions before the preference test.

Conditioned taste aversion (CTA). For 3 adaptation days, we gave rats 30 min of access to 15ml dH_2O from a single lick spout in a test chamber. Training sessions were identical to adaptation sessions, but we replaced the dH_2O with 0.15% saccharin, and gave an intraperitoneal injection of 0.15M LiCl (20ml/kg) to each rat immediately after the drinking session. On the next day, rats again had access to 0.15% saccharin. The difference in consumption compared with the training day quantified the acquired aversions to the saccharin.

Taste Cortical Inactivation. Immediately preceding particular sessions, rats were held in the lap of an experimenter, who inserted infusion cannulae into the surgically-implanted intracranial cannulae. Muscimol (MP Biomedicals, LLC, Ohio) or saline vehicle was infused bilaterally into TC at a rate of 0.25µl/min for 2 min (total 0.50 µl infusant). Infusion cannulae stayed in place for an additional minute, such that infusant could diffuse away from the cannula tips. The functional spread of infusions delivered using this protocol is less than 1.5mm[9].

Statistics. Comparisons of food consumption in the STFP tests required 2-way mixed effects ANOVAs, with raw g of consumed food serving as input to the analysis. Group (experimental vs control) was the between-subject variable, and food (demonstrated vs undemonstrated) was the within-subject variable. Significant interaction terms underlay all described effects of anosmia and cortical inactivation, revealing that the group manipulation changed food preference. Simple effects tests that explicated which groups learned and which didn't supplemented the anovae.

Single-neuron pre-lidocaine, during-lidocaine, and post-lidocaine taste activity was summarized in peri-stimulus time histograms (10-msec bins, smoothed for visualization but not for analysis), evaluated with paired t-tests: the last second of pre-stimulus firing was compared to the first second of post-stimulus firing.

Cannula tract histology. Rats were deeply anesthetized and perfused transcardially with 10% saline followed by 10% formalin. Brains were removed and refrigerated in 30% sucrose/10% formalin solution for several days. Coronal sections (40 µm) were cut on a cryostat and mounted to slides. Sections were stained with cresyl violet to visualize cannula tracks under light microscope. Whole coronal slices were saved to digital image, and the x-y coordinates of the cannula tips were measured in relative to the –aca- anterior commissure –anterior part and the intersection corpous callosum, and then mapped on the corresponding bregma from the Patson and Waxinos Rat Atlas[10].

Fluorescent muscimol histology. Rats were deeply anesthetized, and fluorescent Muscimol (1µg/µl, BODIPY, TMR-X conjugate, Invitrogen, CA) was infused into TC at a rate of 0.25µl/min for 2 min (total volume 0.50 µl). Cannulae were left in place for an additional 2 minutes, to allow infusant to diffuse away from the tips. Thirty-five minutes later, rats were perfused with ice-cold saline followed by 80-100 ml of 4% paraformaldehyde (PFA) in 1.00 M phosphate buffer (also ice-cold). Brains were removed and maintained at 4°C in PFA + 30% sucrose until sectioning. Fifteen consecutive vibratome-cut coronal slices (100 µm thick) through TC were collected in 0.1 M PBS, beginning either with the apparence of cannulae tracks or intersection of corpus callosum. Slices were stained with NeuroTrace® 435/455 blue fluorescent Nissl (1:300; Invitrogen,CA) following the manufacturer's protocol[11]. The extent of muscimol diffusion and the nissl stains were imaged using 4X an Olympus-1X-81 inverted fluorescent microscope fitted with rhodamine and and CyGFP filters respectively. Images for the whole brain coronal sections

were captured using the Orca-ER CCD digital camera (Hamamatsu, Japan) and Velocity software (Improvision, Lexington, MA).

Supplementary references

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Supplementary table

Table 1. Sample size (subjects)

Treatment	Training	Testing
Anosmia		
Triton X	15	13
Saline	13	15
4th days after Triton X	—	10
5th days after Triton X	—	11
Ageusia		
Topical lidocaine	9	—
Saline	10	—
Taste cortex inactivation		
Muscimol	7	7
Saline	6	7
Double Muscimol	10	*
Double Saline	13	*
Somatosensory cortex inactivation		
Muscimol	3	—
Mesh Screen		
Naïve	8	—
Muscimol	6	—
Electrophysiology		
Topical lidocaine	—	3
Total =166	**100**	**66**

* Same animals

Supplementary figures

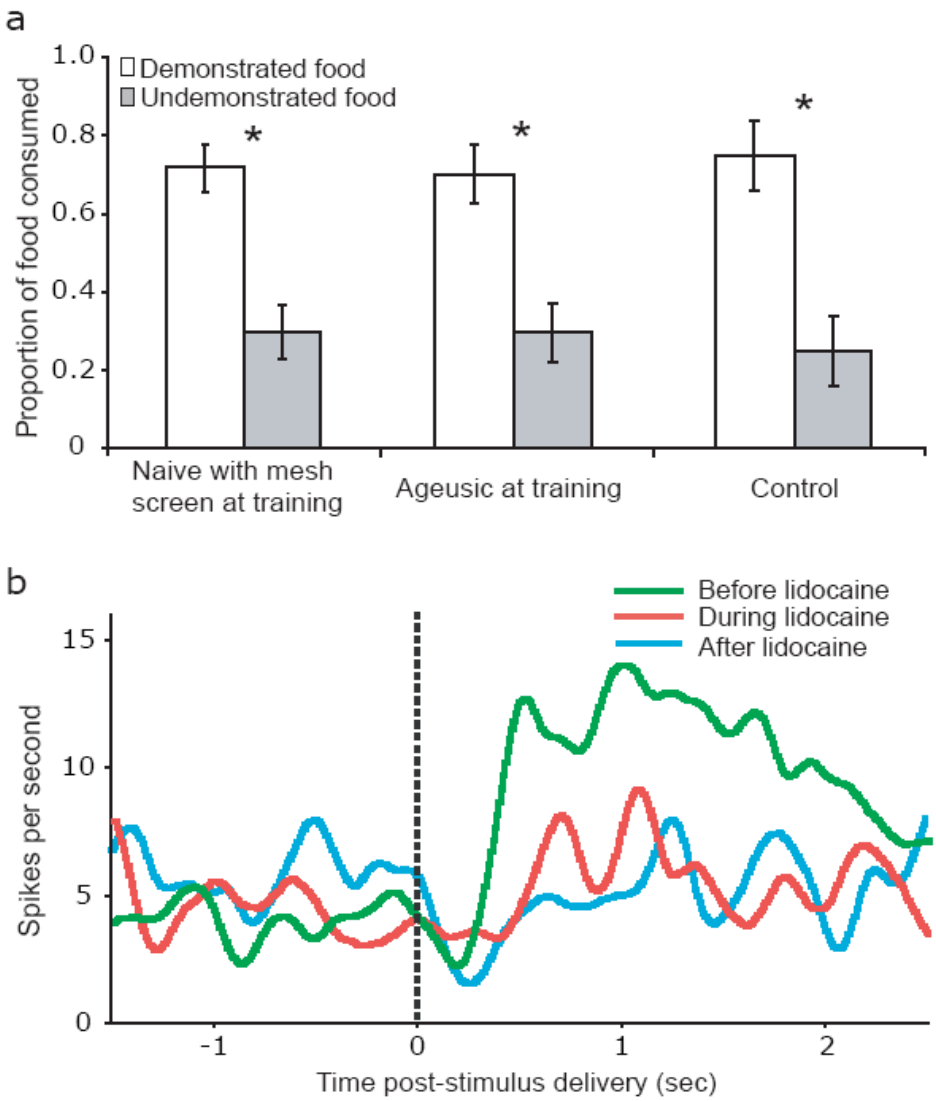

Fig. S1: Taste stimuli are unnecessary for STFP. **a**. Subject rats separated from demonstrators by a mesh screen that prevented taste, visual, or somatosensory contact (left pair of bars) later showed a strong, normal (compared to control subjects, right pair of bars) preference (y-axis) for the demonstrated food. Similarly, subjects interacting normally with the demonstrators, but only after topical lingual applications of lidocaine rendered them ageusic (i. e., unable to taste, middle pair of bars), developed the normal preference. **b**. To confirm that the lingual lidocaine swabs did in fact block taste transduction, we recorded taste responses of taste cortical single neurons before (green), during (red), and well after (blue) the procedure. Peri-stimulus time histograms of one neuron (out of 51 total recorded neurons) are shown. The x-axis is time post-stimulus delivery in sec (the vertical dashed line is the time of stimulus delivery), and the y-axis is spikes per sec. The significant excitatory taste response in the pre-lidocaine record vanishes with lidocaine application, and does not recover within 25 minutes. Of the 29% of recorded neurons that responded to taste delivery, all were largely eliminated by lidocaine application; 5-10% of these had begun to recover by 25 min after application (well after the 15-min training was done). * (here and elsewhere) $p < 0.05$.

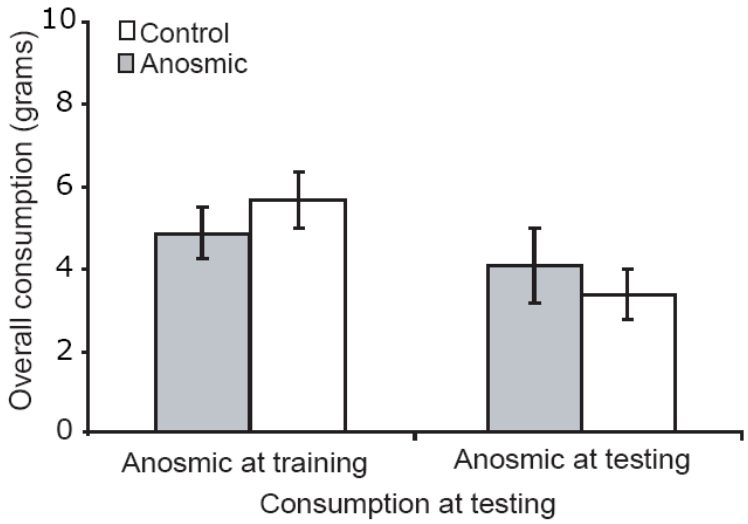

Fig. S2: Anosmia did not hinder normal eating. The total amount of food consumed in the testing session (i. e., without regard for preference) was similar for rats receiving control and detergent nasal infusions. Nasal infusions just before testing sessions briefly reduced desire to feed (right pair of bars), but this effect was short-lived (i. e., by 2 days after infusion subjects were eating more, left pair of bars) and similar for infusions of detergent and saline.

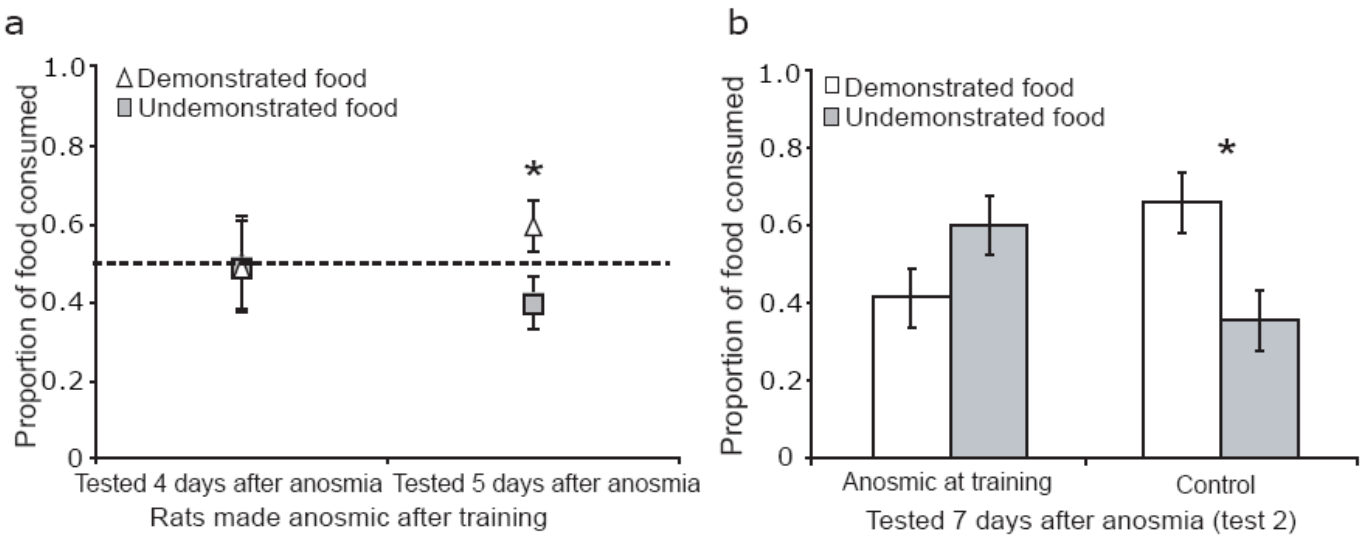

Fig. S3: Anosmia itself, and not some secondary impact of the deciliation procedure, rendered rats unable to acquire and express STFP. **a**. Subjects have begun to recover from anosmia within 5 days. When rats were rendered anosmic immediately following training, they were unable to express preferences in a test done 4 days later (left); a group of subjects tested 5 days after deciliation (right) did show a significant preference, however. **b**. Rats made anosmic just before training (left pair of bars) were still unable to express a preference for the demonstrated food even 7 days later, well after the nasal epithelium had recovered. Control rats retained preferences learned in the training session across this 8-day interval (right pair of bars).

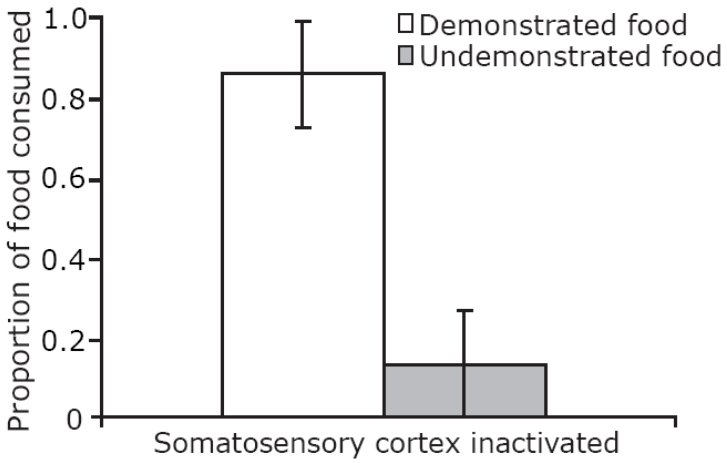

Fig. S4: Muscimol infusions into somatosensory cortex do not impact STFP. In a subset of subject rats, pre-training muscimol infusions were placed in oral somatosensory cortex, dorsal to taste cortex. These rats subsequently expressed a preference for demonstrated (grey bar) over undemonstrated (white bar) food that was indistinguishable from control rats ($F_{interaction}(1,15)=1.8$, $p>0.2$). Thus, we conclude that it was the inactivation of taste cortex, and not the infusion of muscimol, that impacts performance on the STFP task.

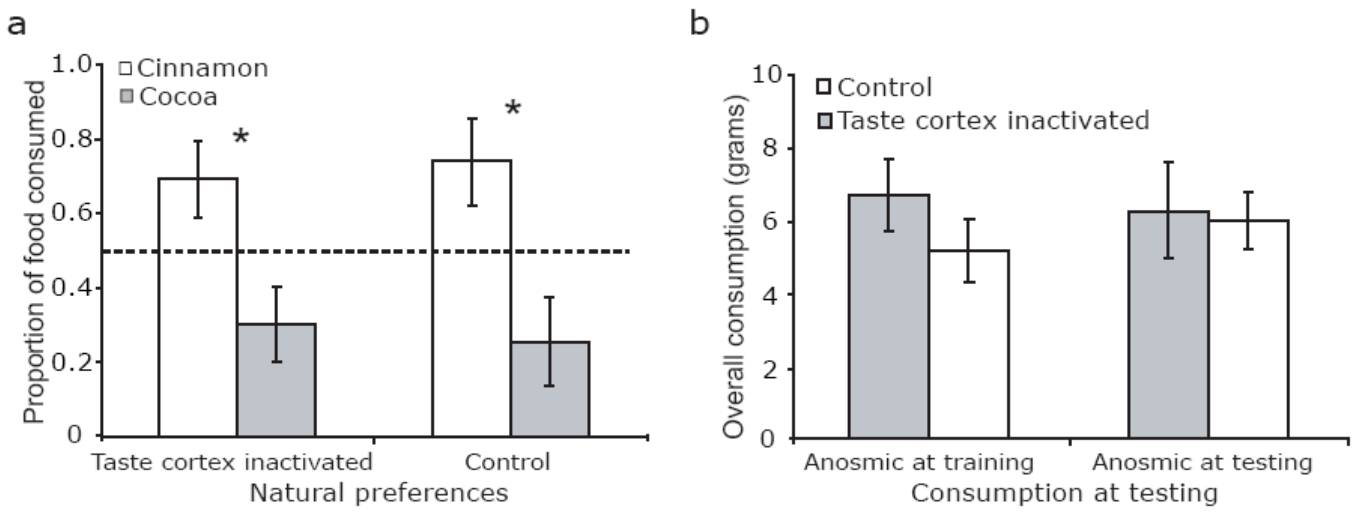

Fig. S5: Taste cortex inactivation did not alter basic ability to feed. **a**. When naïve, untrained rats were offered a choice, inactivation of taste cortex (left pair of bars) did not change rats' innate preference (right pair of bars) for cinnamon over cocoa. **b**. Neither inactivation of taste cortex during training (left pair of bars) nor testing (right pair of bars) altered subject rats' overall meal size in the testing session.

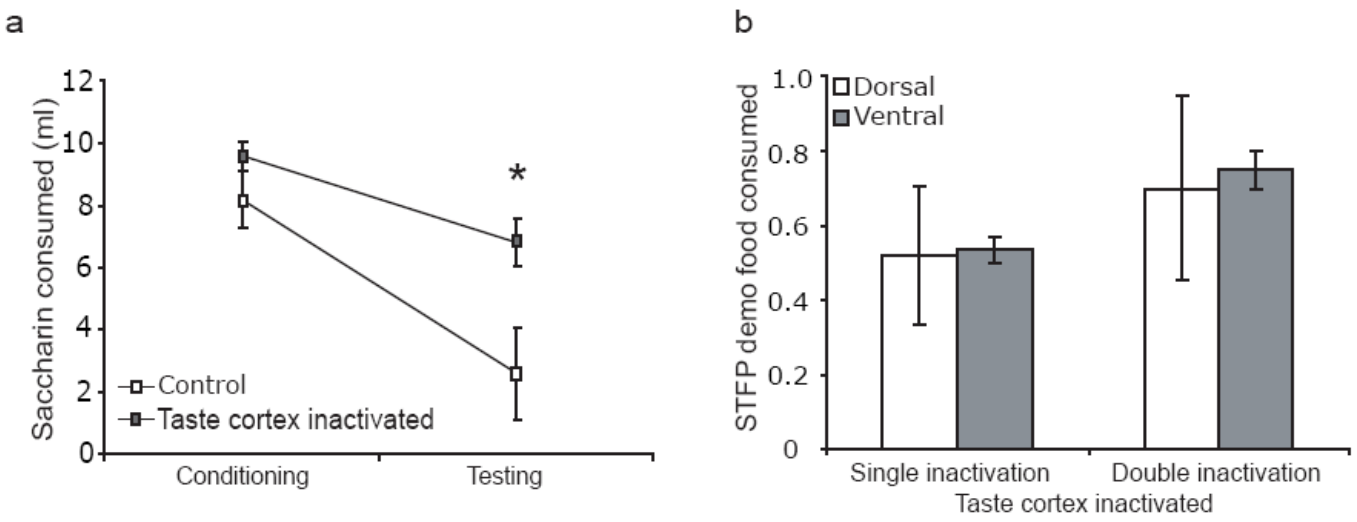

Figure S6: The site of our inactivations is taste cortex. **a**. We infused either muscimol or vehicle into taste cortex of a subset of rats that had been used for STFP, and then immediately offered the rats the chance to drink from bottles containing 0.15% saccharin (cortical inactivation did not interfere with saccharin consumption in the training session; $t(20)<1$). Following this conditioning session, all rats were given intraperitoneal injections of the emetic LiCl, sufficient to cause gastric malaise. Offered saccharin again on the day following training, control rats (empty squares) demonstrated a new-formed aversion to the formerly palatable taste (i. e., they drank little of the proffered fluid). Rats that had been trained with taste cortex inactivated (filled squares), meanwhile, were significantly less averse to the saccharin following training ($t(20)=-4.6$, $p<0.005$). These results confirm that the STFP-relevant region of cortex is indeed *bona fide* taste cortex. **b**. Muscimol infusion sites were sorted on the basis of whether the rat received infusions during either training or testing sessions (left pair of bars) or both (right pair of bars), and whether the infusion was into dorsal or ventral TC (white and grey bars, respectively; see Figure 2a). The impact of the muscimol infusions on STFP (summarized in terms of the % of consumed chow that contained the demonstrated flavor) was unaffected by precise infusion site: regardless of whether the infusion was dorsal or ventral in TC, rats receiving muscimol in one session failed to prefer the demonstrated chow, and rats receiving muscimol in both sessions did prefer the demonstrated chow.

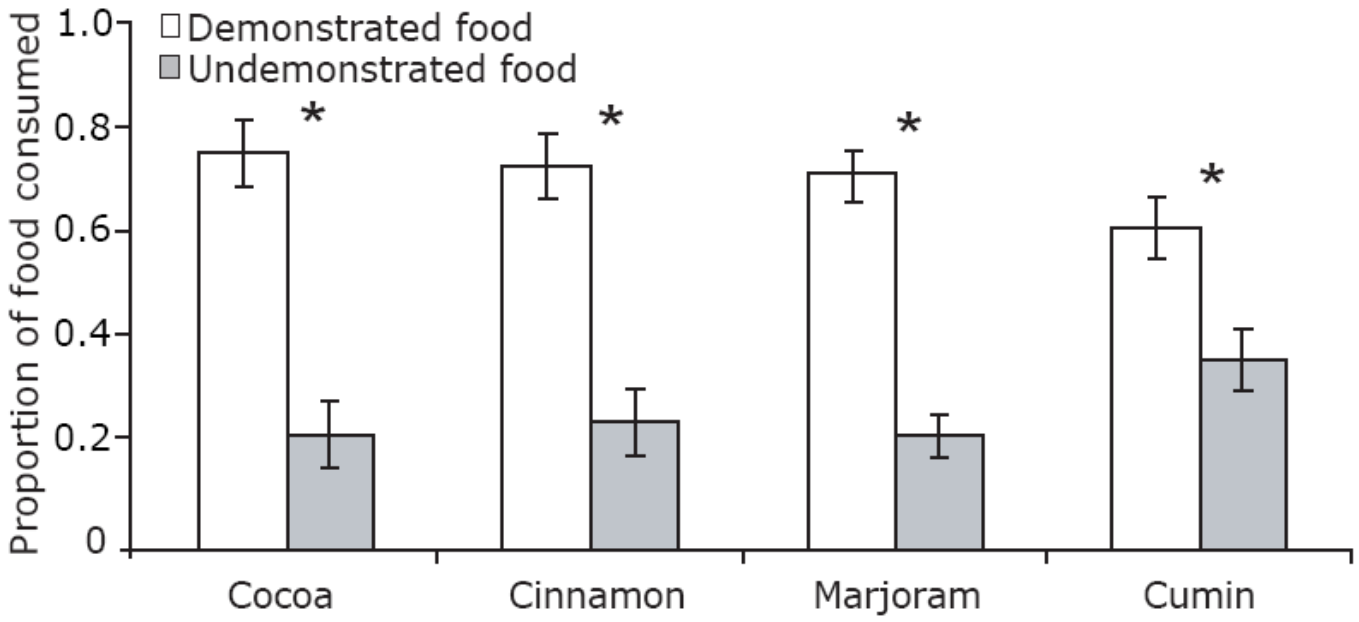

Fig. S7: STFP acts identically on all proffered spices. We broke our STFP testing data down by specific food (x-axis) and examined the impact of training (y-axis) on each. Despite the fact that each food had a distinct palatability to the naïve rat (e. g., Fig. S5a), the impact of training was remarkably similar for all—the preference for demonstrated food was significant in each case, and no between-taste differences approached significance. Analysis of raw consumption in grams yielded identical results. We conclude that our effects do not reflect artifactual effects related to particular spices or spice pairings.