Supplementary Information

The stability of tastant detection by mouse lingual chemosensory tissue requires Regulator of G protein Signaling-21 (RGS21)

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Supplementary Methods

Tracheal ring contractility

Five mm sections of tracheal rings were excised, cleaned of surrounding tissue, rinsed in physiological salt solution, and mounted in a myobath chamber between a fixed point and a force transducer (World Precision Instruments), as previously described (DeVallance *et al.* 2018). Tracheal rings were pre-stretched, and allowed to equilibrate for 1 hour in Krebs Henseleit Buffer aerated with 95% O_2 and 5% CO_2 at 37°C. After equilibration, tracheal baseline tension was adjusted to 1 g. For relaxation studies, rings were pre-contracted with methacholine (1×10^{-6} M, Sigma-Aldrich A2251), which was maintained during addition of multiple doses of isoproterenol or denatonium benzoate. Dilation was calculated as percent contractility for each dose of relaxant from the following equation: % contractility = $((z-x)/(z-y)) \times 100$, where z = tension after methacholine, x = tension following a given dose of relaxant, and y = baseline tension.

Urine specific gravity measurements

A handheld manual refractometer (Reichert Model #1310400A) was used to measure the salinity of urine output from mice and averaged over three consecutive days prior to two-bottle choice testing.

LiCl exposure-induced NaCl threshold recognition test

Singly housed mice were exposed to two bottles containing distilled water for two days to acclimate to two drinking spouts. Mice were then given two bottles containing 150 mM LiCl for 24 hours, a 24-hour recovery period with water, then another 24-hour period with two bottles containing 150 mM LiCl. After a second 48 hour period with 2 bottles of water, the mice received 48 hour tests with a choice between water and an ascending series of NaCl concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 150 mM NaCl), as performed in two bottle preference testing.

Supplementary Figures

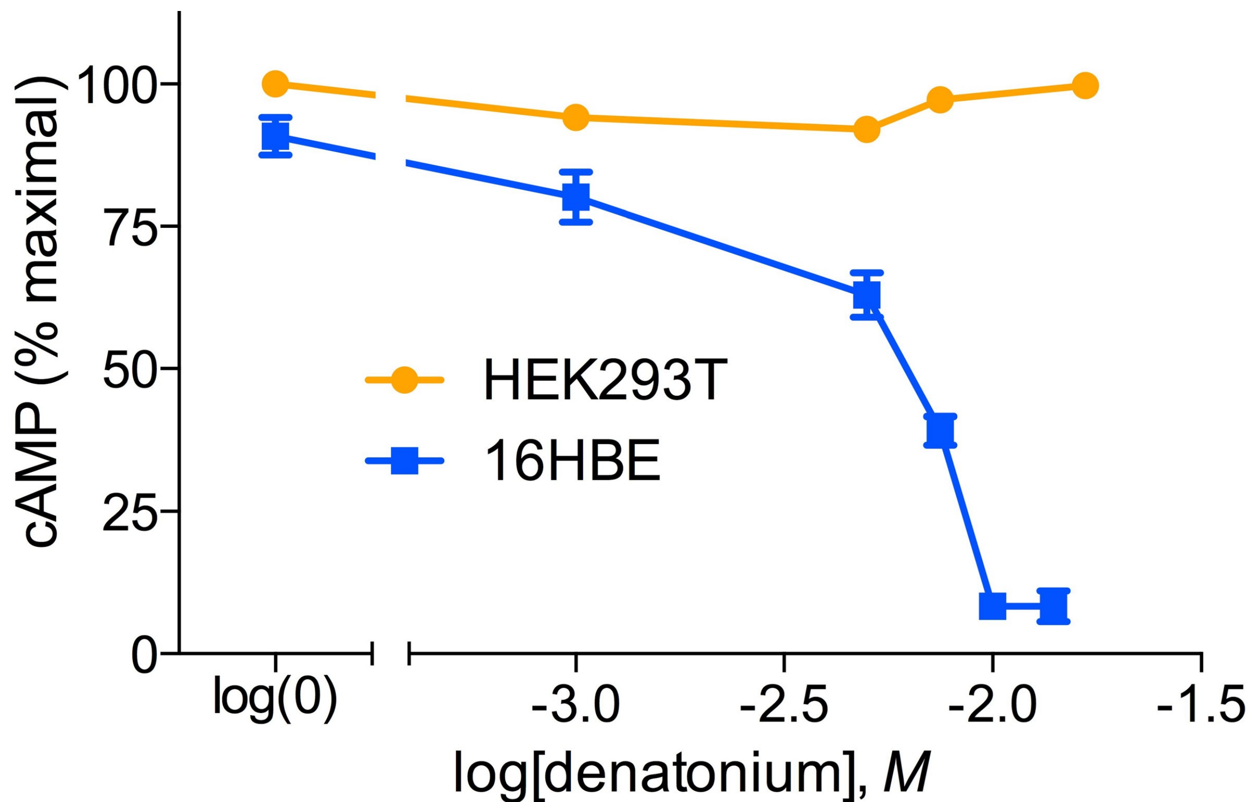

Figure S1. Establishment of the EC_{50} for denatonium benzoate in inhibiting cAMP production within the 16HBE cell line. Monolayers of the 16HBE cell line (or the HEK293T cell line as a negative control unresponsive to denatonium benzoate) were transiently transfected with GloSensor cAMP biosensor cDNA. Inhibition of forskolin-stimulated cAMP production (1 μM forskolin) by treatment with indicated concentrations of denatonium-B was determined 24 hours post-transfection by detection of GloSensor-dependent luminescence.

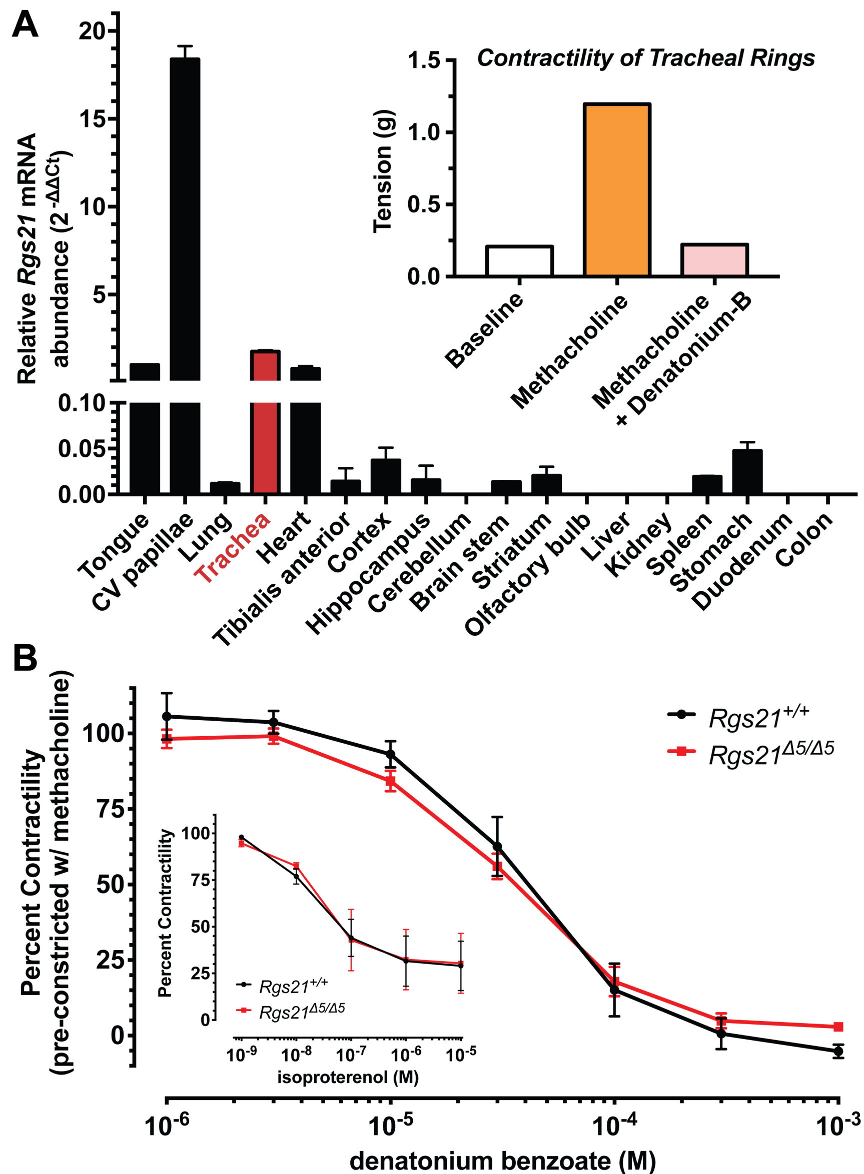

Figure S2. Tracheal ring relaxation *ex vivo* by denatonium benzoate is unaffected by *Rgs21* gene status. (**A**) Identification of tracheal expression of *Rgs21* gene locus by qRT-PCR across a panel of indicated organs and tissues from wild-type C57BL/6J mice (as normalized to the detection of 18S rRNA). *Inset bar graph*, Contractility of mouse tracheal rings *ex vivo*. Methacholine was applied at 1 μM, in the absence or presence of denatonium benzoate at 10 mM. (**B**) Relaxation of tracheal ring constriction by indicated concentrations of denatonium benzoate is unaffected by the loss of *Rgs21* expression (*i.e.*, compare responses of $Rgs21^{+/+}$ [n = 3] vs $Rgs21^{\Delta5/\Delta5}$ [n = 3] littermates; mean ± SEM plotted). *Inset line graph*, Control responses of tracheal rings to relaxation by indicated concentrations of isoproterenol.

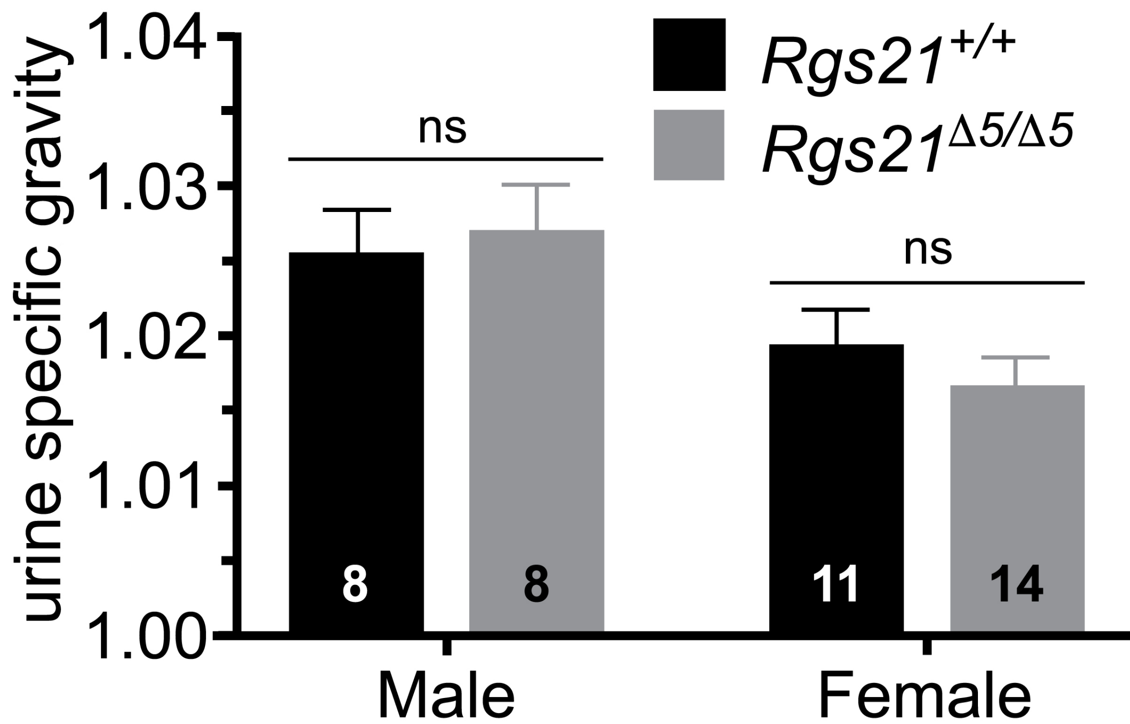

Figure S3. Urine specific gravity is unaffected by *Rgs21* genotype in adult male and female C57Bl/6J mice (numbers of animals tested as indicated within bars; ns, not significant when tested using Student t-test across genotype).

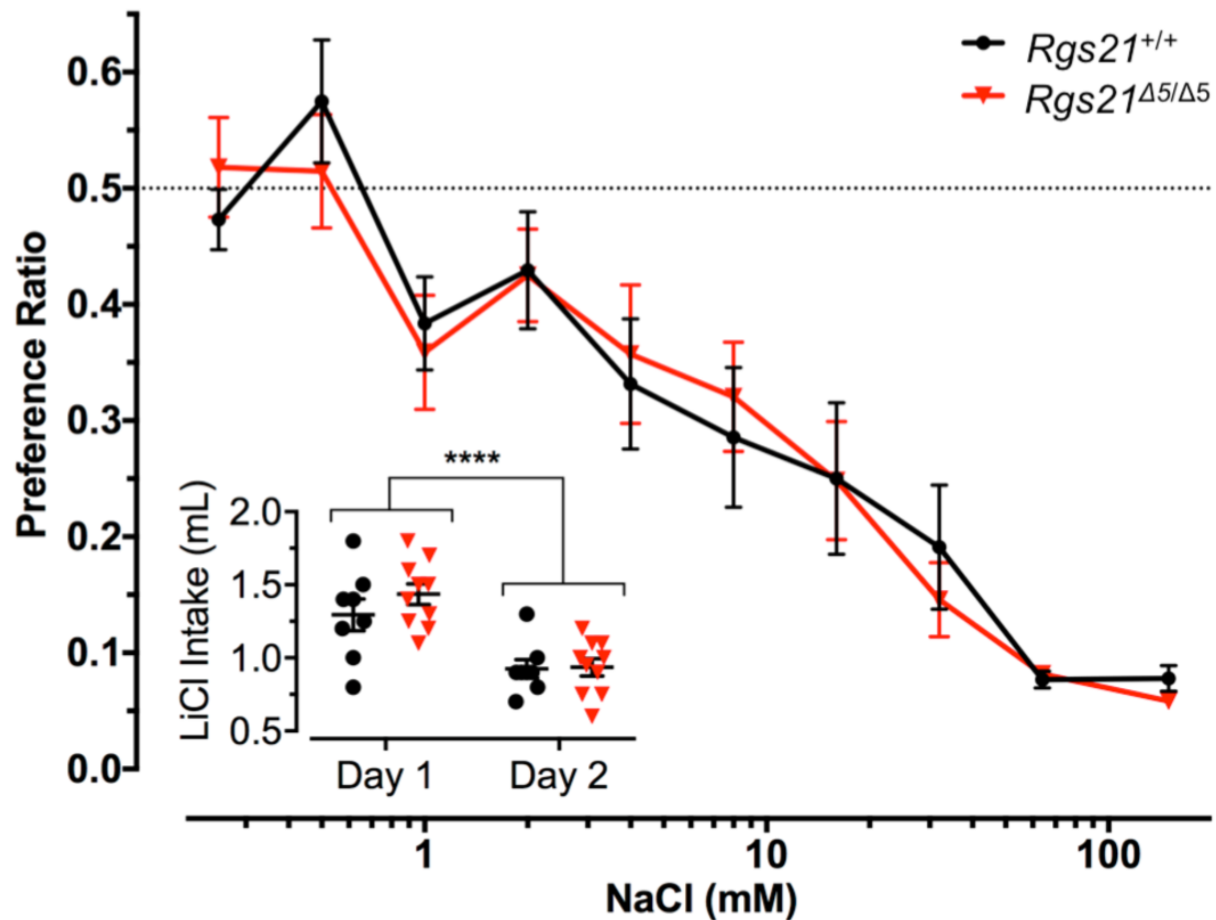

Figure S4. Constitutive *Rgs21*-null (*Rgs21*$^{Δ5/Δ5}$) mice display normal NaCl threshold recognition. Following exposure to LiCl in their drinking water, which causes mice to become temporarily ill and tastes similar to NaCl, mice will avoid NaCl. This LiCl pre-exposure (*inset*) allows the determination of the lowest concentration of NaCl that tastes salty to mice. *Rgs21*$^{+/+}$ [n = 8] and *Rgs21*$^{Δ5/Δ5}$ [n = 10] mice avoided NaCl similarly after LiCl pre-exposure, with no significant difference between the groups at any concentration, suggesting that RGS21 is not involved in the detection of low concentrations of NaCl.

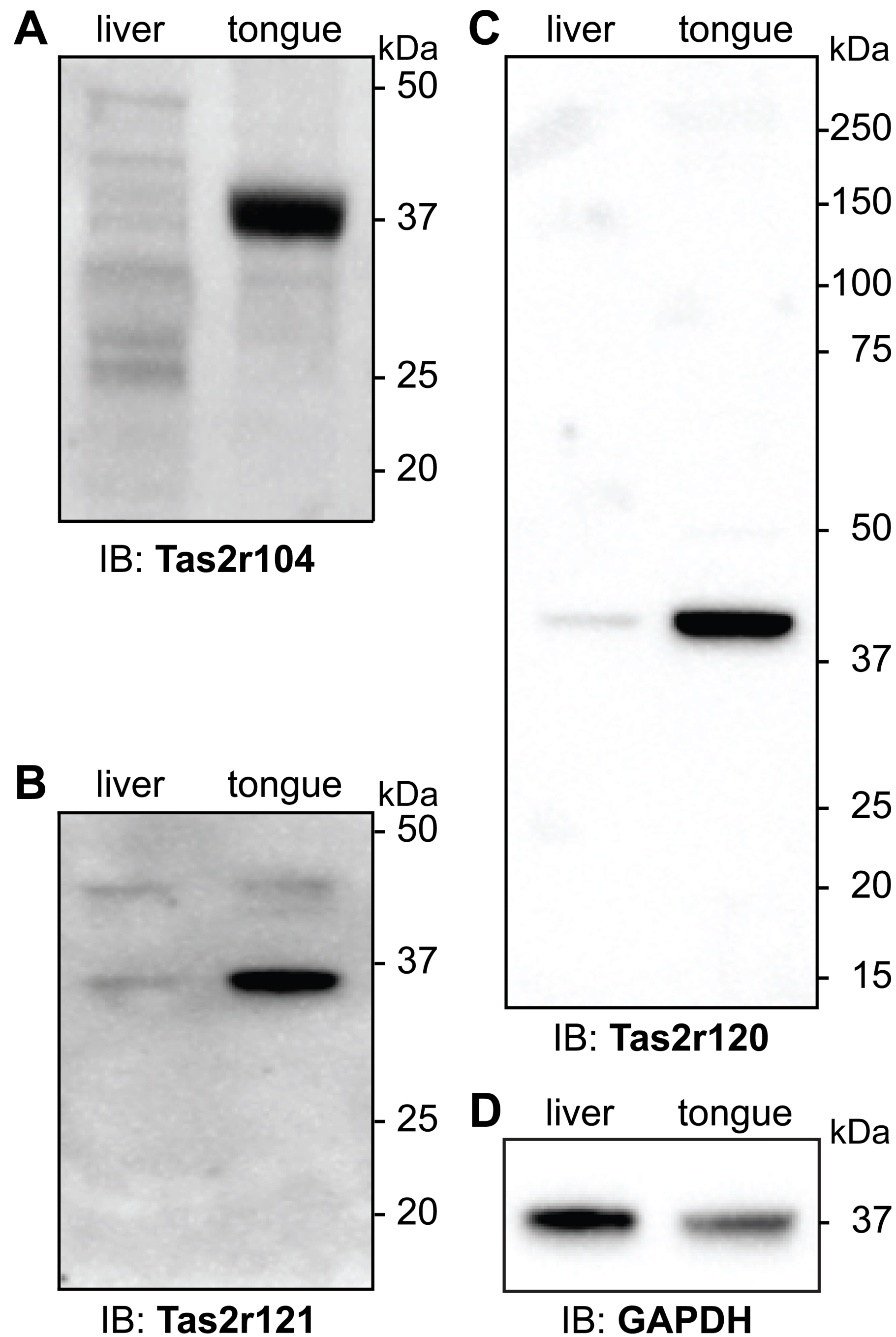

Figure S5. Validation of commercial anti-Tas2r antibodies by immunoblotting of isolated mouse liver *vs* mouse tongue tissue lysates resolved by standard SDS-PAGE and detected by chemiluminescence. (**A**) Immunoblotting (IB) for expression of Tas2r104. (**B**) Immunoblotting for expression of Tas2r121. (**C**) Immunoblotting for expression of Tas2r120. (**D**) Immunoblotting for expression of GAPDH as a tissue lysate loading control.

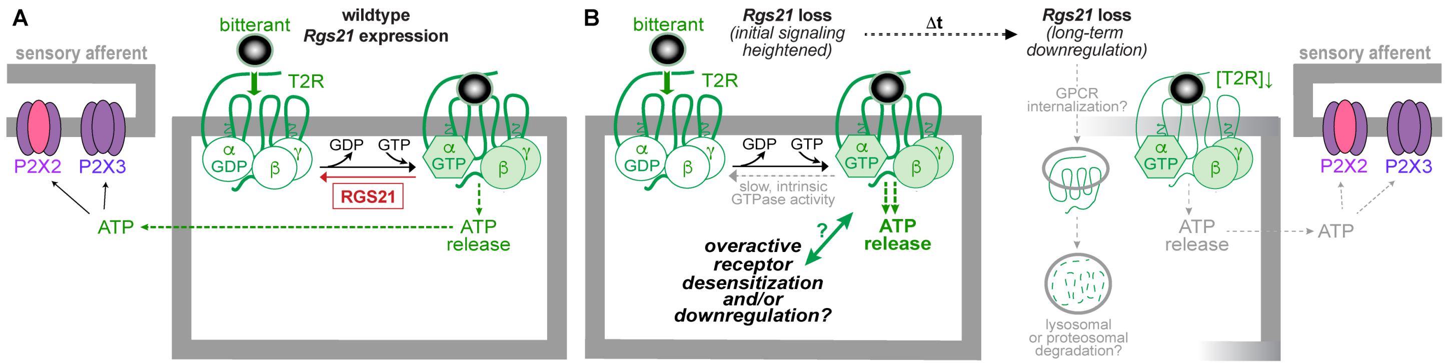

Figure S6. A model of long-term changes to explain how the loss of RGS21's negative regulatory activity ultimately leads to *decreased* (not increased) sensitivity to bitterants. (**A**) Bitterant binding to T2R proteins at the cell surface leads to activation of receptor guanine nucleotide exchange (GEF) activity and, in turn, GTP loading of the heterotrimeric Gα subunit, resultant downstream signaling by both freed Gβγ and Gα·GTP subunits, and (ultimately) ATP release, which signals via P2X2 and P2X3 ionotropic purinergic receptors on the sensory afferent juxtaposed to the taste receptor cell. In the taste receptor cells of wild-type mice (*i.e.*, with wild-type RGS21 expression), RGS21-mediated GTPase-accelerating (GAP) activity inactivates the G protein heterotrimer that is originally activated by bitterant-bound T2R GEF activity. (**B**) Initially after loss of RGS21 expression, bitterant-stimulated T2R signaling is more active, leading to overly active T2R desensitization and/or downregulation (including, perhaps, receptor internalization and degradation, as illustrated) that leads, over time ("Δt"), to reduced cell-surface levels of T2R and, hence, reduced sensitivity to bitterant stimulation. Desensitization of later steps in the tastant signal transduction pathway, such as the purinergic receptors on the afferent nerve fibers, might also arise from an initial phase of chronic prolonged bitterant/T2R signaling.

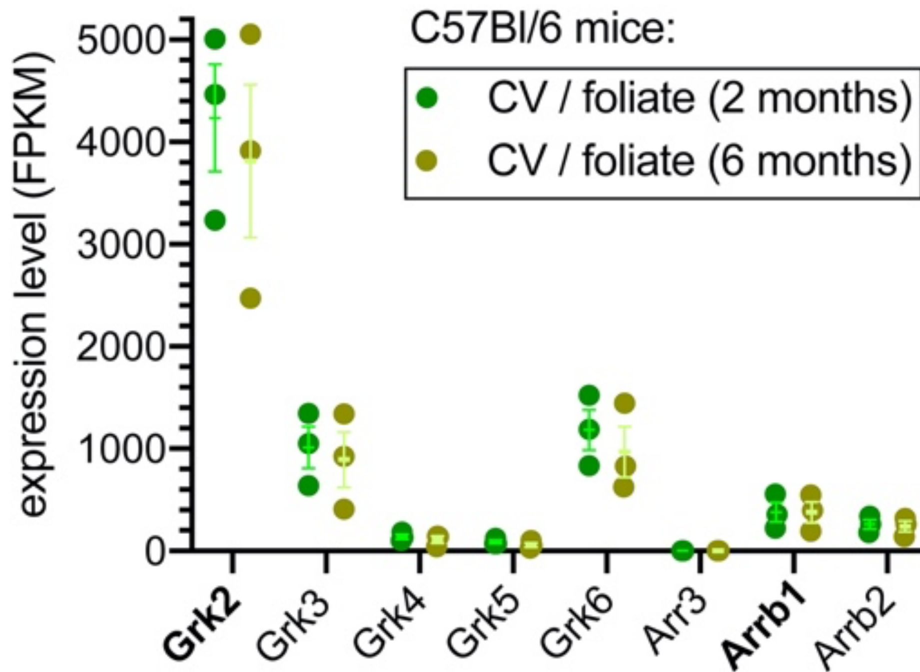

Figure S7. *Grk2* (encoding the G protein receptor kinase βARK1/GRK2) and *Arrb1* (encoding β-Arrestin1) are among the GPCR desensitization machinery genes expressed in mouse tastant-responsive lingual tissue. Data from NCBI id GSE85308 representing RNA-seq analysis of isolated taste bud cells from circumvallate (CV) and foliate papillae of 2 month-old and 6 month-old C57Bl/6 mice, as previously described (Shandilya *et al.* 2016). Bars represent mean ± SEM for the three replicates plotted as circles.