Expression of *Calca* gene-derived peptides in the murine taste system

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Abstract

Taste cell regeneration and taste signaling are regulated by myriad growth factors and signaling molecules secreted by neurons and taste papillae - resident cells. The Calcitonin Related Polypeptide Alpha (*Calca*) gene is a source of four biologically active peptides with varied physiological roles. Alternative splicing of the *Calca* messenger RNA generates either prepro calcitonin gene related peptide (CGRP) or preprocalcitonin encoding transcripts. Proteolytic processing of preprocalcitonin generates procalcitonin, calcitonin and katacalcin. Calcitonin is a ligand for the G-protein coupled receptor calcitonin receptor (CALCR) while CGRP is a ligand for the CGRP receptor (CGRP1R) formed by the calcitonin receptor like receptor (CALCRL)-receptor activity modifying protein 1 (RAMP1) complex. Interestingly, procalcitonin too, is a ligand for the CGRP1R where it can antagonize CGRP. CGRP expression in taste and trigeminal neurons has been documented and is posited to regulate taste signaling. Single cell and bulk RNASeq of taste papillae revealed that the preprocalcitonin but not the *Cgrp* transcript is expressed in *Tas1r3*- expressing type II taste cells, while CGRP1R subunits are expressed in taste stem/progenitor cells and by subsets of fibroblasts and immune cells in the lingual mesenchyme. We confirmed this expression pattern using quantitative polymerase chain reaction (qPCR) and histological techniques. qPCR of geniculate and nodose-petrosal ganglia revealed that both express *Cgrp* and CGRP1R subunit mRNAs, but not preprocalcitonin and *Calcr*. This interesting expression patterns suggests that procalcitonin and CGRP might reciprocally regulate the CGRP1R in the taste papillae and potentially influence taste signaling, taste cell regeneration and the taste microbiome.

Keywords

Neuropeptide, Procalcitonin, CGRP, Gustation

53 **Introduction**

54 Taste cell regeneration, taste neuron pathfinding and taste signaling are regulated by myriad
55 growth factors and signaling molecules.[1,2] Among the growth factors regulating regeneration, R-
56 spondin 2 is primarily derived from the taste nerve, while sonic hedgehog is derived from both
57 taste nerves and type IV taste cells.[3-7] Other growth factors such as Wnts, noggin, jagged, bone
58 morphogenetic proteins, insulin like growth factors etc. are presumed to be produced locally by
59 taste and mesenchymal cells in the papillae.[7-11] Molecules such as nerve growth factor, brain-
60 derived neurotrophic factor, semaphorins and ephrins are required for taste neuron pathfinding,
61 while another set including ghrelin, glucagon like peptide 1, cholecystokinin, neuropeptide Y,
62 leptin and vasoactive intestinal peptide are known to regulate taste signaling.[9,12-21] The above
63 list is far from exhaustive, and it is likely that many more peptide signaling molecules remain to
64 be discovered. The *Calca* gene is a source of four such molecules (Figure S1A).[22,23] Tissue
65 specific alternative splicing of *Calca* transcript generates mRNAs coding for either calcitonin
66 gene related peptide (preproCGRP, proteolytically processed to alpha CGRP) or
67 preprocalcitonin (prePCT).[21] PrePCT is proteolytically processed to generate procalcitonin
68 (PCT), which is further processed to generate calcitonin and katacalcin.[22,23] All four belong to a
69 larger group of peptides that include beta CGRP, amylin, adrenomedullin, and intermedin, which
70 are ligands for receptors formed by the G protein coupled receptors calcitonin receptor (CALCR)
71 or calcitonin receptor like receptor (CALCRL) by themselves or in combination with one of three
72 receptor activity modifying proteins (RAMP1, RAMP2 or RAMP3).[24,25] For example, calcitonin is
73 a ligand for CALCR without an associated RAMP subunit, whereas CGRP binds both CALCR+
74 RAMP1 and CALCRL+RAMP1 (this being its primary receptor, henceforth designated CGRP1R;
75 Figure S1A).[25,26] CGRP is an exceptionally well-studied neuropeptide; it is expressed in virtually
76 all peripheral sensory neurons, many central neurons, and some non-neuronal cells.[25,27] It
77 serves a wide variety of functions including nociception, neurogenic inflammation, immunity,
78 vasodilation, wound healing, regulation of the microbiome etc.[27-29] It is a potent neuro-immune

79 modulator and was recently shown to modulate antigen sampling by M cells in the Peyer's
80 patch.[27-30] It displays antimicrobial activity against several gram-negative and gram-positive
81 bacteria and Candida albicans, which adds a further dimension to its immunomodulatory and
82 wound healing roles.[31,32] From a clinical standpoint, its prominent role in triggering migraine has
83 generated significant scientific and clinical interest. This has led to the development of small
84 molecule- and monoclonal antibody-based CGRP1R antagonists to treat migraines.[27,33,34]
85 Interestingly, clinical studies have suggested that one-quarter of migraine patients have altered
86 taste perception.[35] Indeed, CGRP was shown to shape taste transduction by inducing 5-HT
87 secretion by type III taste cells in a phospholipase C-dependent manner.[36]
88 Calcitonin and katacalcin are key regulators of calcium and phosphate levels in blood and
89 bone.[24,37,38] PCT is a much less studied neuropeptide. It is not widely secreted in the steady
90 state; it may regulate bone density by suppressing osteoclast (macrophage) migration and
91 maturation.[24,39] Interestingly, sepsis-associated cytokine storm is preceded by ubiquitous
92 upregulation of PCT expression, leading to its adoption as an early sepsis marker.[40-44] PCT is a
93 partial agonist of CGRP1R and exerts its mediator role in sepsis through this receptor, and
94 partially antagonizes CGRP at this receptor.[45-47] However, its biological role(s) in both the steady
95 state and in sepsis remains enigmatic. Analysis of single cell RNASeq (scRNASeq) and bulk
96 RNASeq of the circumvallate papillae (CVP) and fungiform papillae (FFP) showed that the
97 prePCT encoding *Calca*- transcript is highly expressed in *Tas1r3*- expressing type II taste cells
98 in the CVP but only weakly in FFP, while subunits of the CGRP1R are expressed in taste stem
99 cells and lingual mesenchymal fibroblasts and subtypes of immune cells. The CGRP transcript
100 and *Calcr* are not expressed in taste papillae. This was confirmed using qPCR, RNAScope and
101 immunohistochemistry. On the other hand, the nodose- petrosal- jugular (NPJ) and geniculate
102 ganglia that innervate taste papillae were shown to express transcripts for *Cgrp* and CGRP1R
103 subunits, but not the prePCT transcript and *Calcr*. This intriguing expression pattern suggests

104 that taste cell derived PCT and taste nerve derived CGRP may reciprocally modulate CGRP1R
105 signaling in the taste papillae.
106
107 **Methods**
108 ***Animals.***
109 8-10 weeks old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) and Tas1r3-GFP[48],
110 Lgr5-GFP[49] and Gad1-GFP[50] mice strains were used for this study. Animals were housed in a
111 specific pathogen free vivarium with a 12-h light/dark cycle and open access to food and water.
112 All animal experiments were performed in accordance with the National Institutes of Health
113 guidelines for the care and use of animals in research and reviewed and approved by the
114 Institutional Animal Care and Use Committee at University of Nebraska-Lincoln (protocols: 2610
115 and 2366) and the Monell Chemical Senses Center (protocols: 1163, 1151).
116
117 ***Bulk RNASeq of taste papillae.***
118 10 µm thick coronal sections of fresh frozen tongue from C57BL/6 mice containing FFP or CVP
119 were prepared using a cryostat. Sections were stained with hematoxylin& eosin, and pools of
120 ~100 taste buds from CVP (n=2 pools from 2 mice) and ~20-30 from FFP (n=3 pools from 3
121 mice) were excised using laser micro dissection, and full length bulk RNASeq libraries were
122 prepared using the Ovation RNASeq system V2 (Tecan biosystems, Morgan Hill, CA) per
123 manufacturer instructions. Indexed illumina sequencing libraries were prepared and sequenced
124 in a Hiseq 2000 sequencer (Illumina Inc, San Diego, CA). Raw sequences were aligned to the
125 mouse reference genome (version GRCm38.p3) using the STAR program with default settings
126 and Gencode M14.gtf as the splice junction annotation file, and the reads mapping to genes
127 were counted using the featureCounts package.[51,52] Normalization and differential expression
128 analysis was done using the DESeq2 package in R/Bioconductor.[53] Sashimi plots to show
129 alignment of reads were generated using the integrative genome viewer.[54]

130 ***Isolation and scRNASeq of GFP labeled taste cells.***

131 scRNASeq of taste cells from Tas1r3-GFP cells from CVP has been previously reported.[55]
132 scRNASeq of GFP expressing cells from CVP of Gad1-GFP (n=9) and Lgr5 GFP (n=5) mice
133 and FFP of Tas1r3-GFP (n=5) mice was done using the same protocol. Briefly, mice were
134 euthanized by exposure to CO2 followed by cervical dislocation. The tongue was quickly
135 dissected and washed in chilled Tyrode's solution, and a protease mixture was injected under
136 the lingual epithelium. After a 12 min incubation at 37 °C, the lingual epithelium was peeled,
137 washed, and subsequently incubated in Ca2+-free Tyrode's for 30 min at room temperature and
138 then replaced with DMEM. Taste buds were triturated with a glass pipette, and individual GFP
139 labeled cells were collected by aspiration using a micro capillary tube controlled by a
140 micromanipulator attached to a fluorescence microscope. aRNA amplification, illumina library
141 preparation and deep sequencing were done as previously described.[55] Reads alignment,
142 counting, normalization and differential expression analysis was done as described above for
143 bulk RNASeq.

144

145 ***RNAScope Hiplex assay.***

146 RNAscope assay was done using the Hiplex fluorescent assay kit for mice (Advanced Cell
147 Diagnostics Inc., Newark, CA, Cat. no. 324443) with indicated probes (Table S1) as previously
148 described using the manufacturer's instructions.[56] Positive and negative control probes were run
149 in parallel to test probes to ensure proper hybridization and imaging conditions were attained in
150 our experiments. Confocal images were captured using a Nikon A1R-Ti2 confocal laser
151 scanning microscope using NIS-Elements A1R software image acquisition and analysis
152 software, using 40/60X objectives. Images were taken using a sequential channel series setting
153 to minimize cross-channel signal, and the channels used were: GFP 488, TxRed 550, Cy5
154 650 nm. Z-series stack with 10 images per stack was captured at a step size of 1 μm.
155 Acquisition parameters [i.e., gain, offset, photomultiplier tube (PMT) setting] were held constant

156 for experiments. Colocalization counts were made using QuPath software.[57] Cell boundaries
157 were detected automatically based on DAPI staining. The fidelity of each cell boundary was
158 confirmed by manual inspection. The number of fluorescent spots in each channel (that
159 corresponds to individual mRNA molecules) per cell were extracted and used for colocalization
160 counting. Data from more than two non-consecutive sections from two mice were pooled.
161
162 ***Immunohistochemistry***
163 Standard immunohistochemical techniques were used as previously described.[56,58] Briefly, 12
164 μm thick sections from tongues drop were prepared using a cryostat. Frozen sections were
165 rehydrated with PBS. Nonspecific binding was blocked with SuperBlock Blocking Buffer
166 (Thermo scientific, Waltham, MA, Cat. no. 37580) at room temperature for 1 h. Sections were
167 incubated with primary antibodies overnight at 4 °C in a humidified chamber. After three 15-min
168 washes with PBST, slides were incubated for 1 h at room temperature with the indicated
169 fluorescent secondary antibodies in blocking buffer. All double-immunofluorescent labeling was
170 done with combinations of the secondary antibodies along with DAPI (1:1,000; Invitrogen™,
171 Thermo scientific, Waltham, MA, Cat. no. D1306) to label cell nuclei for cell counting. The
172 primary and secondary antibodies and their concentrations used in this study are listed in Table
173 S2.
174 Double labeling with two antibodies made from the rabbit (LRMP+PCT and T1R3+PCT), was
175 done as described before.[59] After blocking, the sections were incubated in succession: first
176 primary anti-rabbit antibody overnight at 4 °C, first fluorescence Fab fragment secondary for 1 h
177 at room temperature, unlabeled anti-rabbit antisera [AffiniPure Fab fragment donkey anti-rabbit
178 IgG (H+L)] for 3 h at room temperature to ensure that all binding sites in the first primary
179 antibody are occupied, second primary anti-rabbit antibody overnight at 4 °C, and then second
180 fluorescence Fab fragment secondary for 1 h at room temperature. Controls for the double
181 immunohistochemistry experiments with two rabbit primary antibodies were done without

182 donkey Fab fragment incubation following the first secondary antibody and omitting the second
183 primary antibody (Figure S4 I & II, control A) or with donkey Fab fragment incubation and
184 omitting the second primary antibody (Figure S4 I & II, Control B) to show adequate blocking of
185 rabbit IgG by donkey Fab fragment. Confocal imaging was done as described above for
186 RNAScope. Colocalization for each taste cell marker was done using images from at least 2
187 nonconsecutive CVP and FOP containing sections (n=1 mouse). Only those taste cells for
188 which the entire cell body and nucleus could be visualized were counted.
189
190 ***PCR and qPCR.***
191 Total RNA was isolated from freshly dissected geniculate and nodose-petrosal jugular ganglia
192 complex (NPJ, since it is hard to dissect the desired petrosal portion from the NPJ), taste
193 papillae or NT epithelium using the Quick-RNA Microprep kit (Zymo Research Corp, Irvine, CA,
194 Cat. no. R1050) with on-column DNA digestion. cDNAs were prepared from total RNA samples
195 using SuperScript IV VILO Master Mix kit (Thermo Fisher, Waltham, MA, Cat. no. 11756050).
196 End point PCR and qPCR were done as previously described.[59] Exon-exon junction spanning
197 primers were designed whenever possible to avoid amplification from any contaminating
198 genomic DNA. A minimum of three biological replicates were used for all cDNA samples. The
199 ratio of the log10 of the average δ-cycle threshold (Ct) value (difference between Ct values of
200 *Bact* and each gene of interest was plotted.[59] Primers used are shown in Table S3.
201
202 **Results**
203
204 ***Bulk RNASeq and PCR show the expression of Calca transcripts and receptors in taste***
205 ***papillae and ganglia.***
206 Analysis of bulk RNASeq data from CVP taste buds isolated by laser microdissection showed
207 that the *Calca* transcript is strongly expressed in CVP, but only weakly in the FFP (not shown).

208 Alignment of the RNASeq reads to the *Calca* genomic locus showed that no reads mapped to
209 the preproCGRP specific exon 5, while large number of reads aligned to prePCT specific exon 4
210 and to the shared exons 1-3 (Figure 1A). This observation was confirmed using end point PCR
211 using primers specific to the *Cgrp* and prePCT transcripts (Figure S1B), which showed robust
212 amplification for the prePCT transcript in CVP and foliate papillae (FOP) and very weak
213 expression in the FFP. *Cgrp* transcript is expressed at very low levels or were undetectable in all
214 three taste papillae. We could readily detect transcripts for *Calcrl* and *Ramp1* in all three taste
215 papillae and for *Ramp2* in CVP, but not for *Calcr* and *Ramp3*. The *Calcb* transcript that encodes
216 beta CGRP is expressed in all three taste papillae. None of the transcripts are expressed in
217 non-taste lingual epithelium (NT, Figure S1B). Next, we checked the expression of these
218 transcripts in the geniculate ganglia that innervate the FFP and the NPJ ganglia complex, the
219 petrosal portion of which innervate the CVP and FOP. The prePCT transcript and *Calcr* are not
220 expressed in either ganglia, while the *Cgrp*, *Calcrl, Calcb*, *Ramp1*, and *Ramp2* transcripts were
221 detected in both (Figure S2B). Next, we quantified the expression of these transcripts in CVP,
222 FOP, geniculate and NPJ ganglia using qPCR. Consistent with the end point PCR results,
223 robust expression of prPCT is detected in both papillae and weak expression of *Cgrp* is
224 detected in the CVP but not FOP (Figure 1B). Conversely, *Cgrp*, but not prPCT transcript is
225 strongly expressed in both taste ganglia (Figure 1C). Robust expression of *Calcrl, Calcb and*
226 *Ramp1* were detected in both the taste papillae and ganglia, while *Calcr and Ramp3* are not
227 expressed in any sample. Weak *Ramp2* expression is detected in the two ganglia, while it is not
228 expressed in the taste papillae (Figure 1B-C).

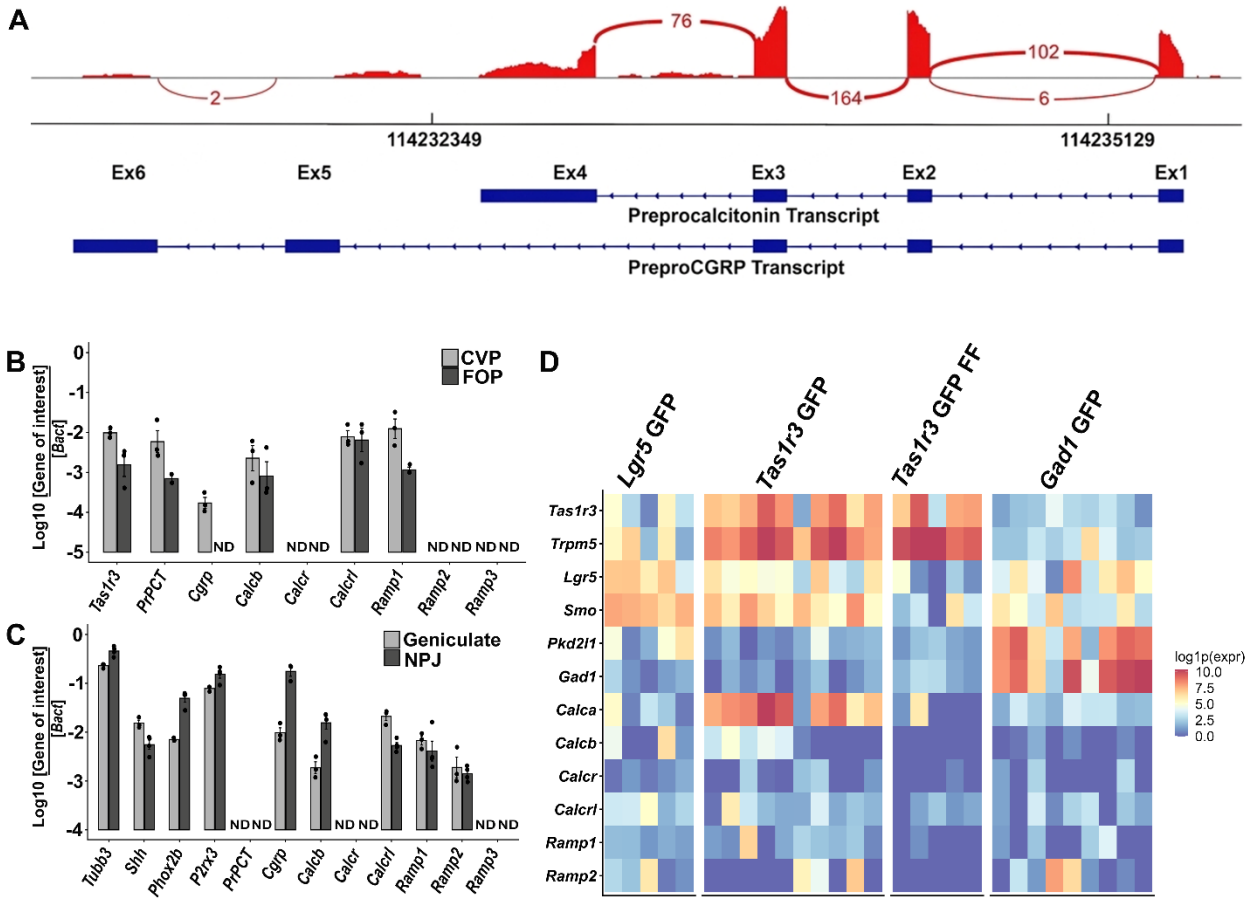


Figure 1. Expression of *Calca* transcripts and receptors for Calca-derived peptides quantified using RNASeq and qPCR. A) Sashimi plot of the *Calca* gene locus (transcribed from bottom strand in right to left direction) in a bulk RNASeq library from CVP. The reads aligning to exons 1-6 (Ex1-Ex6, right to left) are shown in red, with the height of the red bars showing the strength of expression, and the number of reads aligning across splice junctions indicated in the loops between exons. Very few reads align to the preproCGRP specific exon 5, while large number of reads align to prePCT specific exon 4 and the shared exons 1-3. **B- C shows qPCR profiling of *Calca and Calcb* transcripts and their receptors in cDNA from taste papillae and sensory ganglia**. **B)** Strong expression of *PrPCT, Calcb*, *Calcrl* and *Ramp1* is observed in both CVP and FOP. Weak expression of *Cgrp* is observed in CVP, while it is undetectable in FOP. *Calcrl and Ramp1* are expressed in both CVP and FOP while *Calcr, Ramp2 and Ramp3 are* not detected in either CVP or FOP. *Tas1r3* is used as a control to demonstrate the quality of taste cDNA. Expression in FFP was not quantified by qPCR due to the preponderance of non-taste epithelium in peeled

242 FFP samples. **C)** qPCR of above transcripts in geniculate and NPJ ganglia. *Cgrp* and *Calcb* are expressed
243 in both ganglia, with stronger expression observed in the nodose-petrosal ganglion. *Calcrl, Ramp1 and*
244 *Ramp2* are expressed in both ganglia and similar levels. Expression of *Ramp3*, *PrPCT* and *Calcr* is not
245 observed in either ganglion. The taste ganglion marker genes *Tubb3, Shh, Phox2b*, and *P2rx3* are used to
246 demonstrate the quality of ganglia cDNA. The expression of each gene is plotted as the logarithm of the
247 ratio between its cycle threshold value and that of the house keeping gene *Bact*. ND= not detected. **D)**
248 **scRNASeq data showing expression of transcripts for *Calca, Calcb* and their receptors in taste cells.**
249 Data from Lgr5-GFP (n=5 cells), Tas1r3-GFP (n=10), Tas1r3-GFP cells from FFP (n=5) and Gad1-GFP
250 (n=9) cells are shown. Marker genes for type II cells (*Tas1r3, Trpm5*), Lgr5+ cells (*Lgr5, Smo*), and Type III
251 cells (*Pkd2l1, Gad1*) are also shown as controls.

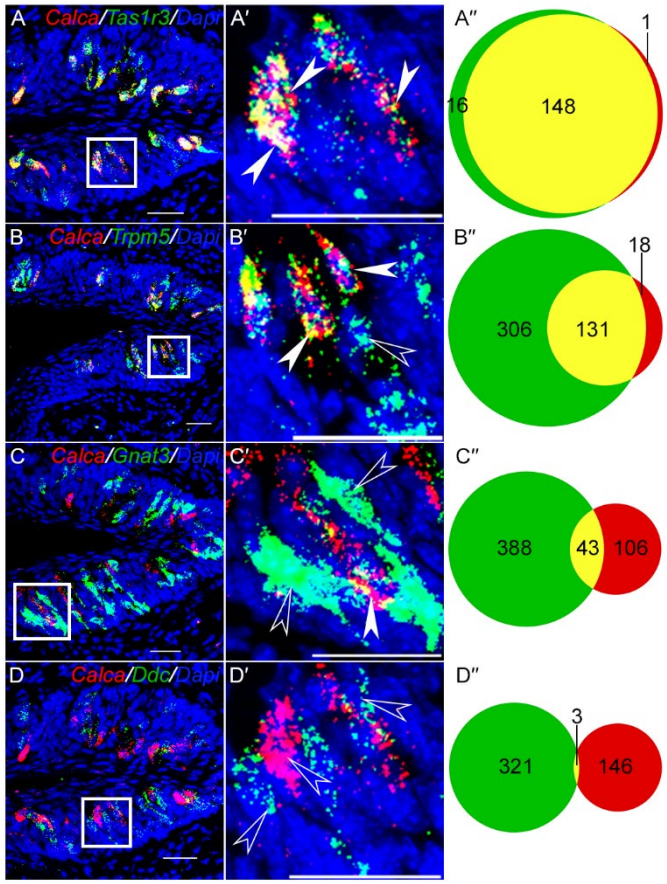
252

253 ***scRNASeq of taste cells identifies Calca and CGRP1R subunit expressing taste cells.*** To
254 identify taste cells that express *Calca* and CGRP1R subunits, we turned to scRNAseq dataset
255 from GFP expressing taste cells isolated from CVP of Lgr5-GFP and Gad1-GFP mice and both
256 CVP and FFP of Tas1r3-GFP mice. Robust expression of the *Calca* transcript (alternative
257 splicing info cannot be extracted from 3' biased scRNASeq data) is observed from Tas1r3-GFP
258 cells (sweet and umami receptor expressing, type II) in CVP but not in Tas1r3-GFP cells in the
259 FFP, and in Lgr5-GFP (stem/progenitor) and Gad1-GFP (Type III) cells from CVP. The CGRP1R
260 subunits *Calcrl* and *Ramp1* are expressed at moderate levels in Lgr5-GFP cells and in Tas1r3-
261 GFP and Gad1-GFP cells (Figure 1 D).

262

263 ***Histological analyses confirm the expression of Calca, Calcrl and PCT in taste cells.*** Next,
264 we turned to histological analyses to confirm the cell type specific patterns of *Calca* and *Calcrl*.
265 RNAscope Hiplex analysis of CVP confirmed the scRNASeq results: Using a *Calca* probe
266 capable of detecting both mRNA isoforms, strong coexpression with the sweet taste receptor
267 subunit *Tas1r3* is observed, with 148/164 (90%) of *Tas1r3* expressing cells coexpressing *Calca,*

268 and 148/149 (99%) *Calca* expressing cells coexpressing *Tas1r3* (Figure 2A- A″). *Calca* is also
269 coexpressed the canonical type II taste cell marker *Trpm5,* with 131/437 (30%) of *Trpm5*
270 expressing cell coexpressing *Calca*, while 131/149 (88%) of *Calca* expressing cells coexpress
271 *Trpm5* (Figure 2 B- B″). Most type II cells that do not express *Calca* appear to be bitter taste
272 receptor cells, as less than one tenth of *Gnat3* expressing cells (primarily bitter taste cell
273 marker in CVP) expressed *Calca* (Figure 2C- C″).[60,61] *Calca* is not expressed in type III taste
274 cells marked by *Ddc*. (Figure 2D- D″).



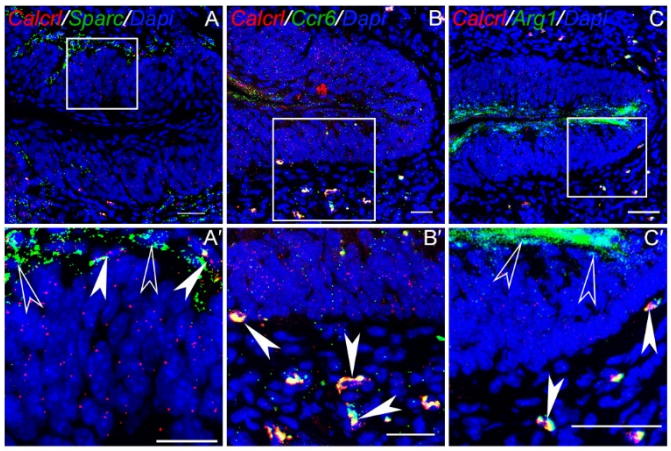
275

276 **Figure 2. Analysis of *Calca* mRNA expression in CVP**. RNAscope Hiplex fluorescence assay was used
277 to determine the coexpression of *Calca* with the taste cell markers *Tas1r3* (A-A′), *Trpm5* (B-B′), *Gnat3* (C-
278 C′) and *Ddc* (D-D′). The areas highlighted in white boxes in A-D are magnified in A'-D'. Filled white
279 arrowheads highlight double positive cells, and open white arrowheads highlight single positive cells. A″-
280 D″ are venn diagrams showing the number of taste cells that co express or singly express the indicated

281 marker genes and *Calca* (Data are from two non-consecutive sections from two mice). Strong
282 coexpression (yellow) of *Calca* is observed with *Tas1r3* and *Trpm5*, less strong coexpression is observed
283 with *Gnat3* and negligible coexpression is observed with *Ddc*. Scale bars = 30 μm. DAPI is used as a
284 counterstain for nuclei.

285

286 *Calcrl* staining is observed in unidentified mature taste cells and basal (presumably *Lgr5*+
287 stem/progenitor) cells in the CVP taste buds (Figure 3A, A′). Significant staining for *Calcrl* is also
288 observed in mesenchymal cells close to basal cells of taste buds that stained strongly for the
289 fibroblast marker *Sparc* (Figure 3A, A′). In agreement with a published scRNASeq dataset from
290 tongue immune cells, *Calcrl* staining was also observed in innate lymphoid cell 3 (ILC3) marker
291 *Ccr6* (Figure 3B, B') and ILC2 marker *Arg1* (Figure 3 C,C').[62] As expected, *Calca* expression is
292 very weak in the FFP, but appear to colocalize primarily with type II taste cells (Figure S2 A-D').
293 *Calcrl* expression is similar to that in CVP taste buds, with strong expression in mesenchymal
294 fibroblasts and weaker expression in basal and mature cells in the taste buds (Figure S2 E,E').

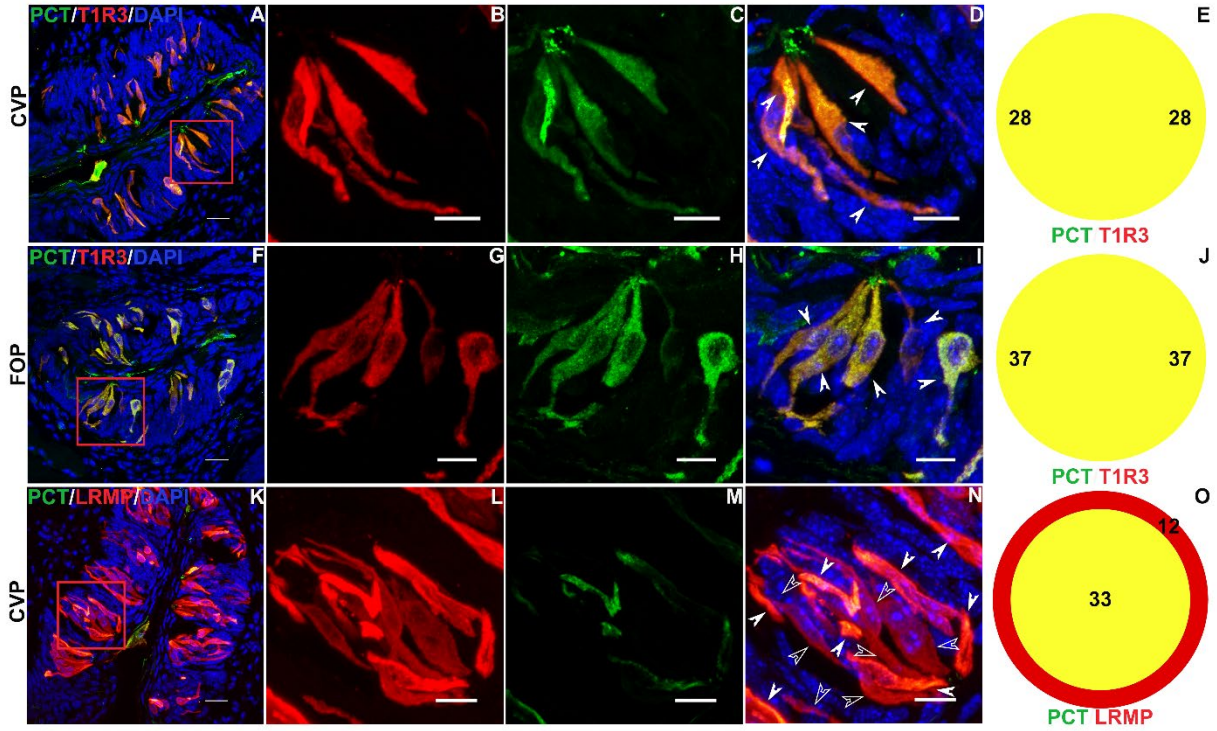


295

296 **Figure 3. Co expression of *Calcrl* with fibroblast and innate lymphoid cell marker genes**. RNAscope
297 Hiplex fluorescence assay was used to determine the coexpression of *Calcrl* with indicated marker genes.
298 Areas inside white boxes in A-C are magnified in A'-C'. A & A′ shows the expression of *Calcrl* in fibroblasts
299 marked by *Sparc* adjacent to basal taste cells. B & B' shows the expression of *Calcrl* in *Ccr6*+ ILC3 and C

& C' shows its expression in *Arg1*+ ILC2 population of immune cells next to the taste buds. Filled white arrowheads highlight double positive cells, and open white arrowheads highlight single positive cells. Scale bars in A-C are 100 µm and A'-C' = 30 µm. DAPI is used as a counterstain for nuclei.

Complementary results were obtained using double labeled immunohistochemistry. Using a PCT specific antibody, we saw strong co expression of PCT with the sweet taste receptor subunit T1R3 (100% in both directions) in CVP (Figure 4 A-E) and FOP (Figure 4 F-J). In addition, PCT is strongly coexpressed with the pan-type II marker LRMP, with all PCT expressing cells expressing LRMP and 33/45 (73%) of LRMP expressing cells coexpressing PCT in the CVP (Figure 4 K-O). In case of LRMP, colocalization is observed primarily with cells that stained strongly with LRMP antibody. The weaker LRMP positive cells primarily expressed GNAT3, which, as alluded to before, is primarily expressed in bitter taste receptor cells in the CVP (Figure S3).



314 **Figure 4. PCT is expressed in type II taste cells.** Double labelled immunofluorescence confocal
315 microscopy of CVP and FOP sections with antibodies against PCT (green) and type II taste cell markers
316 T1R3 (red; A–D, F-I) or LRMP (red; K–N). Nuclei are counterstained with DAPI (blue). A, F, K are lower
317 magnification images and dashed red boxes indicate regions shown at higher magnification in respective
318 panels to the right. (D, I, N) Double positive cells are highlighted with solid arrows and single positive cells
319 with hollow arrows. Colocalization counts are shown in Venn diagrams (E,J,O). Scale bars = 20 μm. DAPI
320 is used as a counterstain for nuclei.

321

322 ***CGRP expression in lingual taste and non taste papillae.*** Next, we used
323 immunohistochemistry with antibodies against CGRP and TUBB3 to examine the expression of
324 CGRP in neuronal fibers that innervate the lingual epithelium. In agreement with previous
325 reports, strong CGRP expression was observed in fibers that innervate the CVP, with a large
326 proportion of TUBB3 positive nerve fibers coexpressing CGRP.[36] Processes in the CVP
327 mesenchyme are strongly labeled, as are many fibers that innervated taste buds (Figure 5 A-D).
328 A similar pattern of expression is observed in the FOP and FFP (Figure 5 E-H and I-L).
329 Interestingly, CGRP is also expressed in the fibers innervating the non-taste epithelium, albeit
330 less densely compared to the taste epithelium (Figure 5 M). However, strong CGRP expression
331 is observed in fibers innervating the filiform papillae (Figure 5 N-P). CGRP staining in all parts of
332 the tongue is punctate, likely reflecting localization to vesicles.

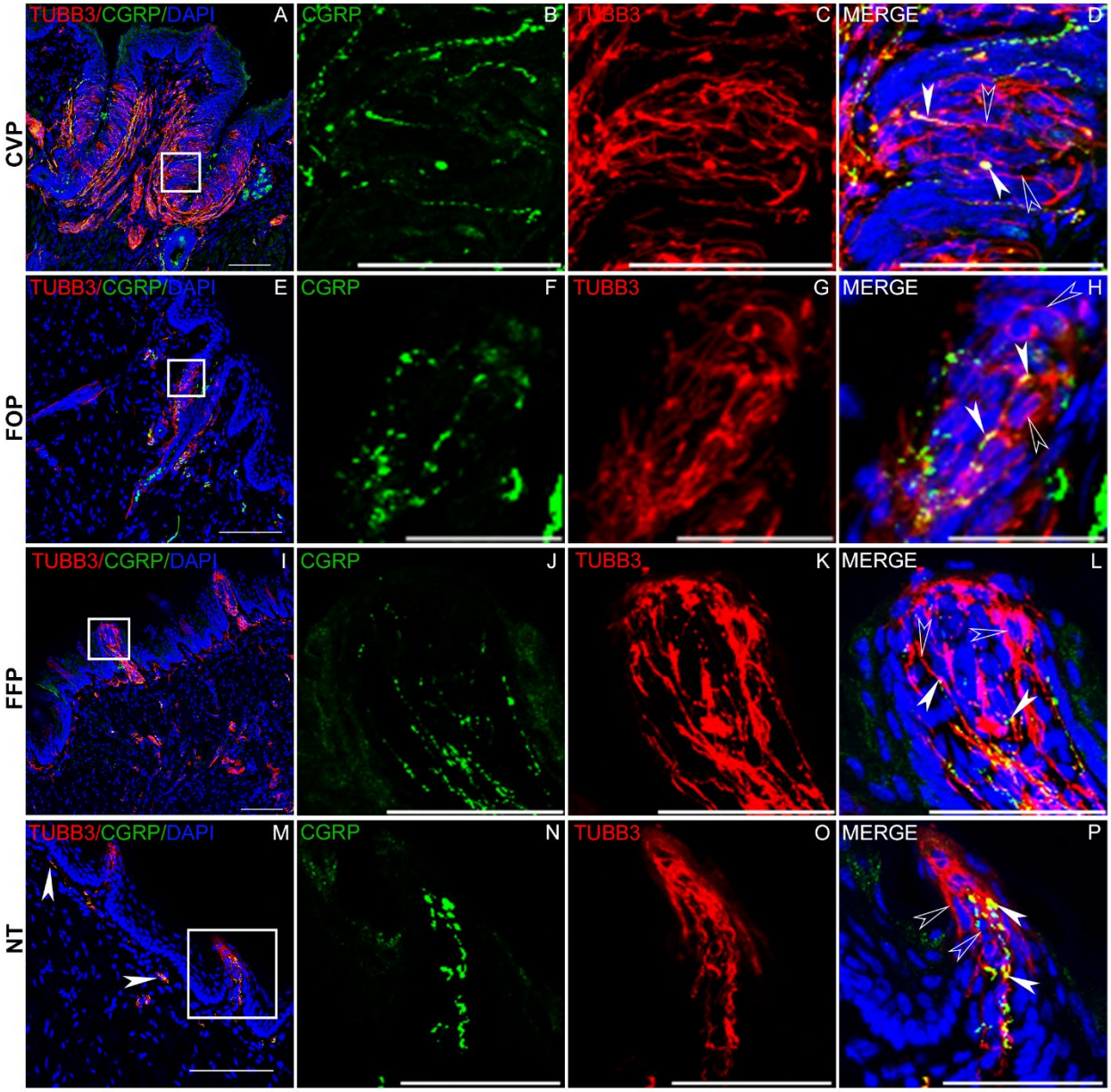


Figure 5. Expression of CGRP in lingual neurons. Double labelled immunofluorescence confocal microscopy with antibodies against CGRP (green) and TUBB3 (pan neuronal marker protein, red) was used to visualize CGRP expression in CVP (A-D), FFP (E-H), FOP (I-L) and non-taste lingual epithelium (NT, M-P). Areas inside white boxes in A, E, I and M are magnified in the respective panels to the right. Strong CGRP expression is seen in a large subset of neuronal fibers in the mesenchyme as well as within taste buds in all three taste papillae. Filled white arrow heads show colocalization, and open white arrowheads show TUBB3 only fibers. Neuronal innervation is weak in the NT epithelium, but the filiform papillae are

341 strongly innervated by neuronal fibers, many of which are CGRP positive. Scale bars in A, E, I and M are
342 100 μm and those in the remaining panels are 30 μm. DAPI is used as a counterstain for nuclei.

343

344 **Discussion**

345 Neuropeptides and growth factors are essential for taste function, but it is likely that many key
346 neuropeptides required for taste function have not yet been identified. The *Calca* gene derived
347 peptides CGRP, calcitonin, and procalcitonin play key roles in health and disease.[24,27,33,38-40,63]
348 Using a number of techniques probing the expression of the corresponding mRNAs and
349 proteins, we show that the prePCT encoding transcript is strongly expressed in CVP and FOP,
350 almost exclusively in the *Tas1r3* expressing type II (primarily sweet and umami receptor
351 expressing) taste cells. Curiously, *Calca* expression (both prePCT and *Cgrp* mRNAs) is very
352 weak in FFP (Figures S1B, S2). The related *Calcb* gene is expressed in all three taste papillae.
353 All three taste papillae express the CGRP1R subunits *Calcrl* and *Ramp1* (Figures 1, 3, S1B,
354 S2). Interestingly, the taste neurons in the geniculate and NPJ ganglia that innervate the
355 papillae expresses the *Cgrp* but not the prePCT transcript and express both the CGRP1R
356 subunits (Figure S1B, Figure 1C). A small amount of *Cgrp* transcript is detected in the CVP
357 using both endpoint and qPCR, although it is likely derived from the nerve endings that
358 innervate the taste papillae rather than taste cells themselves (Figure S1B, Figure 1). This is
359 supported by the absence of *Cgrp* transcript in the bulk RNASeq data from CVP, derived from
360 laser microdissected taste buds devoid of contamination from nerve bundles in the CVP core
361 that would be present in the CVP samples used for PCR experiments (Figure 1A). CGRP
362 expression in trigeminal neurons is well studied, and it is very likely that trigeminal neurons that
363 innervate the taste papillae also express CGRP, although this has not been experimentally
364 demonstrated to our knowledge.[64] Thus, it is possible that the bulk of alpha CGRP in the taste
365 papillae is nerve derived. Indeed, abundant staining for CGRP is observed in nerves innervating

366 the taste papillae and non-taste lingual epithelium (Figure 5). Previous immunolocalization
367 studies of CGRP in rodent, pig and human taste papillae show CGRP expression restricted to
368 nerve bundles and their termini in and around CVP taste buds.[36,65-67] PrePCT is processed to
369 generate PCT and then to calcitonin and katacalcin (Figure S1A). We were able to detect the
370 robust expression of prePCT transcript using end point and qPCR (Figures 1 and S1). Although
371 the RNAScope probe for *Calca* does not distinguish between the CGRP and PCT encoding
372 mRNA isoforms, we obtained strong staining in TAS1R3- and LRMP- expressing taste cells with
373 the PCT antibody (Figures 2, S2 and 4). However, these experiments do not allow us to
374 determine if calcitonin and katacalcin are produced by taste cells. Since both peptides are part
375 of PCT, they cannot be readily distinguished using antibody staining. Notably, their biological
376 roles are distinct from that of PCT; both are well known hormones produced by the parathyroid
377 gland that bind to the calcitonin receptor to regulate bone and serum calcium and phosphorus
378 levels.[24,37,38] PCT on the other hand, is an early marker of sepsis, which is only expressed at
379 very low levels in healthy individuals.[39-42,45] Notably, we did not detect the calcitonin receptor
380 mRNA (*Calcr*), in the taste papillae or the geniculate and NPJ ganglia using qPCR and only low
381 levels were detected in the scRNASeq data (Figures 1, S1). Thus, the available evidence
382 indicates that calcitonin and katacalcin are either not produced by taste cells and nerves or will
383 not be biologically active in case they are produced. PCT on the other hand, can stimulate
384 CGRP1R, and it also antagonizes CGRP at this receptor.[45,47] Considering abundant CGRP1R
385 receptor expression in CVP, FOP, and taste ganglia, PCT is the only peptide generated from
386 taste cell expressed prePCT transcript capable of exerting its biological role.

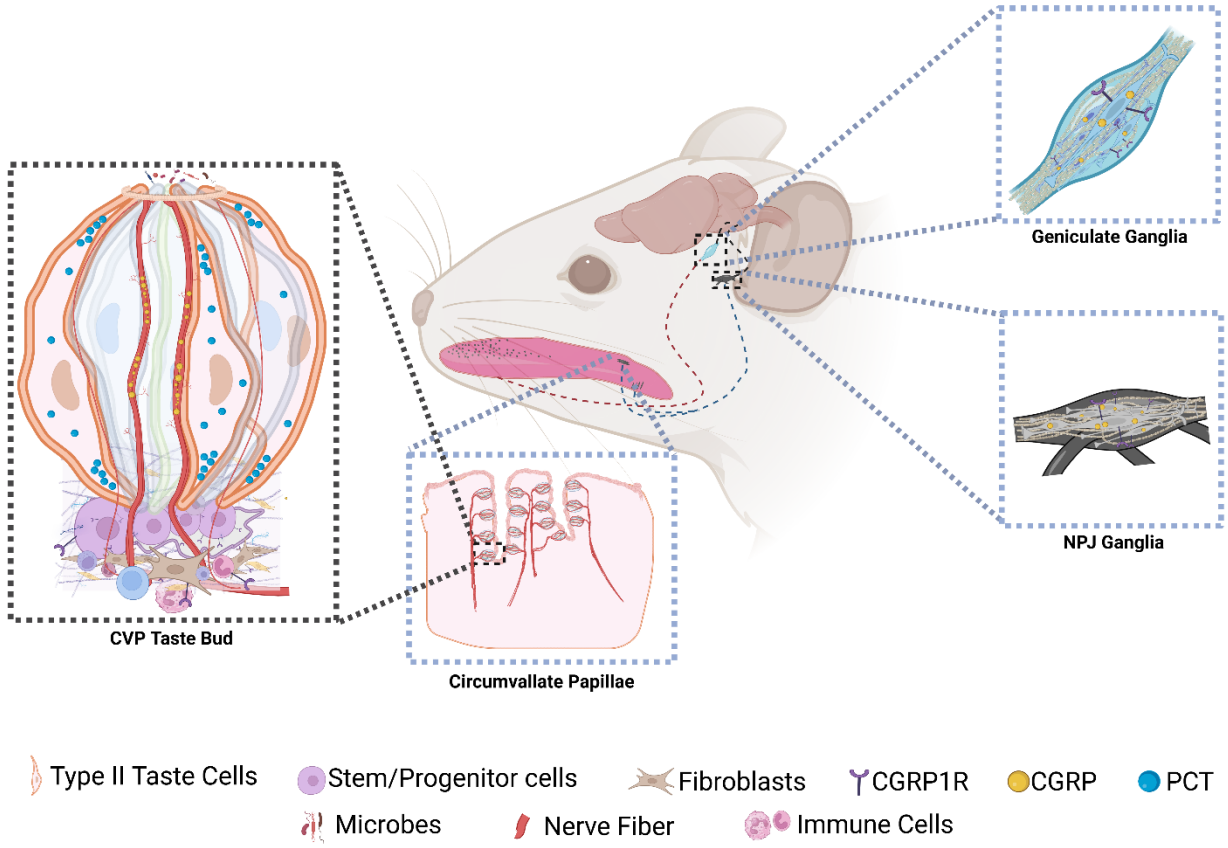


Figure 6. Model for the roles of PCT and CGRP in taste papillae. Lingual CGRP is produced primarily by the taste (geniculate, petrosal portion of NPJ) and trigeminal (not shown) ganglia and delivered to the tongue through the nerve fibers. In CVP and FOP, taste buds are arranged along the walls of trenches that limit their cleansing by salivary flux, likely causing higher bacterial biofilm formation which might proceed to infection if unregulated. PCT produced by type II taste cells in the CVP and FOP, and nerve-derived CGRP can bind to the CGRP1R expressed in some mature taste cells, stem/progenitor cells, mesenchymal fibroblasts and ILC2 and ILC3 cells. They may also be secreted to the taste papillae trenches and modulate the microbiome by virtue of their microbicidal or microbiostatic effects. Illustration created in BioRender. Siddiqui, A. H. (2026) https://BioRender.com/d1kq5qf

Our model for the likely roles of PCT and CGRP in the taste system is illustrated in Figure 6. There is a large body of literature about the biological roles of CGRP. One study that looked at the effects of CGRP in isolated taste buds using calcium imaging and bioassay showed that

401 CGRP may regulate taste signaling by regulating 5-HT signaling by type III cells.[36] We (and the
402 original study) did not detect CGRP1R expression in type III cells. We found that *Calcrl* is
403 expressed in taste stem cells using scRNASeq and RNAScope (Figures 1, 3, S2). Moderate
404 *Ramp1* expression in *Lgr5*+ cells is detected in the scRNASeq data, and we could detect it in
405 taste papillae using qPCR as well (Figure 1). CGRP regulates stem cell maintenance, and
406 CGRP1R expression in Lgr5+ cells in CVP and basal (likely stem/progenitor) cells in the FFP
407 taste buds and adjoining fibroblasts raises the possibility that taste nerve derived CGRP
408 regulates taste stem cells directly, and indirectly through the fibroblasts (Figures 1, 3, S2).
409 CGRP can also regulate immunity at the taste cells through its effects on immune cells,
410 epithelial cells and fibroblasts, and can shape the oral microbiome directly through its
411 microbicidal effects as described above (Figure 6). Intriguingly, CGRP1R is expressed in taste
412 bud- adjacent ILC2 and ILC3 (Figure 3 B-C'). CGRP1R signaling in lungs is a critical regulator
413 of ILC activity, and it plausible that they might play a similar role in taste cells.[68-70] It is also
414 capable of regulating microfold cells that mediate microbial transcytosis in the Peyer's patch.[30]
415 We have shown that type II taste cells might mediate immune surveillance similar to microfold
416 cells, and it is possible that CGRP plays a similar role in taste papillae.[56] The amount of PCT
417 produced by the taste cells will not be sufficient to elevate it's circulating levels in the body, and
418 it likely exerts its effects by paracrine signaling within the taste papillae (Figure 6). Sepsis-
419 associated cytokine storm is preceded by ubiquitous PCT expression, leading to its adoption as
420 an early sepsis marker.[40] However, very little is known about its role in normal physiology. In the
421 skeletal system, it may regulate bone density by suppressing osteoclast (macrophage)
422 migration and maturation.[39] It's expression in CVP and FOP but not FFP might provide some
423 clues to its function in taste cells. Unlike the FFP, the CVP and FOP have deep trenches around
424 which the taste buds are arranged. The trenches are relatively less exposed to salivary flux and
425 may be more hospitable to the oral microbiota, including those responsible for halitosis,
426 transient lingual papillitis etc.[71,72] This raises the possibility that PCT might regulate the taste

427 papillae microbiome in much the same manner as CGRP (Figure 6). In addition, it has the
428 potential to regulate taste signaling and taste cell regeneration by regulating stem/progenitor cell
429 expressed CGRP1R. As stated before, PCT is a partial agonist of the CGRP1R, and it can
430 partially antagonize the effects of CGRP at this receptor. Thus, it is plausible that PCT and
431 CGRP reciprocally shape the biological effects CGRP1R signaling in the taste papillae (Figure
432 6). In light of this, we hypothesize that the taste papillae might be a suitable model system to
433 determine the biological roles of PCT and its cross talk with CGRP.
434

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446

447 **Conflict of Interest**
448 The authors declare no conflict of interest.
449

450 **Author Contributions**
451 SRP and AHS: performing experiments, acquiring the data, data analysis, interpretation of the
452 results, creation of the figures, and revision of the manuscript. PJ and RFM: funding acquisition,

supervision of data acquisition, interpretation of results and revision of the manuscript. SKS: funding acquisition, conceptualization and design of the study, supervision of data acquisition, interpretation of results, writing of the manuscript.

Data availability.

The underlying bulk and scRNASeq data are being uploaded to NCBI's short read archive and will be available very soon.

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