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Enhancement of allyl isothiocyanate-evoked responses of mouse trigeminal ganglion cells by the kokumi substance γ-glutamyl-valyl-glycine (γ-EVG) through activation of the calcium-sensing receptor (CaSR)

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Abstract

Some γ-glutamyl peptides including glutathione (γ-Glu-Cys-Gly) and γ-glutamyl-valyl-glycine (γ-Glu-Val-Gly=γ-EVG) are reported to increase the intensity of basic tastes, such as salty, sweet, and umami, although they have no taste themselves at tested concentrations. The mechanism of action of γ-glutamyl peptides is not clearly understood, but the calcium sensing receptor (CaSR) may be involved. Glutathione and γ-EVG enhance the pungency of some spices, and the present study investigated the effects of γ-EVG on the responses of trigeminal ganglion (TG) cells to thermosensitiveTRP channel agonists. Single-cell RT-PCR revealed that most CaSR-expressing cells co-expressed TRPV1 (sensitive to capsaicin) and TRPA1 (sensitive to allyl isothiocyanate=AITC). Intracellular Ca^{2+} imaging showed that pretreatment with γ-EVG excited 7% of trigeminal ganglion (TG) cells and increased the amplitude of their responses to AITC, but not to capsaicin or menthol. The enhancing effect of γ-EVG was prevented by a CaSR inhibitor. The results indicate that γ-EVG increases AITC pungency by activating a subset of trigeminal ganglion cells that co-express CaSR and TRPA1.

Keywords

Trigeminal ganglion cell; Calcium-sensing receptor (CasR); Calcium imaging; Immunohistochemistry; TRPA1; TRPV1; *Kokumi* substance

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Declaration of Competing Interest

The authors declare no financial conflict of interest regarding the results or interpretation of the reported experiments.

CRediT authorship contribution statement

E. Carstens and T. Akiyama, designed the study. T. Akiyama, E.. Curtis, M. Iodi Carstens performed the research. T. Akiyama and E. Carstens analyzed the data and wrote and edited the paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2022.114063.

1. Introduction

It has been reported that some γ-glutamyl peptides referred to as kokumi substances increase the intensity of basic tastes, such as salty, sweet, and umami although they have no taste themselves at tested concentrations [1]. Kokumi means “rich taste” and imparts senses of richness, body, complexity and “mouthfulness” (viscosity, mouth-and tongue-coating) and increases the continuity or “lastingness” of flavors [1–6]. γ-glutamyl peptides, such as γ-Glu-Leu, γ-Glu-Val, and γ-Glu-Cys-β-Ala-from edible beans, while tasteless at the concentrations used, increased flavor complexity and lastingness when added to salty-umami solutions or model chicken broth [2]. γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His-identified in matured Gouda cheese enhanced the mouthfulness and continuity of flavor when added to the re-constituted matured cheese extract [4]. γ-Glu-Val-Gly (γ-EVG) enhanced the basic tastes of salty, sweet, and umami and increased the intensities of thickness, continuity and mouthfulness when added to chicken soup [1]. In animal studies, kokumi peptides increased rat chorda tympani nerve responses to salty and umami [7] or to sweet, umami and fat taste solutions [8] and enhanced preference of rats for umami, sweet and fat solutions [8]. Although the mechanism of action of γ-glutamyl peptides is not clear, the calcium sensing receptor (CaSR) is considered to be involved. Ohsu et al. [1] have reported the following. [1] All known CaSR agonists, such as calcium lactate, protamine, polylysine, cinacalcet, and L-histidine, increase taste intensity when added to salty and umami solutions. [2] Glutathione activates the human CaSR, as do several γ-glutamyl-peptides, such as γ-Glu-Ala, γ-Glu-Val, γ-Glu-Cys, γ-Glu-α-aminobutyryl-Gly (ophthalmic acid), and γ-Glu-Val-Gly. Moreover, these compounds increased taste intensity when added to a salty and umami solution, despite having no intrinsic taste at the concentrations tested. [3] The CaSR agonist activity of these γ-glutamyl peptides positively correlated with sensory effects. [4] The sensory effect of GSH and γ-EVG was suppressed by a CaSR inhibitor, NPS-2143. These observations suggested that such substances are sensed through the CaSR in humans.

It has been reported that adding glutathione [9] and γ-EVG [10] increased the flavor and pungency of spices. Spices contain various pungent compounds, and the effects of glutathione and γ-EVG on the spice must be investigated according to these individual compounds. Pungent compounds such as capsaicin in chili pepper (*Capsicum annuum*) and allyl isothiocyanate (AITC) in *wasabi* (*Eutrema japonicum*) or horseradish (*Armoracia lapathifolia*) activate transient receptor potential (TRP) channels located in the trigeminal nerve [11]. As CaSR has been found in the rat trigeminal ganglia [12], it is possible that γ-glutamyl peptides increase the pungency of spices by interacting with CaSRs in human trigeminal nerve.

In the present study we tested the hypothesis that mouse trigeminal ganglion (TG) neurons co-express CaSR and TRPA1/TRPV1, and that responses of such TG neurons to AITC or other TRP channel agonists are enhanced by γ-EVG. This study was motivated by the recent report that γ-glutamyl peptides enhance the perceived pungency of AITC and piperine in humans in a manner blocked by a CaSR inhibitor [10]. This hypothesis was tested in two approaches. The possibility that CaSR is expressed in mouse TG and trigeminal nerve fibers and coexpressed with TRPA1 and TRPV1, the receptors for AITC and capsaicin,

respectively, was investigated by immunochemical methods. We also used calcium imaging to investigate the effects of γ-EVG on the responses of mouse TG neurons to AITC and other TRP channel agonists.

2. Materials and methods

All experimental protocols and procedures in animal experiments were approved by the University of California Davis Institutional Animal Care and Use Committee. The study was conducted following the regulations of the National Institutes of Health Guide for Care and Use of Laboratory Animals and adheres to ARRIVE guidelines.

2.1. Chemicals

γ-Glu-Val-Gly (γ-EVG; Flavor and Extract Manufacturers Association of the United States-GRAS no. 4709; Joint Food and Agriculture Organization / World Health Organization Expert Committee on Food Additives food flavoring no.2123) was obtained from Ajinomoto Co., Inc. (Tokyo, Japan). Anserine (food additive grade) was purchased from Yaizu Suisan Kagaku Industry (Shizuoka, Japan). Allyl isothiocyanate (AITC) of food additive grade was obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). NPS-2143, a CaSR inhibitor, was synthesized as described [13]. Deionized water for test solutions and mouth rinse was supplied by a Mill-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Determination of CaSR activity of anserine using HEK-293 cells

CaSR agonist activity was determined using HEK-293 cells by the method of [1]. Briefly, human CaSR cDNA was inserted into pcDNA 3.1 expression vectors in Opti-MEM I medium (Thermo Fisher Scientific, Carlsbad, CA, USA), mixed with FuGENE 6 (Roche Applied Science, Penzberg, Germany), and poured onto HEK-293 cells grown at a sub-maximum concentration. After 24 h culture in 96-well plates, the cells were incubated with 5 μM Calcium-5 (Calcium-5 assay-kit, Molecular Devices, San Jose, CA, USA) for 45–60 min, and measurements were conducted using an Image Analyzer (FlexStation, Molecular Devices) and its associated software. Activation of the CaSR expressed in HEK-293 cells leads to an increase in intracellular calcium ions and this increase was measured using Calcium-5 dye. The binding of Ca^{2+} ion to Calcium-5 results in an increase in dye fluorescence which is excited at 485 nm and emits at 525 nm. The concentration dependence of the fluorescence intensity was analyzed.

2.3. Mice

C57BL/6 J adult mice (≥ 8 weeks old, male) were sacrificed by CO_2 asphyxiation, followed by cervical dislocation. Tongue and TG tissues were removed and immersed in cold Tyrode's solution.

2.4. Immunohistochemistry

Sacrificed mice were perfused with ice-cold phosphate buffer saline, then 4% paraformaldehyde. The TG and tongue were removed and cryoprotected with 10–30% sucrose/0.1 M phosphate buffer at 4 °C. Cryosections cut at 16 μm were prepared and embedded in Protein Block (Dako, Glostrup, Denmark) with 1% Triton X-100 for 2 h

at room temperature. Sections were then incubated overnight at 4 °C with rat anti-CaSR antisera [14] and rabbit anti-protein gene product 9.5 antibody (PGP9.5, Ultraclone, Cambridge, UK), followed by incubation with AlexaFluor-labeled donkey anti-rat IgG and goat anti-rabbit IgG (Thermo Fisher Scientific) secondary antibody (1:1000). Negative controls without the primary antibodies were processed in parallel. Images were obtained with a laser-scanning confocal microscope (FV-300, Olympus, Tokyo, Japan).

2.5. Trigeminal cell isolation

TGs were collected bilaterally from male mice and placed into dishes containing Hanks balanced salt solution (HBSS, Thermo Fisher Scientific, Carlsbad, CA) or Tyrode's solution (135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 10 mM sodium pyruvate, 5 mM $NaHCO_3$ at pH 7.3, 316–323 mOsm). The TG were minced with fine spring scissors, incubated in 20 units/ml papain (PAP, Worthington Biochemical Corp. Lakewood, NJ, USA) and 0.67 mg/ml L-cysteine (Sigma) for 10 min at 37 °C. The TG was then transferred to 3 mg/ml collagenase type 2 (CLS-2, Worthington) for 10 min at 37 °C. The ganglia were then macerated with fire-polished glass pipettes and debris was removed by filtering the cell suspension with 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) into a cell culture well. The dispersed TG cells were cultured in Ham's F12 with 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan) on poly-D-lysine/laminin-coated glass coverslips (Becton Dickerson) for 18–24 h.

2.6. Calcium imaging

Cultured TG cells were incubated in Tyrode's solution with 2.5 μM Fura 2-AM (Thermo Fisher Scientific) and 0.1% bovine serum albumin. Fura 2 loaded TG cells were mounted on a recording chamber (GLP-26, Warner Instruments, Hamden, CT, USA) and viewed with an Olympus IX71 or Nikon TS100 inverted microscope with 20 × water-immersion objective lens. Images were acquired ratiometrically of excitation 340 and 380 nm. The fluorescent light with 510–550 nm was collected with an ORCA-R2 camera (Hamamatsu Photonics, Hamamatsu, Japan). Imaging analysis was performed using AquaCosmos 2.6 (Hamamatsu Photonics) or Simple PCI (Compix Inc, Cranberry Township, PA, USA). Solutions were delivered by a solenoid-controlled valve system (VC-6, Warner Instruments or ValveLink AutoM8, Automate Scientific, Berkeley CA USA) at a flow rate 1 ml/min.

2.7. RT-PCR

TG and other tissues were dissected from sacrificed mice [14]. The total RNA was extracted using RNeasy Plus Micro kit (Qiagen, Hilden, Germany). The purified RNA was denatured, and first-strand cDNA was synthesized using random primer and reverse transcriptase (Super Script III, ThermoFisher Scientific). cDNA was used as a template in 20 μl of PCR mixture with Taq polymerase (Promega, Madison, WI, USA). The PCR conditions were as follows: 94 °C for 2 min and 35 cycles at 94 °C for 30 s, 58 °C for 20 s, and 72 °C for 45 s. The PCR products were analyzed by gel electrophoresis with GelRed staining (Biotium, Fremont, CA, USA). The primers used are shown in Table 1.

For single-cell RT-PCR, individual TG cells were collected with 30 μm diameter glass micropipettes and expelled into lysis solution of Array-Pure Nano-scale RNA Purification

Kit (epicenter, Madison, WI, USA). Extracted RNA was amplified and converted to cDNA using Message-BOOSTER cDNA kit (epicenter). PCR was performed with Power SYBR Green Master Mix (ThermoFisher Scientific) in 20 μl aliquot of PCR mixture for 40 cycles in parallel with cDNA from trigeminal ganglion as a positive control sample. All PCR primers, except β-actin, were separated by at least one intron on the corresponding genomic DNA and were designed using NCBI Primer-BLAST [25]. The primers are shown in Table 1.

2.8. Statistical analyses

Statistical analyses were conducted using JMP 9.0 (SAS Institute Inc., Cary, NC, USA). Differences were assessed using analysis of variance (ANOVA), Kruskal-Wallis followed by Dunn's, or paired or unpaired *t*-tests. *P*-values < 0.05 were considered significant.

3. Results

3.1. CaSR is expressed in TG, DRG and tongue

CaSR in mouse tissue was assayed by RT-PCR and immunohistochemistry. For the RT-PCR experiment, CaSR was detected in TG, dorsal-root ganglia (DRG), and taste buds containing circumvallate papillae (CV), but not in tongue epithelium that did not contain taste buds (Fig. 1A). PLCβ2, a type II taste cell marker molecule, was observed only in taste buds-containing CV (Fig. 1A)[16]. Snap25, a presynaptic SNARE component molecule found in type III taste cells [16] and neurons [17], was observed in TG and DRG and faintly in CV (Fig. 1A). TRPA1 and TRPV1 were both detected in TG and DRG with TRPA1 also detected faintly in CV (Fig. 1A).

CaSR protein expression in trigeminal neurons was assayed by immunohistochemistry. CaSR immunoreactivity was observed in a subset of large (≥ 15 μm) diameter trigeminal neuronal cells with large rounded nuclei (Fig. 1B). The CaSR immunoreactive cells were observed in a subset of TG neuronal cells. Out of 189 TG cells, 24 cells (12.7%) expressed CaSR. CaSR immunoreactivity was also observed in a subset of protein gene product 9.5 (PGP-9.5)-positive nerve fibers in fungiform papillae of tongue tip (Fig. 1C) [18]. The terminals of those fibers were located beneath the keratin layer of the stratified squamous epithelium (Fig. 1C).

3.2. Some CaSR-positive cells co-express TRPA1 and TRPV1 channels

The expression of CaSR and TRP channels were assayed by single-cell RT-PCR in dissociated TG cells to investigate the molecular basis for γ-EVG enhancement of AITC-induced response by trigeminal neurons. Single-cell RT-PCR was performed in 44 isolated TG cells that responded to KCl-stimulation. Aliquots of cDNA derived from the cell were tested in parallel for CaSR, TRPV1, and TRPA1 expression [18,19]. There was a positive association of CaSR with TRPA1 and TRPV1 expression (Fig. 1D, Supplemental Table). CaSR was found in four of the 44 cells (9%). Three of four CaSR-positive TG cells were positive for both TRPA1 and TRPV1. CaSR was also detected in one cell that was negative for TRPA1- andTRPV1.

3.3. γ-EVG enhances AITC-evoked response in TG cells

The effect of γ-EVG on TG cell responses to AITC was investigated by calcium imaging. A total of 198 TG cells were imaged. for responsiveness to AITC, capsaicin, menthol, and γ-EVG (Figs. 2,3,5,6) and an additional 88 TG cells for responses to AITC and γ-EVG (Fig. 4). Examples of TG cell responses to various chemicals are shown in Fig. 2A. The TG cell indicated by the red trace responded to all tested substances including γ-EVG, EVG, AITC, or menthol.

The overall percentages of TG cells that responded to the 4 tested chemical stimuli are shown in Fig. 2B. Seven% of the TG cells responded to γ-EVG (100 μM) as well as 2 or 3 of the TRP channel agonists. Of the TG cells unresponsive to γ-EVG, 24% responded to capsaicin only, 2% to AITC only and 8% to menthol only, while the remaining 18% responded to two or more TRP channel agonists. Forty-one% of the cells did not respond to any chemical tested (Fig. 2B).

We next tested the effect of pretreatment of γ-EVG on TG cell responses elicited by TRP agonists, beginning with AITC. We first tested if repeated responses to AITC exhibit tachyphylaxis. Fig. 3A shows an individual example, and Fig. 3B shows that the mean peak responses of 13 TG cells tested did not significantly differ between the first and second applications of AITC. We then tested if pretreatment with γ-EVG affected responses to AITC. Fig. 3C shows an individual example in which application of γ-EVG elicited a response, followed by the TG cell's responses to two applications of AITC the first of which was larger than the second. Thirteen of 18 TG cells tested responded directly to application of γ-EVG, while 5 cells did not respond. Fig. 3D shows peak TG cell responses to AITC before (none) and after application of γ-EVG. The red symbols indicate peak responses to AITC following γ-EVG for TG cells that responded to γ-EVG (γ-EVG+) while the blue symbols are responses to AITC following γ-EVG for TG cells that did not respond to γ-EVG (γ-EVG−). Responses to AITC were significantly enhanced following γ-EVG only in those cells that responded to γ-EVG (Fig. 3D). Fig. 3E shows the percentage of TG cells that responded to AITC. About 19% of TG cells responded to AITC; examples are shown in Fig. 3A and F.

We next tested if γ-EVG enhanced responses of TG cells to a 2nd application of AITC. Three TG cells responded to the first application of AITC followed by γ-EVG and then to a 2nd application of AITC. An example is shown in Fig. 3E. Two TG cells were initially unresponsive to AITC, but after responding to γ-EVG they then responded robustly to a second application of AITC. An example is shown in Fig. 3G. Thus, there was an increase in the percentage of TG cells that were excited by AITC following γ-EVG (Fig. 3F, red bar). In addition, the amplitudes of TG cell responses to the 2nd application of AITC were significantly greater when preceded by γ-EVG (Fig. 3H).

The enhancement of AITC-evoked responses by γ-EVG was abolished by 3 μM NPS-2143, an inhibitor of CaSR. Fig. 4A–D shows responses of 3 TG cells to AITC. As noted above, a subset of TG cells responded to 100 μM γ-EVG. A lower concentration of 10 μM γ-EVG did not induce an apparent response (Fig. 4B), but the amplitude of response to subsequent application of AITC was enhanced by pretreatment with10 μM γ-EVG (Fig. 4B). In 9 tested

cells the response to AITC was significantly enhanced (141%) by pretreatment with γ-EVG, (Fig. 4E). Co-application of 3 μM NPS-2143 prevented any enhancement by γ-EVG of 6 TG cells' responses to AITC (Fig. 4D, E). Consistent with the preceding experiments (Fig. 3), ~19% of TG cells responded to AITC with ~10% of these responding to γ-EVG (Fig. 4F).

γ-EVG did not enhance TG cell responses to capsaicin or menthol. The capsaicin data are shown in Fig. 5. There was a significant decrease in the response to a second successive application of capsaicin (Fig. 5A, B). The response to a first application of capsaicin was not altered when preceded by the application of γ-EVG, regardless of whether the cell responded to γ-EVG or not (Fig. 5C, D). Moreover, γ-EVG did not alter the response to a 2nd application of capsaicin (Fig. 5E,G,H) nor did application of γ-EVG have any large effect on the percentage of TG cells responsive to capsaicin (Fig. 5F). Likewise, γ-EVG had no effect on TG cells responses to menthol (Fig. 6). There was no significant change in the magnitude of response to a 2nd application of menthol (Fig. 6A,B). Application of γ-EVG had no effect on TG cells' responses to the first (Fig. 6C,D) or 2nd (Fig. 6E,G,H) application of menthol and did not affect the percentage of menthol-sensitive TG cells (Fig. 6F).

4. Discussion

In a parallel human study, CaSR activation by the kokumi substances glutathione and γ-EVG enhanced pungency elicited by AITC and piperine, while anserine (β-alanyl-N-1-methylhistidine), a peptide that has no CaSR activity, did not affect AITC-evoked pungency [10]. In the present study CaSR was detected in TG and circumvallate papillae (CV), with TRPA1 being co-expressed in both tissues. Importantly, the responses of TG cells to AITC were significantly enhanced following application of γ-EVG only in γ-EVG-responsive TG cells but not in cells unresponsive to γ-EVG. We observed that 19% of the mouse TG cells responded to AITC, which is comparable with previous estimates [20]. TrpA1 is a high affinity receptor for AITC, and its reactivity is conserved in many species including rodents and human [21–23]. Single-cell RT-PCR experiments revealed a comparable percentage of TRPA1-positive TG cells, a small percentage of which co-expressed CasR (Fig. 1D). In the calcium imaging experiments, γ-EVG directly excited 7% of TG cells, comparable to the observation that CaSR was expressed in ~9% of TG cells. Moreover, the enhancement of AITC-evoked responses by γ-EVG was abolished by the CaSR inhibitor, NPS-2143. Thus, these results support the hypothesis that γ-EVG acts via the CaSR to sensitize the responses of TRPA1+ TG cells to AITC. This sensitization might explain why the kokumi substances glutathione and γ-EVG enhanced the pungency of AITC.

TG cells were activated by capsaicin acting at TRPV1 and by menthol acting via TRPM8, but the responses were not significantly affected by preapplication of γ-EVG. Since TRPV1 is coexpressed in CaSR-expressing TG cells it is conceivable that capsaicin pungency is enhanced by kokumi substances. Although the current data do not support this, the number of observations is admittedly small. A recent human study did report that γ-EVG enhanced pungency elicited by piperine [10] which has been shown to act at hTRPV1 [24]. It would be worthwhile to test if γ-EVG enhances pungency elicited by capsaicin in humans.

The only other relevant physiological study that we are aware of recorded calcium responses of GCaMP6-positive TG neurons in the mandibular division (V3) to application of γ-EVG [25]. In that study, γ-EVG at 100 μM delivered to the oral cavity activated a very small percentage (0.6%) of TG cells. This difference from our present results likely reflects methodological differences. Importantly, responses to γ-EVG were attenuated by the CasR inhibitor NPS-2143. Individual TG cells were sometimes activated by other kokumi substances but there was no consistent pattern in overlap of responsive cells possibly due to insufficient concentrations or desensitization of CaSR. Because kokumi substances elicit sensations of thickness and mouthfeel, the food thickeners xanthum gum and gluckmannan were also tested. They activated a small percentage of TG cells but in a diffuse manner, and there was no enhancing effect of γ-EVG.

In conclusion, the present data provide a potential basis for the enhancement of AITC-induced oral pungency by showing that the kokumi substance γ-EVG enhances responses of TG cells to application of AITC. The enhancement of TG cell responses to AITC by γ-EVG was prevented by pretreatment with the CaSR inhibitor NPS-2143, which also prevented the enhancement of AITC-induced oral pungency by two kokumi substances (glutathione and γ-EVG) in a human study [10]. CaSR was found to be expressed in TG cells as well as circumvallate papillae of the tongue, and the AITC receptor TRPA1 was coexpressed by CaSR-expressing TG cells. These results support the idea that kokumi substances act via CaSR to enhance TG cellular responses to the TRPA1 agonist AITC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AITC	allyl isothiocyanate
γ-Glu-Val-Gly	γ-EVG γ-glutamyl-valyl-glycine; form)
CaSR	calcium-sensing receptor
TRPV1	Transient Receptor Potential cation channel subfamily V member 1
TRPA1	Transient Receptor Potential cation channel subfamily A member 1

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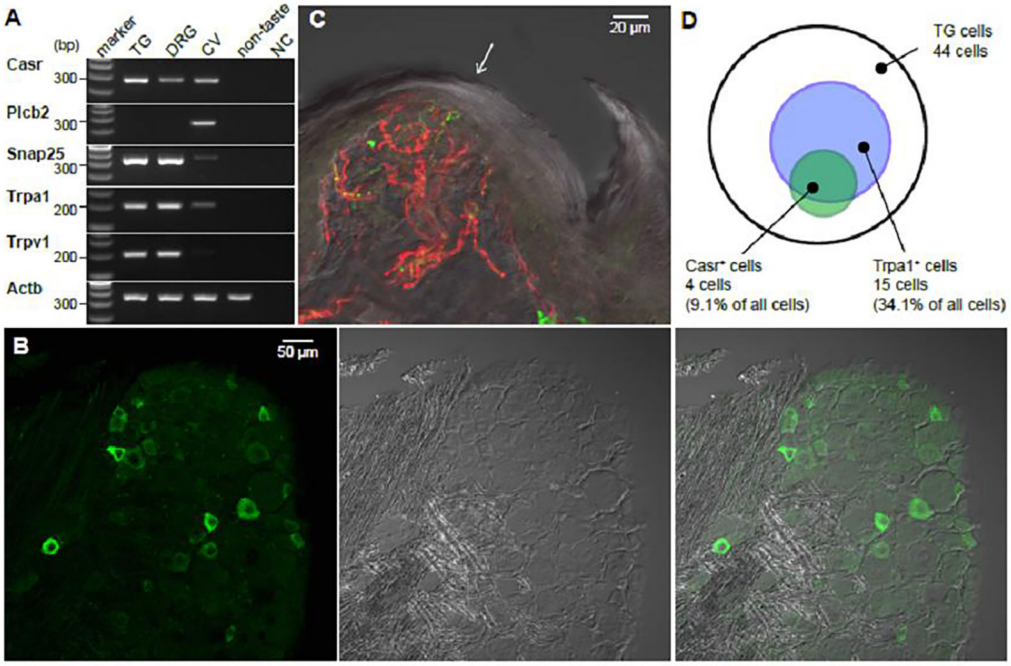

Fig. 1.

Trigeminal nerve express CaSR. (A) RT-PCR for CaSR detection in mouse trigeminal ganglion (TG), dorsal root ganglion (DRG), taste bud-enriched circumvallate papilla (CV), and non-taste bud lingual epithelium (non-taste). NC is cDNA absent negative control. (B) Immunohistochemistry for CaSR in mouse TG. CaSR immunofluorescence is seen in a subset of TG cells. Immunofluorescent (left), differential interference contrast (DIC, center) and merged images (right) are shown. (C) Immunohistochemistry for CaSR in a fungiform papilla of mouse tongue. Confocal immunofluorescent signals of CaSR (green) and PGP9.5 (neuronal marker, red) were superimposed on the DIC image. (D) Venn diagram showing coincident expression of TRPA1 and Casr in TG cells.

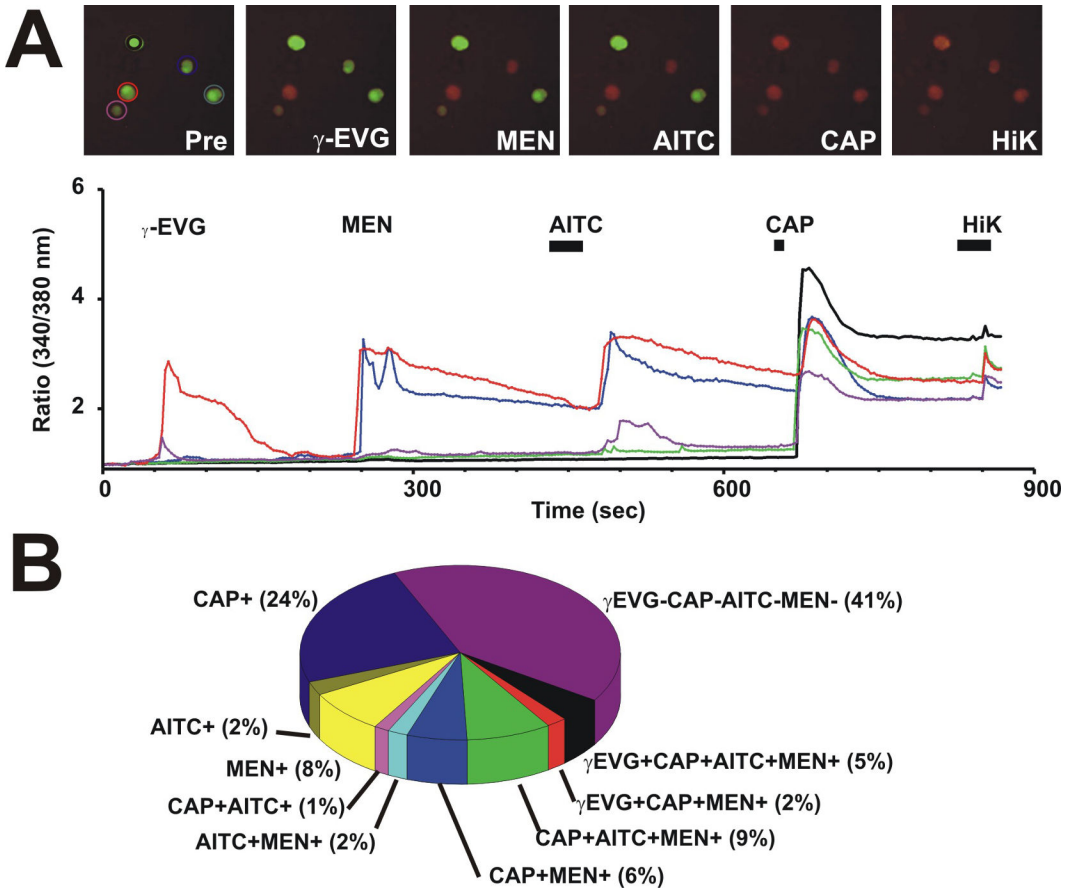

Fig. 2.

γ-EVG excites a subset of nociceptive trigeminal ganglion neurons. A. Fluorescence microscopic images (upper row) show examples of trigeminal ganglion (TG) cells that responded to γ-EVG followed by menthol (MEN), allyl isothiocyanate (AITC), capsaicin (CAP), and high potassium (HiK). in a series of recordings. Graph (below) plots F340/F380 nm ratio as a function of time. Color of circles around neurons (Pre micrograph) correspond to traces of graphs below. Horizontal bars indicate time of application of each substance. B. Pie chart shows percentages of TG neurons responding to one or more of the th substances γ-EVG, MEN, AITC, and/or CAP.

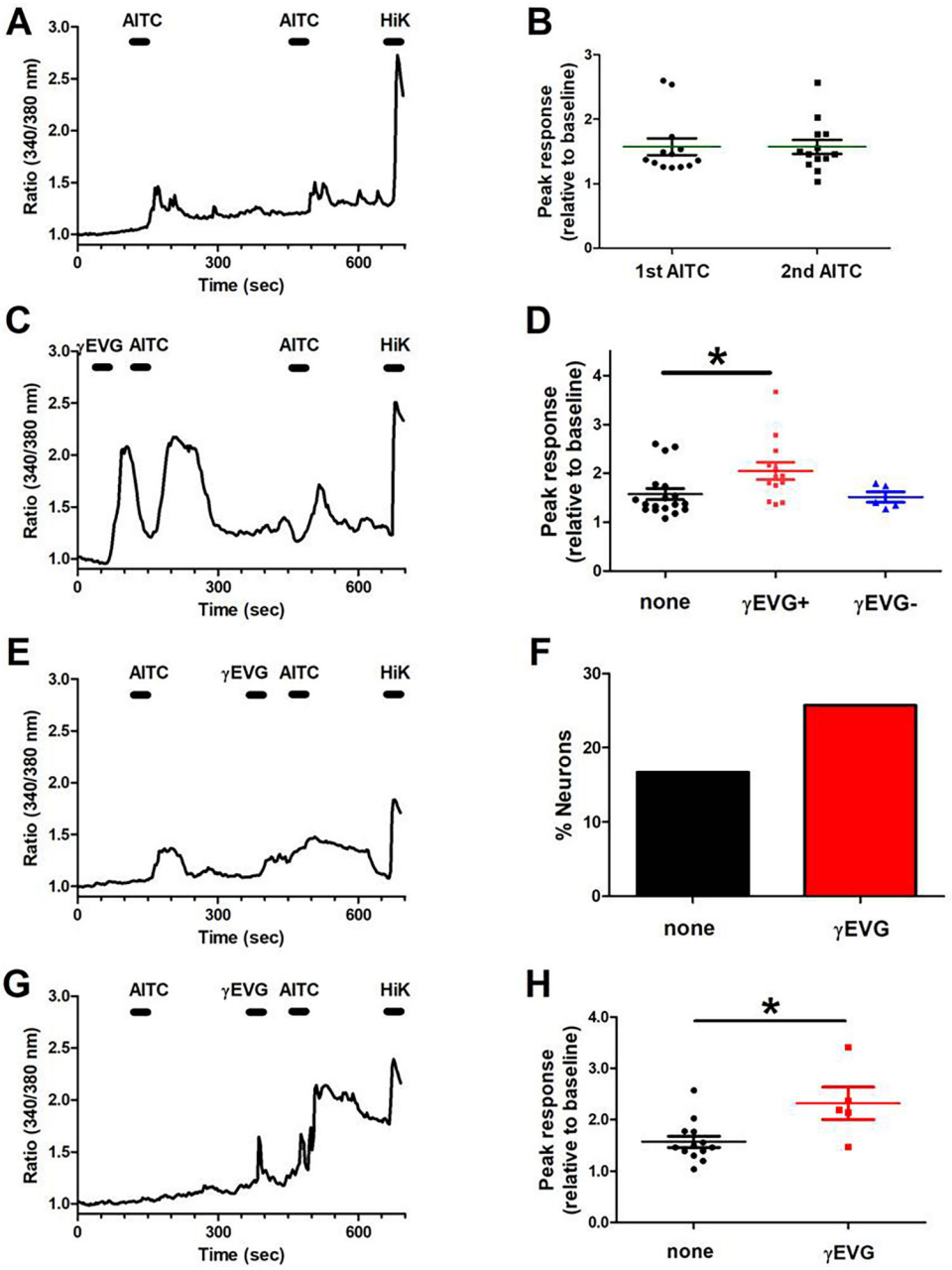

Fig. 3.

TG cell responses elicited by AITC were enhanced by γ-EVG pretreatment. A. Example of TG cell responses to two successive applications of AITC followed by HiK. B. Scatter plot shows baseline-corrected responses of 13 TG cells to two successive applications of AITC. Long horizontal line indicates mean and shorer horizontal lines show SEM. C. Individual example of TG cell responses to γ-EVG (100 μM) followed by two successive applications of AITC. D. Scatter plot shows that baseline-corrected responses of TG cells to an initial application of AITC ($n = 18$; black symbols) were significantly enhanced when γ -EVG was co-applied with a second application of AITC (red symbols; $n = 13$; *$p <$ 0.05, Kruskal-Wallis followed by Dunn's). Blue symbols indicate responses of 5 TG cells to

γ-EVG, $n = 5$. E. Example of TG cell's responses to 1st AITC and 2nd AITC preceded by γ-EVG. TG cell responded to 1st and 2nd application of AITC F. Incidence of responses of TG cells to AITC. Black bar: no pretreatment. Red bar: γ-EVG pretreatment. G. Individual example of TG cell that responded to the 2nd application of AITC preceded by γ-EVG, but not to 1st application of AITC. H. Enhancement of AITC-evoked responses by γ-EVG. Scatter plots of baseline-corrected responses of TG cells to second application of AITC. (*$p < 0.05$, unpaired t-test). None, $n = 13$. γ-EVG, $n = 5$.

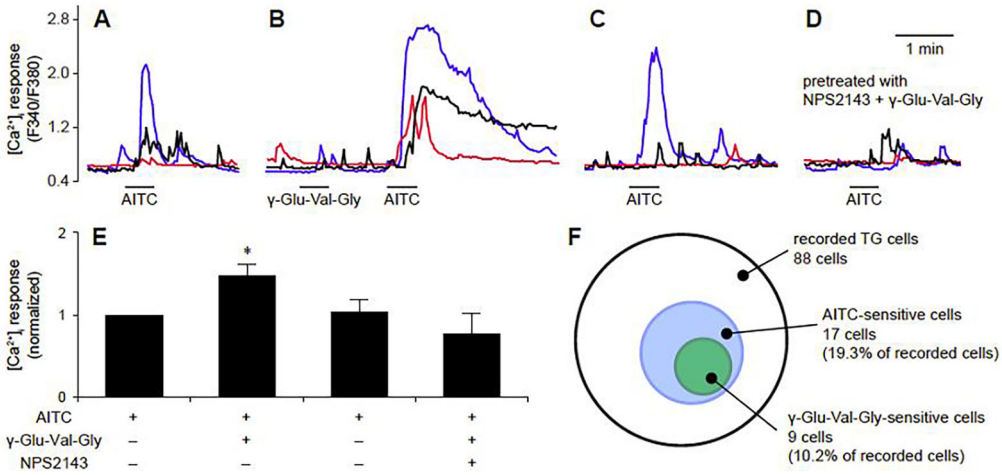

Fig. 4.

TG cell responses elicited by allyl isothiocyanate (AITC) were enhanced with γ-Glu-Val-Gly-pretreatment. Examples of 3 trigeminal ganglion (TG) cell responded to AITC in a series of recording. Graph plots F340/F380 nm ratio of Fura 2 as a function of time. Horizontal bars indicate time of application of each substance. The cells treated with AITC alone (10 μM, A, C), treated with γ-Glu-Val-Gly (10 μM, B) before AITC stimulation. (D) Trace shows that TG cells treated with both NPS-2143 (3 μM) + γ-Glu-Val-Gly (10 μM) before AITC stimulation. (E) Summary of AITC-evoked intracellular Ca2+ responses in the presence and absence of γ-Glu-Val-Gly. Bars show mean ± SEM (n = 6 TG cells). The responses were normalized to responses in first stimulation with AITC. Enhanced response with γ-Glu-Val-Gly-was significantly reduced with NPS-2143. (F) Venn diagram showing coincident responses of TG cells to AITC and γ-Glu-Val-Gly.

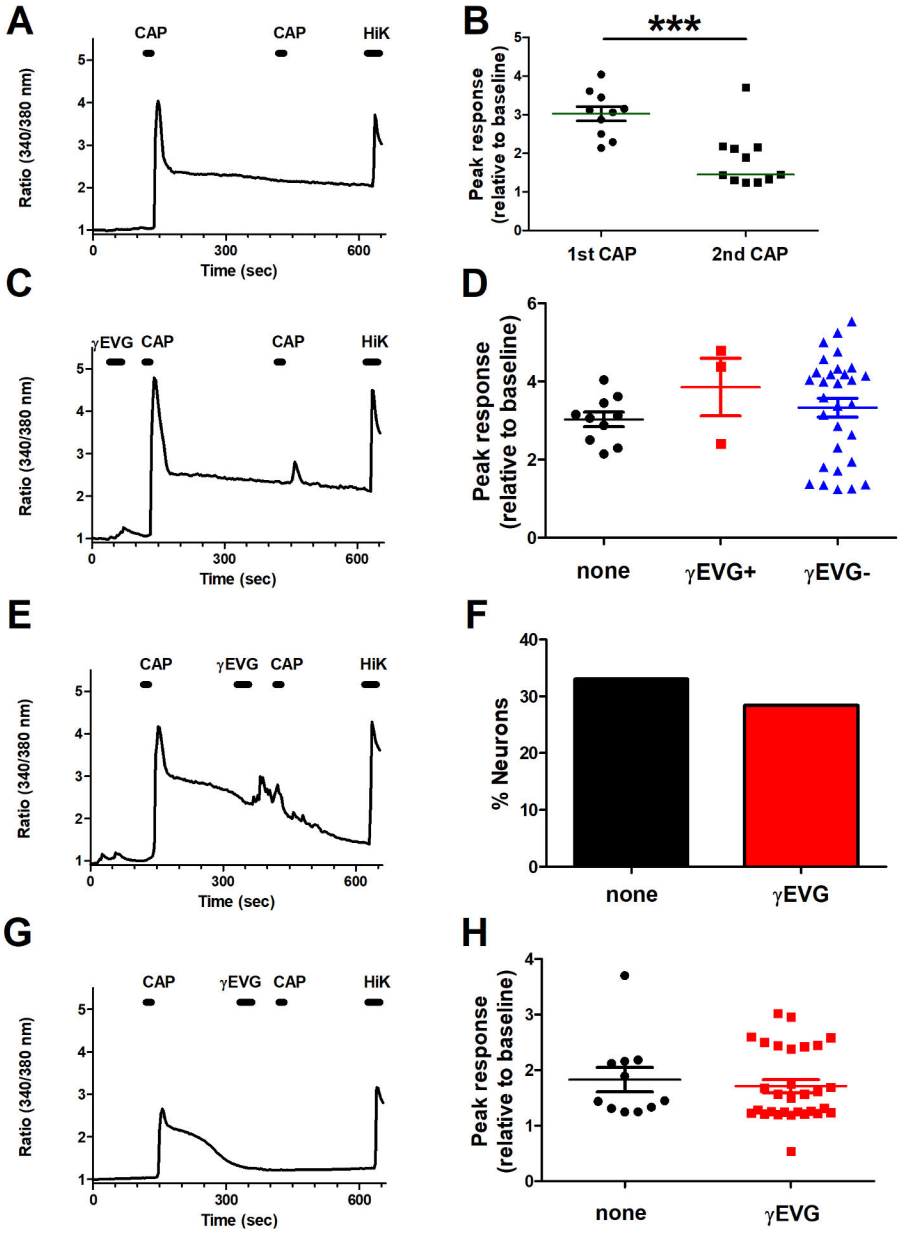

Fig. 5.

TG cell responses elicited by capsaicin (CAP) were not affected by γ-EVG pretreatment. A. Example of TG cell responses to two successive applications of capsaicin followed by HiK. Second application of capsaicin did not elicit a response. B. Scatter plot shows baseline-corrected responses of 11 TG cells to two successive applications of capsaicin (black symbols). Format as in Fig. 3B. The mean response to the 2nd capsaicin application was significantly smaller compared to the first ($p < 0.05$, paired t-test). C. Individual example of TG cell responses to γ-EVG followed by two successive applications of capsaicin. D. Scatter plot shows that baseline-corrected responses of TG cells to an initial application of capsaicin ($n = 10$; black symbols) were unaffected when γ -EVG was applied prior to a second application of capsaicin regardless of whether the cell responded directly to

γ -EVG (γ-EVG+, red symbols; *n* = 3) or not (γ-EVG−, blue symbols, *n* = 30). E. Example of TG cell's responses to 2 applications of capsaicin as well as to γ-EVG. F. Incidence of responses of TG cells to capsaicin. Black bar: no pretreatment. Red bar: γ-EVG pretreatment. G. Individual example of TG cell that responded to the 1st application of capsaicin but not to γ-EVG or a second application of capsaicin. H. Lack of effect of γ-EVG on TG cell responses to capsaicin.. Scatter plots of baseline-corrected responses of TG cells to 1st application of capsaicin (none; black symbols; *n* = 11) and 2nd application of capsaicin immediately after application of γ-EVG. (γ-EVG; red symbols; *n* = 30).

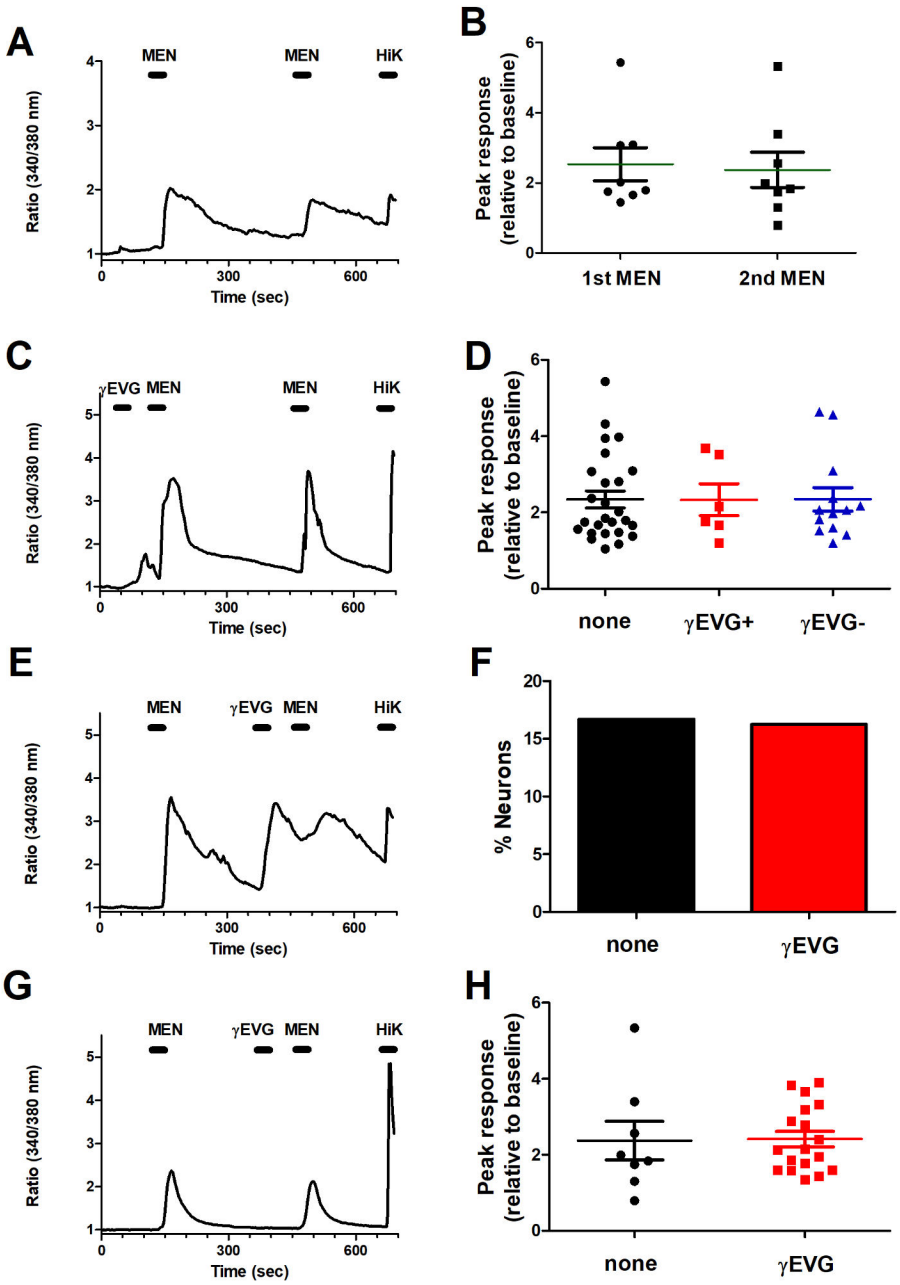

Fig. 6.

TG cell responses elicited by menthol (MEN) were not affected by γ-EVG pretreatment. A. Example of TG cell responses to two successive applications of menthol followed by HiK. Second application of menthol elicited a slightly smaller response. B. Scatter plot shows baseline-corrected responses of 8 TG cells to two successive applications of menthol (black symbols). Format as in Fig. 3B. The mean response to the 2nd menthol application was not significantly different compared to the first ($p > 0.05$, paired t-test). C. Individual example of TG cell responses to γ-EVG followed by two successive applications of menthol. D. Scatter plot shows that baseline-corrected responses of TG cells to an initial application of menthol ($n = 26$; black symbols) were not significantly affected when preeded by γ -EVG in either

TG cells that responded to γ -EVG (red symbols) or not (blue symbols). E. Example of TG cell's responses to 1st application of menthol and 2nd application of menthol preceded by γ-EVG. F. γ -EVG had no effect on the incidence of responses of TG cells to menthol. Black bar: no pretreatment. Red bar: γ-EVG pretreatment. G. Example of a different TG cell's responses to successive applications of menthol. Cell did not respond to γ -EVG prior to second menthol. H. Lack of effect of γ –EVG on response of TG cells to 2nd application of menthol. Scatter plots of baseline-corrected responses of TG cells to second application of menthol without (None, $n = 8$) or with prior application of γ-EVG (γ –EVG, $n = 18$).

Table 1

Oligonucleotide primers used in RT-PCR.

Gene	accession number	forward primer (5′-3′)	reverse primer (5′-3′)	product (bp)
Casr	NM_013803	GTCTGTGGAGTGCATCAGGTA	ACGGTGTTACAGGTGTCGAA	125
Plcb2	NM_177,568	TGAGAGCAACATGGCCCTTA	GTAAGATCTGCCCAGGTGTCA	87
Snap25	NM_011428	CATGGAGAAGGCTGATTCCAA	AAGGAGCTCTCTTGTCCAACA	133
Trpa1	NM_177,781	GTCCAGAAGCATGCGTCATTG	GTTCAAGGGCTCCACGAGAAG	139
Trpv1	NM_001001445	CCTGCATTGACACCTGTGAGA	GTTCAAGGGCTCCACGAGAAG	104
Actb	NM_007393	CACCCTGTGCTGCTCACC	GCACGATTTCCCTCTCAG	328