**Metabotropic Glutamate Receptors Are Involved In The Detection Of IMP And L-
Amino Acids By Mouse Taste Sensory Cells**

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Abstract

G-protein coupled receptors are thought to be involved in the detection of umami and L-amino acid taste. These include the heterodimer T1r1+T1r3, taste and brain variants of mGluR4 and mGluR1, and calcium sensors. While several studies suggest T1r1+T1r3 is a broadly tuned L-amino acid receptor, little is known about the function of mGluRs in L-amino acid taste transduction. Calcium imaging of isolated taste sensory cells (TSCs) of T1r3-GFP and T1r3 knock-out (T1r3 KO) mice was performed using the ratiometric dye Fura 2 AM to investigate the role of different mGluRs in detecting various L-amino acids and inosine 5' monophosphate (IMP). Using agonists selective for various mGluRs such as (*RS*)-3, 5-dihydroxyphenylglycine (DHPG) (an mGluR1 agonist) and L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) (an mGluR4 agonist), we evaluated TSCs to determine if they might respond to these agonists, IMP, and three L-amino acids (monopotassium L-glutamate, L-serine and L-arginine). Additionally, we used selective antagonists against different mGluRs such as (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (an mGluR1 antagonist), and (*RS*)- α -methylserine-O-phosphate (MSOP) (an mGluR4 antagonist) to determine if they can block responses elicited by these L-amino acids and IMP. We found that L-amino acid- and IMP-responsive cells also responded to each agonist. Antagonists for mGluR4 and mGluR1 significantly blocked the responses elicited by IMP and each of the L-amino acids. Collectively, these data provide evidence for the involvement of taste and brain variants of mGluR1 and 4 in L-amino acid and IMP taste responses in mice, and support the concept that multiple receptors contribute to IMP and L-amino acid taste.

Key Words: L-amino acids, IMP, mGluR1, mGluR4, Taste, T1r3 knockout mice

Abbreviations: TSC, Taste Sensory Cells; MPG, Monopotassium glutamate; MSG, monosodium glutamate; Ser, L-Serine; Arg, L-Arginine; IMP, inosine 5' monophosphate; GMP, guanosine 5' monophosphate; mGluR, metabotropic glutamate receptor; iGluR, ionotropic glutamate receptor; HEK cells, Human Embryonic Kidney cells; GFP, Green fluorescent protein; GPCR, G-protein couple receptor; L-AP4, L-(+)-2-amino-4-phosphonobutyrate; CPPG, (RS)- α -cyclopropyl-4-phosphonophenylglycine; SC45647, 2-[[[4-(aminomethyl)phenyl]amino]-[[[(1R)-1-phenylethyl]amino]methyl]amino]ethane-1,1-diol; DHPG, (S)-3,5-Dihydroxyphenylglycine; CNQX, Cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D-(-)-2-Amino-5-phosphonopentanoic acid; AIDA, (RS)-1-Aminoindan-1,5-dicarboxylic acid; MSOP, (RS)- α -Methylserine-O-phosphate, T1R1, Taste receptor type 1 member 1; T1R3, Taste receptor type 1 member 3; NMDA, N-Methyl-D-aspartic acid; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, Analysis of variance. CTA, Conditioned taste aversion.

Animals, including humans, must ingest dietary proteins composed of 20 different L-amino acids either of animal or plant origin. Detection of amino acids and protein in foods is thought to be linked to a unique basic taste called umami, a Japanese term roughly translated as “savory” (Ikeda, 2002). The L- amino acid glutamate is the prototypical compound representative of umami. Certain 5’ ribonucleotide such as inosine 5’ monophosphate (IMP), and guanosine 5’ monophosphate (GMP) also have an umami taste (Kodama, 1913; Kuninaka, 1960; Yamamoto et al., 1991; Wifall et al., 2007). One interesting characteristic of umami taste is the remarkable synergistic enhancement of glutamate signaling when combined with IMP or GMP (Kuninaka et al., 1964; Tamaguchi and Kimizuka, 1979; Maga, 1983; Hiji and Sato, 1996a, b; Yamaguchi and Ninomiya, 2000). In addition to increasing the palatability of food, umami compounds can also cause vagal efferent activation and salivary flow responses that help in mastication and digestion of food (Bellisle et al., 1991; Niiijima 2000; Hodson and Linden, 2006; Bellisle, 2008). Recent research has shown that fortification of meals with an appropriate amount of MSG may improve food intake and therefore has potential for improving the nutritional status and quality of life in elderly and nutritionally deficient patients (Schiffman, 2000; Toyama et al., 2008; Yamamoto et al., 2009). Thus, understanding the receptors and transduction pathways that mediate umami taste and potentially the tastes of L-amino acids in general could be beneficial in regulating ingestive behavior that contributes to good health.

Recent molecular, physiological, and behavioral studies have provided evidence of multiple candidate G-protein coupled receptors (GPCR) in umami taste. The first candidate taste receptors to be identified were two variants of the metabotropic

glutamate receptor 4 (mGluR4), taste-mGluR4 (Chaudhari et al., 1996, 2000) and brain-mGluR4 (Toyono et al., 2002). When cloned, the taste-mGluR4 was found to be missing more than 50% of the N-terminal region compared to the brain-variant of mGluR4 (Chaudhari et al., 2000). Like the better known brain-mGluR4, the truncated taste variant also responds to glutamate and the group III selective mGluR agonist L-(+)-2-amino-4-phosphonobutyrate (L-AP4) (Chaudhari et al., 1996; Hayashi et al., 1996; Bigiani et al., 1997), although the affinity of taste-mGluR4 for glutamate and L-AP4 is more than 100 times lower than that of brain-type receptors (Chaudhari et al., 1996, 2000). Later a heterodimer receptor, T1r1+T1r3, was identified and is now one of the most firmly established as an umami receptor (Li et al., 2002; Nelson et al., 2002). In heterologous expression systems (HEK cells), the human T1R1+T1R3 heterodimer is activated by glutamate, whereas mouse T1r1+T1r3 is activated by several amino acids, but in many cases only when L-amino acid is presented with IMP (Nelson et al., 2002). Further evidence for the role of T1r1+T1r3 as an umami taste receptor comes from studies with knockout (KO) mice in which the *Tas1r1* or *Tas1r3* gene was selectively eliminated (Damak et al., 2003; Zhao et al., 2003; Kuschhara et al., 2013). One KO mice model, having a deletion of the N-terminal extracellular domain but not the seven transmembrane helices of the gene (for *Tas1r3* gene) or vice versa (for *Tas1r1* gene) showed that mice lacking these receptors lose all ability to respond to umami stimuli , thus arguing that T1r1+T1r3 is the only umami receptor (Zhao et al., 2003). However, other studies using an independently derived T1r1 and T1r3 receptor KO mouse model lacking the complete coding sequence of *Tas1r1* or *Tas1r3* gene found only partial taste

loss for umami taste, evidence arguing that other receptors can also contribute to umami taste (Damak et al., 2003; Delay et al., 2006; Kusunohara et al., 2013).

Subsequently, additional candidate umami receptors have been identified in the oral cavity, including full-length variants of mGluR1 (Toyono et al., 2003) as well as a truncated variant of mGluR1 (taste-mGluR1) (San Gabriel et al., 2005, 2009a; Nakashima et al., 2012). Like taste-mGluR4, the truncated taste-mGluR1 also lacks much of the N-terminal extracellular domain and has more than 100-fold lower affinity for glutamate than does the brain-variant of the receptor (San Gabriel et al., 2005, 2009a). The full-length brain-mGluR1 and brain-mGluR4 are now known to be expressed in a subset of taste cells in fungiform, foliate and circumvallate papillae in rats in contrast to the truncated taste variants of mGluR1 and mGluR4 which are mainly expressed in the foliate and circumvallate papillae. Behavioral, nerve recording, and calcium imaging studies with rodents have shown that antagonists for mGluR4 and mGluR1 can selectively block or reduce responses elicited by glutamate (Nakashima et al., 2001; Yasuo et al., 2001; Eschle et al., 2009; Yoshida et al., 2009; Kusunohara et al., 2013).

Even though these studies suggest a role for mGluR4s and mGluR1s in the detection of glutamate, little is known about the involvement of mGluRs in detection of other L-amino acids and IMP. Similar to glutamate, other L-amino acids and IMP are present in free form in food, and thus can be detected by receptors expressed in the taste sensory cells (TSCs). The heterodimer T1r1+T1r3 is considered a broadly tuned L-amino acid receptor, but in HEK cells it does not response to glutamate and IMP individually (Nelson et al., 2002). Rodents as well as humans can detect the taste of

several L-amino acids and IMP. Previously we showed that TSCs of mice lacking the T1r3 receptor generate responses elicited by IMP and several L-amino acids, suggesting the involvement of multiple receptors in L-amino acids and IMP tastes (Pal Choudhuri et al., 2015). Moreover, an mGluR4 antagonist, (RS)- α -cyclopropyl-4-phosphonophenylglycine (CPPG), altered the degree of generalization of a conditioned taste aversion (CTA) between monosodium glutamate (MSG) and L-serine (Ser) and L-arginine (Arg) but not glycine in rats, suggesting that CPPG is capable of altering the taste qualities of these L-amino acids (Eschle et al., 2009). Therefore the question remains which receptor(s) is/are involved in the detection of residual L-amino acid and IMP taste in the T1r3 KO mice?

To address this question, we recorded L-amino acid and IMP-elicited calcium responses of TSCs from T1r3-GFP labeled mice and T1r3 KO mice to identify the involvement of different mGluRs in the detection of various L-amino acids and IMP. We used three L- amino acids: (1) monopotassium glutamate (MPG), (2) Ser, and (3) Arg - all of which are known to elicit different tastes in mice and humans, although CTA experiments with rats suggest these amino acids may also share some taste qualities (Schiffman et al., 1981; Delay et al., 2007; Eschle et al., 2009; Kawai et al., 2012). Additionally at high concentration, Ser elicits umami taste in humans (Kawai et al., 2012). We specifically studied the involvement of mGluR1 and mGluR4 in L-amino acid and IMP detection by examining the effects of selective mGluR1 and mGluR4 agonists on TSCs and the effect of selective antagonists on responses elicited by L-amino acids and IMP. We found that some TSCs that respond to selective agonists also responded to L-amino acids and IMP. Additionally, the selective antagonists for both mGluR1 and

mGluR4 significantly suppressed the responses elicited by L-amino acids and IMP. Our results suggest the involvement of both brain and taste variants of mGluR1 and mGluR4 in detection of IMP and L-amino acids.

Experimental Procedures

Animals used, most of the solutions, taste cells isolation procedure, and calcium imaging procedure used in this study is very similar to as described in Pal Choudhuri et al. (2015). The procedures are described in brief and any new information is described in detail.

Ethical Consideration

All experimental procedures were reviewed and approved by the University of Vermont's Institutional Animal Care and Use Committee (IACUC protocol: 10-038). Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation.

Animals

Adult male and female T1r3-GFP (Damak et al., 2008), and T1r3 KO (Damak et al., 2003) mice were used in these experiments. Mice expressing enhanced green fluorescent protein (eGFP) under the control of the promoter of the *Tas1r3* gene were used primarily to confirm the isolation of TSCs. The T1r3 KO mice were generated on C57BL/6J background, and all 6 exons for the *Tas1r3* gene were eliminated. Breeding stock for T1r3-GFP and T1r3 KO mice were generously donated by Dr. Robert Margolskee (Damak et al., 2003, 2008). Expression of the GFP and the genetic deletion

of *Tas1r3* gene were verified by polymerase chain reaction (PCR) in all mice. All mice were maintained on a 12-h light/12-h dark cycle with food and water provided *ad libitum*.

For clarity, throughout the paper we are using T1r1 and T1r3 to refer to the receptor proteins in mice. In addition, terms such as umami, sweet and bitter are human perceptual constructs and are used primarily for effective communication.

Solutions and Chemicals

Tyrode's solution contained (in mM): NaCl 140, KCl 5, MgCl₂ 1, CaCl₂ 2, HEPES 10, Glucose 10, and Na pyruvate 1. Ca²⁺/Mg²⁺ free Tyrode's contained (in mM): NaCl 140, KCl 5, HEPES 10, Glucose 10, Na pyruvate 1, and EGTA 2. L-amino acids used as test solutions were (in mM): MPG 10, Ser 20, and Arg 10. MPG was used to ensure that responses were not due to the sodium component of MSG, as previously reported by other studies (Sako and Yamamoto, 1999; He et al., 2004). Di-sodium IMP was used at 1 mM. Physiologically relevant stimulus concentrations for each substance were chosen from behavioral and physiological data to ensure that each concentration was above recognition threshold in rodents (mice and rats) (Yoshii et al., 1986; Maruyama et al., 2006; Nakashima et al., 2012), but not high enough to cause any osmotic changes. The artificial sweetener, SC45647 (2-[[[[4-(aminomethyl)phenyl]amino]-[(1*R*)-1-phenylethyl]amino]methyl]amino]ethane-1,1-diol) (100 μM) was used as a sweet stimulus (Nagarajan et al., 1996), and a mixture of cycloheximide (20 μM) and denatonium (2 mM) was used as a bitter stimulus. The L-amino acids were dissolved in Tyrode's solution and made fresh every day. Agonists and antagonists for glutamate receptors were purchased from Tocris (Bristol, UK). Agonists used were: 30 μM – 100 μM (S)-3,5-Dihydroxyphenylglycine (DHPG) and 30 μM - 10 mM L-(+)-2-Amino-4-

phosphonobutyric acid (L-AP4). Antagonists used were: 10 μ M – 40 μ M 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 μ M – 100 μ M D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), 100 μ M (*RS*)-1-Aminoindan-1,5-dicarboxylic acid (AIDA), and 200 μ M – 1 mM (*RS*)- α -Methylserine-*O*-phosphate (MSOP). DHPG is a group I mGluR (mGluR 1 and 5) agonist and L-AP4 is a selective group III mGluR (mGluR 4, 5, 7, and 8) agonist. CNQX is a potent AMPA/kinate antagonist. D-AP5 is a selective NMDA antagonist. AIDA is a selective group I mGluR antagonist and MSOP is a selective group III mGluR antagonist. Stock solutions of agonists and antagonists were prepared as recommended by the manufacturer and kept at -80°C. DHPG and L-AP4 stock solutions were used within one week of preparation and antagonist stock solutions were used within 3 to 4 weeks of preparation. All test solutions were adjusted to approximately pH 7.4 using NaOH or HCl.

Taste Cell Isolation

Taste cells from circumvallate and foliate taste buds were isolated using a protocol previously described by Pal Choudhuri et al. (2015), originally adapted from Behe et al. (1990) and Gilbertson et al. (1997). After euthanasia, tongues were removed and immersed in ice cold Tyrode's solution. An enzyme cocktail containing 0.8 mg/mL collagenase A (Sigma, St. Louis, MO), 1.5 mg/mL dispase II (Roche, Indianapolis, IN), 1 mg/mL trypsin inhibitor (Sigma, St. Louis, MO), and 0.05 mg/mL elastase (Worthington, Lakewood, NJ) was used to detach the lingual epithelium from the underlying mesenchyme. The tongue was then incubated in Tyrode's solution for 20 min followed by incubation in Ca^{2+} / Mg^{2+} -free Tyrode's for another 20 min. In both solutions, oxygen was supplied continuously. The epithelium was gently removed from the underlying

connective tissue and pinned flat with the epithelium surface down on a sylgard-lined petri dish. The tissue was incubated in the enzyme cocktail (without dispase II) for 5 min before being transferred to $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Tyrode's solution for 20-25 min. Taste buds and TSCs were removed from circumvallate and foliate papillae by gentle suction using fire polished glass micropipettes. TSCs were plated into a shallow recording chamber with a glass cover-slip pre-coated with Concanavalin A (Sigma, St. Louis, MO) to promote cell adherence. This protocol enabled us to reliably obtain both isolated taste cells and clusters of taste cells. Typically the cells were viable for 6-7 hours.

Calcium (Ca^{2+}) imaging

Measurements of intracellular Ca^{2+} were obtained using the ratiometric fluorescent dye fura-2 AM (Molecular probes, Invitrogen Corporation, NY). Taste cells were incubated in 5- μM fura-2, AM and 0.05% pluronic F-127 dissolved in DMSO in Tyrode's solution for 25-30 min. The recording session began after bath washing the cells in Tyrode's solution for 10-20 min. Image capturing was done with an inverted fluorescent Nikon TE2000S microscope and C4742-95 digital camera. All solutions were bath applied using a gravity flow perfusion system. Test solutions were applied for 30 s before returning to Tyrode's solution. The antagonist CNQX was allowed to equilibrate with receptors for 10 min before application of agonist. D-AP5 was also applied either 10 min prior to agonist application or with the agonist. At the end of any test solution application, cells were washed in Tyrode's solution for 5 to 9 min. We performed a desensitization study in which single cells were stimulated with the same L-amino acid for 4 to 5 times with varying wash times in between. We found that a wash of 5 to 9 min between stimulus applications was optimal for repeated responses of

similar magnitude, although in some cells desensitization occurred independent of an extended wash. If the final stimulus application in the test sequence did not elicit a response, the cell was stimulated with a compound which previously elicited a response to make sure that the cell was still responsive. For the antagonist experiments, we excluded any cell from the analysis that did not recover during wash after antagonist application. Images were captured every 3 s during stimulus application and every 5 to 15 s during wash. In order to minimize cell damage during long wash periods, we limited image capturing during washes. After a response, images were captured only until the Ca^{2+} level went back to baseline (~2-3 min after the start of test solution application) and image capturing was resumed at least 1 min prior to next stimulus application. Fura2 AM was doubly excited at 340 nm and 380 nm and its emissions were recorded at 510 nm. Simple PCI 6.0 software (Hamamatsu, Sewickley, PA) running on a PC computer was used to capture images. Changes in Ca^{2+} concentrations are reported as F340/F380 plotted over time after background subtraction.

Quantification of Calcium Responses

Increases in intracellular Ca^{2+} evoked by stimulus application were calculated as follows:

$$\Delta F = \frac{F_{\text{Peak}} - F_{\text{Baseline}}}{F_{\text{Baseline}}} * 100\%$$

where ΔF is the percent change above baseline, F_{Peak} is the largest F340/380 ratio within 1 min following the onset of a stimulus application, and F_{Baseline} is the average of the five sampled F340/380 ratios immediately before stimulus application. Our criteria for defining ΔF as a response were: 1) the same stimulus elicited an increase in

intracellular Ca^{2+} at least twice, and 2) each $\Delta F \geq 5\%$. Antagonist data were normalized using the average of Ca^{2+} responses to the stimulus applied immediately before and after the application of the mixture of the stimulus and antagonist.

Statistical Analysis

Data are reported as means $\pm$ SEM. All statistical analyses were conducted with Graphpad Prism software. The statistical significance of differences between groups was calculated using paired or unpaired t-tests following Welch's correction, Wilcoxon matched pair signed rank test, and repeated measure ANOVA followed by Tukey's multiple comparison tests where applicable. An alpha of $P < 0.05$ was used to evaluate each analysis.

Results

Multiple receptors are known to be involved in the detection of umami or glutamate taste. These include T1r1+T1r3 heterodimer, mGluR4 (taste and brain variants), and mGluR1 (truncated and brain variants). While T1r1+T1r3 seems to be a generalist L-amino acid taste receptor, L-amino acids can elicit responses in TSCs of T1r1 KO and T1r3 KO mouse. This suggests other receptors are also capable of detecting L-amino acids (Damak et al., 2003; Maruyama et al., 2006; Kusuvara et al., 2013; Pal Choudhuri et al., 2015). The most likely candidates are the mGluRs already known to be capable of detecting umami stimuli. To test this hypothesis, we used selective agonists and antagonists for mGluR1 and mGluR4 to determine if mGluRs underly calcium responses elicited by different L-amino acids. However, before we

conducted these experiments, we needed to address potential issues related the impact of ionotropic glutamate receptors (iGluRs) on Ca^{2+} responses of TSCs.

In the taste bud, L-glutamate functions as a taste stimulus and potentially as a neurotransmitter. iGluRs are thought to be expressed on the basal end of TSCs where they may play a role in glutamate neurotransmission (Caicedo et al., 2000; Vandenbeuch et al., 2010). These receptors induce an increase in intracellular Ca^{2+} in the presence of micromolar concentrations of glutamate (Caicedo et al., 2000; Huang et al., 2012). In this study we used isolated TSCs and all stimuli were bath applied, thus some responses could be generated by iGluRs expressed in the basolateral membrane. Before the role of mGluRs could be explored, two important questions were addressed to avoid confounding our findings by iGluRs. The first was the potential role of iGluRs in generating responses in isolated TSCs and the second was to identify effective concentrations of iGluR antagonists. Since iGluRs can be activated by micromolar concentrations of glutamate, we first determined if a single TSC can respond to both micromolar (probable neurotransmission function) and/or millimolar (probable taste function) concentrations of glutamate. TSCs were stimulated with 50 μM and 10 mM MPG and the changes in intracellular Ca^{2+} levels were measured (Figure 1A, B). Five percent of the cells (19 of 389; Figure 1) responded (i.e. $\Delta F \geq 5\%$ in response to stimulation) to both concentrations of MPG. However, a greater number of cells responded only to 10 mM glutamate (9%, 36 of 389) (Figure 1). The mean ΔF induced by 50 μM MPG (Mean $\pm$ SEM=8.64 $\pm$ 0.91, n=19) was significantly smaller than the ΔF induced by 10 mM MPG (Mean $\pm$ SEM=42.84 $\pm$ 9.48, n=36) (Figure 1B). Of the 55 cells that responded to 10 mM MPG, 34% (19 of 55) also responded to 50 μM MPG and 66%

did not (Figure 1A). These data suggest that in some TSCs glutamate might have a neurotransmitter function, although they might also be taste responses (see discussion).

To avoid NMDA- and AMPA/kinate-mediated responses in subsequent experiments, it was important to determine if a combination of NMDA (D-AP5) and AMPA/kinate (CNQX) receptor antagonists could be used effectively to block any contributions from iGluRs. Because we intended to use 10 mM MPG to investigate taste responses (see methods), we tested combinations of these selective antagonists mixed with 10 mM MPG. The effectiveness of three antagonistic mixtures were tested: (1) low (10 μ M CNQX + 50 μ M D-AP5), (2) medium (20 μ M CNQX + 100 μ M D-AP5), and (3) high (40 μ M CNQX + 200 μ M D-AP5). Even though the lowest concentration of each of the antagonists has been shown individually to completely block responses elicited by 300 μ M glutamate in rat TSCs (Caicedo et al., 2000; Huang et al., 2012), the low antagonists mixture did not have any effect on Ca^{2+} responses elicited by 10 mM MPG (n=7) in mice TSCs. However, doubling those concentrations (medium antagonists mixture) reversibly and significantly suppressed responses to MPG (n=13 of 25 MPG responsive cells) (Figure 2). The high antagonists mixture also significantly suppressed responses to MPG (n=7 of 12 TSCs responding to 10 mM MPG), but the effect was not reversible (Figure 2). Additionally the amount of antagonism by the high antagonists mixture was similar to that of the medium antagonists mix. Thus, for all subsequent experiments involving mGluR agonists and antagonists, we used the medium antagonists mixture to block any iGluR-mediated responses whenever it was considered necessary.

mGluR1-mediates responses to L- amino acid and IMP

mGluR1 is an excitatory receptor known to be expressed mainly in the postsynaptic somatodendritic region (Charpak et al., 1990; Staub et al., 1992; Crepel et al., 1994; Guerineau et al., 1995; Chavis et al., 1996) as well as perisynaptic regions (Nusser et al., 1994; Mateos et al., 2000) of neurons. A brain version as well as a truncated version of mGluR1 have been found to be expressed in the TSCs (Toyono et al., 2003; San Garbiel et al., 2005, 2009) and are thought to elicit an umami taste (Kusuhara et al., 2013) in rodents. If mGluR1s contribute to the detection of L-amino acids and IMP, then a selective mGluR1 agonist would elicit Ca^{2+} responses in TSCs. In addition, selective antagonists for mGluR1 would block or suppress Ca^{2+} responses elicited by different L-amino acids and IMP.

mGluR1 Agonist

DHPG is a selective agonist for mGluR1 (Ito et al., 1992; Schoepp et al., 1994). In neuronal cells the EC_{50} of DHPG ranges between 10-30 μM depending on cell types (Conn and Pin, 1997; Lin et al., 1997). However, since concentrations above 100 μM can also activate other mGluRs, we tested DHPG at 30, 50 and 100 μM to determine an effective dose for subsequent testing (Conn and Pin, 1997; Lin et al., 1997). At 30 μM , DHPG did not elicit any responses in TSCs ($n=180$) (Figure 3A). At 50 μM , DHPG evoked responses in about 2% ($n=3$ of 180 cells) of the TSCs with a mean amplitude of Ca^{2+} responses of $7.28 \pm 1.16\%$ (Mean $\pm$ SEM). At 100 μM , DHPG elicited Ca^{2+} responses in 12 TSCs (6%) with a mean ΔF amplitude of $10 \pm 1.3\%$ (Mean $\pm$ SEM) (Figure 3A). Consequently, 100 μM DHPG was chosen for subsequent experiments.

Next we tested whether a single TSC would show a Ca^{2+} response to DHPG and an array of L-amino acids. Each TSC was stimulated with 5 different stimuli: (1) MPG

(10 mM), (2) MPG (50 μ M), (3) DHPG (100 μ M), (4) Ser (20 mM), and (5) Arg (10 mM). At least one stimulus was capable of eliciting a Ca^{2+} response in 19 of 156 (12%) TSCs. Of the 19 responsive cells, 17 cells (89%) responded to 10 mM MPG and 6 cells (31%) responded to 50 μ M MPG (Figure 3B). Since reports have shown that 10 μ M of CNQX and 50 μ M of D-AP5 can completely block the Ca^{2+} responses elicited by 300 μ M glutamate (Caicedo et al., 2000), responses elicited by 50 μ M MPG in presence of CNQX and D-AP5 mixture are probably due to the activation of the brain version of mGluRs expressed in TSCs. In addition to MPG, TSCs also responded to DHPG (n= 6 of 19 cells; 31%), Ser (n=4 of 19 cells; 21%), and Arg (n=5 of 19 cells; 26%) (Figure 3B, Table 1). Because we were targeting TSCs responsive to MPG and DHPG, our data are biased towards MPG responsive cells. However, like our previous study, not every L-amino acid elicited a response in the same TSC (Pal Choudhuri et al., 2015). Of the 19 cells presented in Table 1, only 1 cell responded to all of the stimuli. Additionally there were cells that responded to IMP and Arg, but not to MPG. The rest of the cells exhibited variable response patterns to the array of stimuli, similar to what was reported previously (Pal Choudhuri et al., 2015).

mGluR1 Antagonist

Next we recorded TSC responses to L-amino acids and IMP with the selective class I mGluR antagonist AIDA (Pellicciari et al., 1995; Moroni et al., 1997). AIDA blocks both mGlu1 and mGluR5. However, at this time there is no evidence that mGluR5 is expressed in TSCs. For all the L-amino acids and IMP, AIDA significantly and reversibly suppressed TSC responses (Figure 4A, B, C). The responses to 10 mM MPG, 20 mM Ser, and 1 mM IMP were reduced by 66% [n=29, $F(2,51)=78.42$, $P < 0.0001$], 69%

[n=12, $F(1,13)=27.57$, $P < 0.001$], and 65% [n=12, $F(1,14)=30.64$, $P < 0.0001$], respectively (Figure 4E, F, G). As a control, we also tested whether AIDA has an effect on a non-amino acid stimulus. Specifically, AIDA did not have any effect on Ca^{2+} responses (n=5) elicited by the potent artificial sweetener, SC45647 (Figure 4D). Additionally, 100 μ M AIDA alone did not elicit any responses in TSCs (n=5).

Since previous studies have shown that mGluR selective agonists can elicit responses in cells expressing T1r1+T1r3, we assumed that it is also possible that selective mGluR antagonists might have an effect on T1r1+T1r3 and limited testing of mGluR1 antagonists to TSCs of T1r3 KO mice. Similar to T1r3-GFP mice, in T1r3 KO cells AIDA also significantly and reversibly suppressed responses elicited by MPG [n=9, $F(2,16)=15.25$, $P < 0.001$], Ser [n=6, $F(2,8)=20.13$, $P < 0.001$], Arg [n=19, $F(2,28)=58.35$, $P < 0.0001$], and IMP [n=9, $F(2,14)=35.22$, $P < 0.0001$] (Figure 5 A, B, C, D, E, F, G, H). These data suggest that at least in the posterior tongue, L-amino acids and IMP can also be detected by mGluR1.

mGluR4-mediates responses to L- amino acid and IMP

mGluR4 receptors belong to the group III mGluR family and are predominantly expressed presynaptically in the CNS (Bradley et al., 1999; Corti et al., 2002; Niswender and Conn, 2010). A brain variant as well as a taste variant of mGluR4 are found in the TSCs and appear to contribute to umami taste (Chaudhuri et al, 1996; Delay et al., 2004; Nakashima et al. 2001; Toyono et al., 2002). If mGluR4 contributes to the detection of L-amino acids and IMP, then a selective mGlu4 agonist such as L-AP4 would elicit Ca^{2+} responses in TSCs and a selective antagonist for mGlu4 such as MSOP would block the responses elicited by different L-amino acids and IMP. However,

L-AP4 can also elicit responses in cells expressing the umami receptor T1r1+T1r3 heterodimer (Nelson et al., 2002). To eliminate any influence of the T1r1+T1r3 heterodimer, we performed all of these experiments using TSCs of T1r3 KO mice.

mGluR4 Agonist

L-AP4, a glutamate analog, is a selective agonist for group III mGluRs (Nakanishi, 1992; Bushell et al., 1995; Tones et al., 1995), but of that group, only mGluR4s have been detected in the TSCs. Behavioral studies in rodents (Chaudhuri et al 1996; Delay et al., 2000, 2004; Nakashima et al., 2001, 2012) suggest that L-AP4 elicits a taste similar to glutamate. We first tested whether L-AP4 can elicit Ca^{2+} responses in TSCs of T1r3 KO mice. We determined the concentration-response relationship for L-AP4 with 0.05, 0.1, 0.5, 1, 3, 5, and 10 mM and found the EC_{50} for L-AP4 to be about 3.7 mM (Figure 6). Therefore, in subsequent experiments we used 4 mM of L-AP4.

Next we determined whether a single TSCs would show a Ca^{2+} response to an array of L-amino acids and L-AP4. Each TSC was stimulated with 5 different stimuli: (1) MPG (10 mM), (2) Ser (20 mM), (3) Arg (10mM), (4) IMP (1mM) and (5) L-AP4 (4 mM). At least one stimulus was capable of eliciting a Ca^{2+} response in 6 of 59 (10%) TSCs. Interestingly, some L-AP4-responsive cells also responded to one or more of the L-amino acids and IMP. Of the 6 responsive TSCs, 4 cells (66%) responded to L-AP4. Along the same line 4, 4, and 3 of the 6 cells responded to MPG, Ser, and Arg, respectively, and 1 cell responded to IMP.

mGluR4 Antagonist

Even though the mGluR4 antagonist, CPPG, has been shown to block glutamate responses in behavioral, nerve recording, and Ca^{2+} imaging studies and is highly potent for mGluR group III, it can also antagonize other mGluRs at higher concentrations (Jane et al., 1996; Toms et al., 1996). On the other hand MSOP is a selective group III mGluR antagonist with no known effect on postsynaptic mGluRs or iGluRs (Thomas et al., 1996). Thus we used MSOP to study the involvement of mGluR4 in detection of L-amino acids and IMP.

MSOP significantly suppressed TSC responses to 4 mM L-AP4 in a dose dependent manner [$n=5$; $F(1,5)=13.37$, $P < 0.05$] (Figure 7A, B). At 500 μM MSOP significantly reduced L-AP4 responses. Since higher concentrations did not suppress the response any further, 500 μM MSOP was used in subsequent experiments. As a control, MSOP was tested to determine if it has any effect on Ca^{2+} responses elicited by a stimulus of another taste quality. MSOP did not have any effect on SC45647-elicited Ca^{2+} responses ($n=4$) nor did it have any effect on TSCs when presented alone at concentrations up to 1 mM ($n=5$).

MSOP significantly and reversibly suppressed responses evoked by the L-amino acids and IMP (Figure 8). MSOP suppressed responses to 10 mM MPG, 20 mM Ser, 10 mM Arg, and 1 mM IMP by 54% [$n=9$, $F(1,11)=5.73$, $P < 0.05$], 63% [$n=6$, $F(1,7)=5.29$, $P < 0.05$], 56% [$n=12$, $F(1,5)=11.15$, $P < 0.01$], and 61% [$n=20$, $F(1,25)=31.14$, $P < 0.0001$], respectively (Figure 8A, B, C, D, E, F, G, H). These data suggest that at least in the posterior tongue, mGluR4 acts as an L-amino acid and IMP receptor.

Discussion

Detection of L-amino acids has been tied to taste receptors associated with umami taste, especially the T1r1+T1r3 heterodimer (Nelson et al., 2002; Li et al., 2002). However, there is a growing body of evidence suggesting additional glutamate receptors, such as the mGluRs, may be involved in detecting umami and that at least some of these receptors are likely to participate in the taste of other L-amino acids. Although T1r1+T1r3 has been proposed to be a broadly tuned L-amino acid receptor, we have previously shown that glutamate-responsive TSCs of wild type mice respond to some amino acids but generally not to all, and that L-amino acid-responsive TSCs may respond to multiple L-amino acids but not necessarily to glutamate (Pal Choudhuri et al., 2015). We also found that L-amino acids can elicit responses in TSCs of T1r3 KO mice. An IMP binding site located in the N-terminal domain of the T1r1 subunit has been proposed (Zhang et al., 2008), but the lack of IMP-induced Ca^{2+} responses in HEK cells expressing T1r1 or the heterodimer T1r1+T1r3 (Nelson et al., 2002) raises questions about its role as an independent IMP taste receptor. Thus we investigated whether mGluRs, specifically mGluR1 and mGluR4, can function as L-amino acid and IMP taste receptors. The principal finding in this study is that these mGluRs are involved in the detection of L-amino acids and IMP. The pharmacological characterization of the responses elicited by L-amino acids and IMP indicate that both mGluR1 and mGluR4 can detect L-amino acids and IMP in TSCs, and are probably the main candidate receptors underlying the Ca^{2+} responses in TSCs of T1r3 KO mice to these stimuli.

The metabotropic glutamate receptors mGluR1 and mGluR4 were originally characterized from brain tissue. In situ hybridization, immunoblot, and

immunohistochemical analysis have shown that these receptors are also expressed in taste buds (Chaudhari et al., 1996; Yang et al., 1999; Chaudhari et al., 2000; San Gabriel et al., 2005, 2009a). For both receptor types, in addition to the brain version, a structurally and functionally distinct form was also found in the taste buds and were termed truncated or taste variants of mGluR1 and mGluR4. The taste-mGluR1 lacks most of the N-terminus VFT domain. Similarly, the taste-mGluR4 also lacks 50% of the receptor's extracellular N-terminus domain. Since the N-terminus VFT domain is considered to be essential for L-glutamate binding, these truncations are likely to influence the affinity of the receptor for glutamate. For both receptors the affinity of the receptor in vitro expression system was lower for the truncated variant than for the brain variant (Chaudhari et al., 2000; San Gabriel et al., 2009a). While brain-expressed variants of the receptors can be activated by micromolar concentrations of glutamate, the truncated receptors responded only at concentrations ≥ 1 mM of glutamate. This reduced sensitivity of the truncated-mGluRs is of physiologic significance as in many behavioral and cellular studies, this corresponds well with the concentrations of agonists needed to elicit taste responses and suggests that the truncated versions of the mGluRs mainly function as taste receptors (Chaudhari et al., 2000; San Gabriel et al., 2009a). Behavioral and cellular studies have showed that micromolar concentrations of glutamate can also elicit significant behavioral responses, suggesting that brain mGluRs also function as taste receptors (Nakashima et al., 2012). Since iGluRs have been reported in tongue epithelium and iGluRs can also be activated by micromolar concentrations of glutamate, we first aimed to characterize and block any iGluR mediated responses to specifically study mGluR activity.

During the early 1990's glutamate or umami taste was hypothesized to involve iGluRs, such as NMDA and non-NMDA type receptors expressed in TSCs (Lin and Kinnamon, 1999; Brand 2000). Later, studies showed that while rats and mice could taste NMDA, their behavior indicated that by itself, NMDA did not elicit a taste similar to glutamate (Chaudhuri et al., 1996; Stapleton et al., 1999; Nakashima et al., 2001; Delay et al., 2004). In addition to being implicated in umami taste, glutamate was also proposed to function as a neurotransmitter activating iGluRs in the taste system (Caicedo et al., 2000; Vandenbeuch et al., 2010; Niki et al., 2011; Huang et al., 2012). To date there is no direct evidence that establishes a neurotransmitter-like function for glutamate for TSCs in the taste buds. There is evidence, however, that glutamate may act as an efferent transmitter in the taste bud. This is based mainly upon the expression of glutamate aspartate transporter in type I TSCs and the expression of vesicular transporters for glutamate, VGLUT 1 and 2, in the nerve fibers surrounding taste buds (Vandenbeuch et al., 2010; Huang et al., 2012). These studies suggest that glutamate may be released by afferent nerve fibers on TSCs, and may modulate taste function. Thus iGluRs may have multiple afferent taste functions, depending upon whether they are expressed in the membrane at the apical end of the TSC (taste) or along the basolateral portion of the membrane (synaptic function).

Since in our study all stimuli were bath-applied, it is possible some responses may be due to activation of iGluRs expressed throughout the plasma membrane of TSCs. In the central nervous system (CNS), micromolar concentrations of glutamate can activate iGluRs and, in the taste system, 30 μ M glutamate can elicit iGluR-mediated responses in TSCs (Caicedo et al., 2000). To block iGluR responses associated with

neural modulation/transmission, 20 μ M CNQX combined with 100 μ M D-AP5 were added to all experimental solutions. These are well above concentrations that effectively block NMDA and AMPA receptors within the CNS and should be similarly effective at blocking iGluRs that might be involved in synapse-associated glutamate activity, enabling us to selectively study the involvement of mGluRs in the detection of L-amino acids and IMP. The iGluR antagonist mixture significantly reduced Ca^{2+} responses elicited by 10 mM MPG in approximately half of the TSCs tested (Figure 2). This finding suggests an iGluR-mediated function in taste buds. Further study would be required to determine if iGluRs might function as L-amino acid taste receptors.

Even in the presence of iGluR antagonists, 35% of the 10 mM MPG responsive cells also responded to 50 μ M MPG, suggesting activation of brain variant mGluRs in TSCs. Threshold experiments reported by Nakashima et al. (2012) have shown that mice can detect glutamate and the selective group III mGluR agonist L-AP4, even at micromolar concentrations, albeit weakly compared to their responses when these substances are presented at millimolar concentrations. CTA studies against L-AP4 and glutamate also gave comparable results (Nakashima et al., 2001; Delay et al., 2002). Moreover, group III and group I glutamate receptor antagonists, CPPG and AIDA were able to block CTAs elicited by micromolar glutamate and L-AP4 (Eschle et al., 2009 Nakashima et al., 2012). Based on these behavioral studies, we can infer that calcium responses elicited by micromolar concentrations of MPG in our study are taste responses mediated by multiple candidate receptors, including: (1) brain expressed variant of mGluR1, (2) brain expressed variant of mGluR4, (3) other brain expressed variants of mGluRs, and/or (4) mGluR heterodimers.

mGluR1 detects L-amino acids and IMP in mouse TSCs

We used selective agonists and antagonists to investigate the role of group I mGluRs in detection of L-amino acids and IMP. For an mGluR1 agonist, we used DHPG, an orthosteric agonist for group I mGluRs with similar potencies at mGluR1 and mGluR5 (Brebet et al., 1995; Wisniewski and Car, 2002). At low concentrations, DHPG is highly selective for group I mGluRs. However, its EC_{50} for group II mGluR is 106 μ M and for group III mGluR is > 1000 μ M (Brebet et al., 1995; Conn and Pin, 1997; Wisniewski and Car, 2002). Although to date there is no evidence that suggests a role for group II mGluRs in umami or L-amino acid detection, Toyono et al. (2007) reported the expression of mGluR2/3 in taste epithelium. Thus we limited the highest concentration of DHPG to 100 μ M. Because the potency of DHPG is similar to that of glutamate (Wisniewski and Car, 2002), we did not expect to see many responses with low concentrations of DHPG. As expected, only 1% and 6% of the TSCs responded to 50 μ M and 100 μ M of DHPG, respectively. Since the truncated taste-mGluR1 is likely to be activated by millimolar concentrations of glutamate, DHPG-activated Ca^{2+} responses are probably mediated by the brain-mGluR1 variant.

Some TSCs that responded to DHPG also responded to Ser and Arg, indicating that these and possibly other L-amino acids also activate mGluR1. All cells that responded to 100 μ M DHPG also responded to 50 μ M MPG and vice versa. But all cells that responded to 10 mM MPG did not necessarily respond to 100 μ M DHPG (Table 1). This was expected since the potency of DHPG is similar to that of glutamate. In a HEK cell expression system, the T1r1+T1r3 receptor was activated by glutamate (50 mM) only in the presence of IMP, whereas some L-amino acids such as Ser and Arg alone

were able to evoke Ca^{2+} responses in those HEK cells (Nelson et al., 2002). Thus, 10 mM MPG elicited responses were probably mediated by the activation of truncated taste-mGluRs with a lower affinity for glutamate compared to the brain-expressed versions of mGluRs. Additionally, some cells that responded to L-amino acids did not respond to MPG or DHPG, suggesting the activation of T1r1+T1r3 or calcium sensors in those instances.

The antagonist data for mGluR1 further strengthened the possibility of a role of mGluR1 in L-amino acid and IMP detection. AIDA, a selective antagonist of mGluR1, significantly and reversibly suppressed responses elicited by MPG, Ser, Arg and IMP in TSCs of T1r3-GFP as well as T1r3 KO mice (Figure 4, 5). AIDA has been shown to have no effect on group II (mGluR2) or group III (mGluR4) receptors expressed individually in baby hamster kidney cells. Additionally, it has no effect on ionotropic glutamate receptors (Pellicciari et al., 1995; Moroni et al., 1997). This suggests that L-amino acids and IMP can activate mGluR1 in TSCs. Although to date, there is no evidence for the expression or activation of mGluR5 on TSCs, we cannot completely rule out that possibility. Still, when considered together, our data strongly suggest that L-amino acids and IMP can evoke responses in TSCs by activation of mGluR1.

L-amino acids and IMP can activate mGluR4 in mouse TSCs

Several behavioral, Ca^{2+} imaging, and nerve recording studies have indicated a role for mGluR4 in the detection of umami taste (Chaudhari et al., 1996, 2000; Delay et al., 2000, 2004; Kusahara et al., 2013). Brain and taste variants of mGluR4 have been shown to be expressed in the posterior portion of the taste epithelium. Evidence for the function of mGluR4 as a taste receptor comes from the fact that the selective mGluR4

agonist, L-AP4, physiologically and behaviorally mimic the response of glutamate (Chaudhari et al., 1996, 2000; Delay et al., 2000; Nakashima et al., 2001; Delay et al., 2004; Nakashima et al., 2012; Kusuvara et al., 2013). Additionally, mGluR4 antagonists CPPG and MPPG block physiological and behavioral responses evoked by glutamate (Eschle et al., 2009; Yasumatsu et al., 2012; Kusuvara et al., 2013). Although L-AP4 is selective for group III mGluRs, it has been shown to activate the T1r1+T1r3 heterodimer expressed in HEK cells in presence of IMP (Nelson et al., 2002). Similar evidence that L-AP4 is able to activate these receptors in vivo have not yet been reported. Even so, responses elicited by L-AP4 in wild type mice may in part result from activation of the T1r1+T1r3 heterodimer, thus may not clearly verify the role of mGluR4 in taste function. In this study a micromolar concentration (100 μ M) of L-AP4 could elicit Ca^{2+} responses in TSCs of T1r3 KO mice. However, the dose-response analysis with L-AP4 revealed a half-maximal effect (EC_{50}) of 3.7 mM (Figure 6). These data indicate that L-AP4 activated the brain as well as the truncated variants of mGluR4 in this study. We further showed that L-AP4 responses can be suppressed by the selective mGluR4 antagonist MSOP.

Interestingly, most of the L-AP4 responsive cells also responded to L-amino acids and IMP and those responses were also significantly suppressed by MSOP, strongly suggesting a role for mGluR4s in L-amino acid and IMP taste responses (Figures 7, 8). Most of the previous studies used CPPG as the mGluR4 antagonist. Although CPPG is a very potent antagonist at mGluR4, it can also effect other mGluRs. Thus we used MSOP which is less potent than CPPG but is highly selective for mGluR4. Additionally it also does not have any known effect on iGluRs. Consequently,

our data provide strong evidence for the activation of mGluR4 by L-amino acids and IMP in TSCs.

From our data it is not possible to identify whether mGluR1 and mGluR4 are expressed in the apical or the basolateral membrane of TSCs. In the central nervous system, mGluR1 is generally expressed on the postsynaptic cells (Charpak et al., 1990; Staub et al., 1992; Crepel et al., 1994; Nusser et al., 1994; Guerineau et al., 1995; Chavis et al., 1996; Mateos et al., 2000) and mGluR4 is predominantly expressed presynaptically (Bradley et al., 1999; Corti et al., 2002; Niswender and Conn, 2010). If glutamate has a neurotransmitter/modulatory function in taste cells and is released by nerve fibers to work on the TSCs, then its function might be mediated by mGluR1 or mGluR4 expressed on the basolateral membrane of the TSCs. However immunoblot and immunohistochemical analyses have shown that both variants of mGluR1 taste-mGluR4 are present in the taste pores, and no immunostaining for mGluR4 was found in taste cells below taste pore in synaptic regions (Toyono et al., 2002, 2003). Additionally, several behavioral and physiological studies have strongly suggested the activation of brain as well as truncated variants of mGluR1 and 4 in glutamate taste detection (Chaudhari et al., 2000; Delay et al., 2002; Eschle et al., 2009; Nakashima et al., 2012; Yasumatsu et al., 2012; Kusahara et al., 2013). The data reported in this study are in agreement with these previous studies and, taken as a whole, these findings argue that both variants of both mGluR1 and mGluR4 are involved in the taste of L-amino acids and IMP.

Neither mGluR1 nor mGluR4 antagonist was able to completely block the responses elicited by L-amino acids or IMP. From our data, it is not possible to

determine if different receptors, i.e., an mGluR1 and T1r1, can generate responses in the same T1r3 KO cell, or if there might be other receptors that act as candidate receptors for IMP and L-amino acids taste in these cells. mGluR2 and mGluR3 are also expressed in the tongue, however, their expression in the taste cells is not yet established. Even so, they may also be potential candidate receptors in T1r3 KO TSCs. Recently, the Ca^{2+} sensor CaSR and the class C GPCR, GPRC6A, which can also detect L-amino acids, were localized to Type I and III taste cells (San Gabriel et al., 2009b; Bystrova et al., 2010). One very intriguing possibility is that taste-mGluR4 and/or some other receptors may form a complex with each other, or with T1rs that responds to L-amino acids and IMP. However, further investigation is needed to examine this hypothesis.

A synergistic response between an umami substance and a 5'-ribonucleotide such as IMP is a characteristic property of umami taste (Delay et al., 2000). We have previously showed that, like MPG, other L-amino acids can also elicit synergistic responses in TSCs when mixed with IMP (Pal Choudhuri et al., 2015). Although the intracellular signaling pathway for synergistic response is not yet understood, one hypothesis is the downregulation of cAMP pathway which in turn causes the downregulation of PKA and subsequent release of phosphorylation and further activation of PLC mediated pathway (Kinnamon and Vandenbeuch, 2009). Since activation of mGluR4 causes downregulation of the cAMP pathway, it might be involved in synergistic responses either by itself or in combination with other receptors. Additionally we have previously demonstrated the occurrence of synergistic responses in the TSCs of T1r3 KO mice. Thus, mGluRs individually, dimerized with T1r1, or

possibly as a component of some form of receptor complex might also be involved in the synergistic responses. Additional research is needed to determine the role of mGluRs, if any, in generating synergistic responses.

Conclusions

In summary we have shown that isolated mouse TSCs can respond to a single L-amino acid and IMP, and to selective agonists of mGluR1 or mGluR4 receptors. Additionally selective antagonists for mGluR1 and mGluR4 suppressed responses elicited by different L-amino acids and IMP. Our data strongly suggest the involvement of mGluR1 and mGluR4 in L-amino acid and IMP taste sensations and provides an explanation for residual L-amino acid and IMP responses in T1r3 KO mice.

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Figure Legends

Figure 1: TSC responses to micromolar (50 μ M) and millimolar (10 mM)

concentrations of glutamate. [A] Cell 1's Ca^{2+} responses are representative of TSCs that responded to millimolar but not micromolar concentrations of MPG. Cell 2's Ca^{2+} responses are representative of TSCs that responded to micromolar and millimolar concentrations of MPG. The bar above each stimulus trace represents the stimulus application period (30 sec). [B] Mean $\pm$ SEM amplitude of Ca^{2+} increases above baseline for responsive cells only, i.e. cells with a $\Delta F \geq 5\%$. n = number of cells. *** P < 0.001.

Figure 2: iGluR Antagonists mixtures partially block responses elicited by 10 mM

MPG. Average effect of three different mixtures of CNQX+D-AP5 on Ca^{2+} responses elicited by 10 mM glutamate. Each antagonist mix was applied 10 min prior to and with MPG. Each bar represents the Mean $\pm$ SEM amplitude of Ca^{2+} responses. n = number of cells. *** P < 0.001, * P < 0.05.

Figure 3: mGluR1 agonists (DHPG) and L-amino acids elicit responses in TSCs of

T1r3-GFP mice. [A] TSC responses to 30, 50, and 100 μ M DHPG (mGluR1 agonist). Dotted line represents the $\Delta F = 5\%$ threshold, above which calcium levels are considered responses. None of the cells responded to 30 μ M and all 12 cells responded to 100 μ M DHPG. [B] Stimuli tested were DHPG (100 μ M) and 3 L-amino acids with different side-chain groups: (MPG (10 mM and 50 μ M), Ser (20 mM), and Arg (10 mM). Bars are Mean $\pm$ SEM amplitudes of Ca^{2+} increases above baseline for responsive cells

only, i.e. cells with a change in $\text{Ca}^{2+} \geq 5\%$. 17 of 19 cells responded to 10 mM MPG. Fewer TSCs responded to each of the other L-amino acids or DHPG and only 1 TSC responded to all stimuli. n = number of cells. **For these experiments, 20 μM CNQX was added to the bath solution, and 100 μM D-AP5 was added to each stimulus.**

Figure 4: Effect of 100 μM AIDA (group I mGluR selective antagonist) on L-amino acids and IMP elicited responses in TSCs of T1r3-GFP mice. The antagonist was tested against MPG (10 mM), IMP (1 mM), and Ser (20 mM). [A, B, C] In TSCs, AIDA partially or completely inhibited responses to IMP, MPG, and Ser, respectively. Traces are representative of responses during pretest stimulation, stimulation with AIDA, and posttest stimulation [D] AIDA had not effect on SC45647 elicited response [E, F,G] Average effect of AIDA on all responses to IMP, MPG, and Ser, respectively. Each bar represents Mean $\pm$ SEM amplitude of Ca^{2+} responses during pretest, test and posttest stimulation. n = number of cells. **** P < 0.0001, *** P < 0.001, ** P < 0.01.

Figure 5: Effect of 100 μM AIDA (group I mGluR selective antagonist) on Ca^{2+} responses elicited by L-amino acids and IMP in TSCs of T1r3 KO mice. The antagonist was tested against MPG (10 mM), IMP (1 mM), Ser (20 mM), and Arg (10 mM). **For these experiments, 20 μM CNQX was added to the bath solution, and 100 μM D-AP5 was added to each stimulus.**

[A, B, C, D] In TSCs, AIDA partially or completely inhibited responses to MPG, IMP, Ser, and Arg, respectively. Representative traces of Ca^{2+} responses during pretest, test,

and posttest stimulation for each L-amino acid and IMP. [E, F, G, H] Average effect of AIDA on all Ca^{2+} responses to MPG, IMP, Ser, and Arg, respectively. Each bar represents the Mean $\pm$ SEM amplitude of Ca^{2+} responses. n = number of cells. **** P < 0.0001, *** P < 0.001, ** P < 0.01.

Figure 6: L-AP4 (group III mGluR selective agonist) elicited responses in TSCs of T1r3 KO mice in a concentration-dependent manner. Concentration-response relationship for L-AP4. Each point represents the Mean $\pm$ SEM Ca^{2+} response for that concentration. X-axis represents log values of L-AP4 concentrations. Increases in L-AP4 concentration elicited dose-dependent increases in Ca^{2+} responses. EC_{50} was calculated to be about 3.7 mM.

Figure 7: MSOP (group III mGluR selective antagonist) partially inhibited L-AP4 (4 mM) mediated responses of TSCs of T1r3 KO mice in a concentration-dependent manner. [A] Representative Ca^{2+} responses in a single cell showing inhibition of 4 mM L-AP4-elicited responses by MSOP in a concentration-dependent manner. L-AP4 was presented alone before and after the three trials with L-AP4+MSOP. [B] Average effect of MSOP on L-AP4 mediated responses. Each point represents Mean $\pm$ SEM response for each stimulus solution. **For these experiments, 20 μM CNQX was added to the bath solution, and 100 μM D-AP5 was added to each stimulus.** n = number of cells. ** P < 0.01, * P < 0.05. ns = no significant difference.

Figure 8: Effect of 500 μ M MSOP (group III mGluR selective antagonist) on L-amino acids- and IMP-elicited responses in TSCs of T1r3 KO mice. The antagonist was tested against MPG (10 mM), IMP (1 mM), Ser (20 mM), and Arg (10 mM). **For these experiments, 20 μ M CNQX was added to the bath solution, and 100 μ M D-AP5 was added to each stimulus.** [A, B, C, D] In TSCs, MSOP partially or completely inhibited responses to MPG, IMP, Ser, and Arg, respectively. In TSCs, MSOP partially or completely inhibited responses to MPG, IMP, Ser, and Arg, respectively. [E, F, G, H] Average effect of MSOP on all responses to MPG, IMP, Ser, and Arg, respectively. Each column represents Mean $\pm$ SEM amplitude of Ca²⁺ responses during pretest, test and posttest stimulus conditions. n = number of cells. **** P < 0.0001, *** P < 0.001, ** P < 0.01.

Figure 1

Figure 2

Low: 10 μ M CNQX + 50 μ M D-AP5

Medium: 20 μ M CNQX + 100 μ M D-AP5

High: 40 μ M CNQX + 200 μ M D-AP5

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table1: Response patterns of 19 TSCs to IMP, L-amino acids, and mGluR1 agonist, DHPG.

TSC	MPG (10 mM)	MPG (50 μM)	DHPG (100 μM)	IMP (1 mM)	Ser (20 mM)	Arg (10 mM)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						

Each row represents an individual cell. Gray cells indicate Ca²⁺ responses to a stimulus solution (column). For these experiments, 20 μM CNQX was added to the bath solution, and 100 μM D-AP5 was added to each stimulus.