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Mouse Strain Differences in Gurmarin-sensitivity of Sweet Taste Responses Are Not Associated with Polymorphisms of the Sweet Receptor Gene, *Tas1r3*

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Abstract

Gurmarin (Gur) is a peptide that selectively inhibits responses of the chorda tympani (CT) nerve to sweet compounds in rodents. In mice, the sweet-suppressing effect of Gur differs among strains. The inhibitory effect of Gur is clearly observed in C57BL/6 mice, but only slightly, if at all, in BALB/c mice. These two mouse strains possess different alleles of the sweet receptor gene, *Sac* (*Tas1r3*) (taster genotype for C57BL/6 and non-taster genotype for BALB/c mice), suggesting that polymorphisms in the gene may account for differential sensitivity to Gur. To investigate this possibility, we examined the effect of Gur in another *Tas1r3* non-taster strain, 129X1/Sv mice. The results indicated that unlike non-taster BALB/c mice but similar to taster C57BL/6 mice, 129X1/Sv mice exhibited significant inhibition of CT responses to various sweet compounds by Gur. This suggests that the mouse strain difference in the Gur inhibition of sweet responses of the CT nerve may not be associated with polymorphisms of *Tas1r3*.

Key words: chorda tympani nerve, gurmarin, mouse strain differences, polymorphisms, sweet taste, *Tas1r3*

Introduction

Gurmarin (Gur), a polypeptide isolated from the plant *Gymnema sylvestre*, is known to selectively suppress behavioral and gustatory neural responses to various sweet compounds without affecting responses to salty, sour and bitter substances in rodents (Imoto *et al.*, 1991; Miyasaka and Imoto, 1995; Ninomiya and Imoto, 1995; Ninomiya *et al.*, 1997, 1998, 1999; Harada and Kasahara, 2000; Lemon *et al.*, 2003; Murata *et al.*, 2003). In mice, the sweet-suppressing effect of Gur differs among strains and taste nerves. Responses to sweet compounds in the chorda tympani (CT) nerve innervating the anterior tongue were inhibited to ~50% of control by Gur in C57BL/6 and C57BL/KsJ (abbreviated C57BL for either) but only slightly if at all in BALB/c (BALB) mice (Ninomiya and Imoto, 1995; Ninomiya *et al.*, 1997). The inhibitory effect of Gur was not clearly evident in responses of the glossopharyngeal (IXth) nerve innervating the posterior tongue even in C57BL mice (Ninomiya *et al.*, 1997). The effect of Gur is normally long-lasting (>2–3 h) but rapidly disappears after rinsing the tongue with either anti-gurmarin serum in rats (Imoto *et al.*, 1991; Miyasaka and Imoto, 1995) or β-cyclodextrin in C57BL mice (Ninomiya *et al.*, 1998). This quick recovery from inhibition by Gur by rinsing the tongue with Gur-binding agents indicates that Gur may directly interact with taste receptors on the taste cell membrane in rodents.

Recently, the taste receptor T1R3 was identified as the gene product of a single locus, *Sac* (*Tas1r3*), on mouse chromosome 4 (Bachmanov *et al.*, 2001; Kitagawa *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Sainz *et al.*, 2001); *Sac* had been shown to influence behavioral and nerve responses to artificial sweeteners, such as saccharin, and to several sugars (Fuller, 1974; Lush 1989; Bachmanov *et al.*, 1997; Li *et al.*, 2001). Differences between taster (C57BL) and non-taster (BALB, various sublines of the 129 strain etc.) strains in behavioral and nerve responses to sweet compounds are thus believed to be caused by polymorphisms in the *Tas1r3* gene (e.g. six amino acid changes, including I60T). The T1R3 protein is proposed to form a heterodimer with T1R2 protein; this combination responds to many sweet compounds (Nelson *et al.*, 2001, 2002). Knockout mice lacking T1R3

showed no preference for artificial sweeteners and had largely diminished behavioral and nerve responses to sugars (Damak *et al.*, 2003; Zhao *et al.*, 2003). This large contribution of T1R3 for reception of sweet compounds raises the possibility that strain differences in sensitivity to Gur, as mentioned above, may also be due to polymorphisms of *Tas1r3*.

To investigate this possibility, our previous study (Shigemura *et al.*, 2005) examined the effect of Gur on CT responses to sucrose in the *dpa*-congenic strain having the same genetic background to BALB mice except the gene segment containing the *dpa* locus derived from C57BL mice. Similar to the *Sac*/*Tas1r3* locus, the *dpa* locus has been proposed to locate on the mouse chromosome 4 and influence behavioral and nerve responses to sweet compounds including a sweet tasting amino acid, D-phenylalanine, and sugars (Ninomiya *et al.*, 1991; Bachmanov *et al.*, 1997). We found clear Gur inhibition of sucrose responses of the congenic strain, suggesting the possibility that Gur-sensitivity in mice may be independent of the *Tas1r3* allele. Since, in a previous study (Shigemura *et al.*, 2005), we examined only one single particular site (I60T, which was digested by a restriction enzyme, DraIII) (Montmayeur *et al.*, 2001), and no other sites (e.g. five other amino acid changes) of the *Tas1r3* allele in the congenic strain, further studies are needed to determine whether mouse strain differences in Gur-sensitivity may relate to polymorphisms of *Tas1r3* or not.

In the present study, therefore, we examined the effect of Gur on CT responses to sweet compounds in non-taster 129 mice, a strain used previously for various genetic studies of *Tas1r3* and shown to possess the non-taster *Tas1r3* allele (e.g. six amino acid changes: Kitagawa *et al.*, 2001; Nelson *et al.*, 2001; Max *et al.*, 2001; Inoue *et al.*, 2004; Reed *et al.*, 2004). Our results indicated that this non-taster strain clearly showed inhibition by Gur of CT responses to various sweet compounds similar to that observed in C57BL mice having the *Tas1r3* taster genotype. This suggests that mouse strain differences in sensitivity to the sweet-suppressing effect of Gur may not be associated with polymorphisms in *Tas1r3*.

Materials and methods

Subjects

All experimental procedures were approved by the committee for Laboratory Animal Care and Use at Kyushu University, Japan. Subjects were adult male and female 129X1/SvJ (formerly 129/SvJ) mice at 8–25 weeks of age (18–30 g body wt, $n = 12$), originally purchased from The Jackson Laboratory (Bar Harbor, ME). This strain possesses the *Tas1r3* non-taster genotype (Kitagawa *et al.*, 2001; Nelson *et al.*, 2001, 2002). Animals were housed in plastic cages under a 12 h light/12 h dark cycle at 20–22°C and 50–55% relative humidity. Two or three mice were housed together in a cage and received food pellets *ad libitum* (MF; Oriental Yeast, Tokyo, Japan) and tap water.

Recordings of responses from the CT nerve

The procedures of dissection and recording of responses of the CT nerve were the same as those previously reported (Yasumatsu *et al.*, 2003). Briefly, under pentobarbital anesthesia (50–60 mg/kg i.p.; Somnopentyl; Schering-Plough Co., Kenilworth, NJ), the trachea of each animal was cannulated, and the mouse was then fixed in the supine position with a head holder to allow dissection of the CT nerve. The hypoglossal nerve was transected bilaterally to prevent tongue movements. The right CT nerve was exposed at its exit from the lingual nerve by removal of medial pterygoid muscle. The CT nerve was then dissected free from surrounding tissues and cut at the point of its entry to the bulla. For whole-nerve recording, the entire nerve was placed on a silver wire electrode. An indifferent electrode was placed in nearby tissue. Neural responses resulting from chemical stimulations of the tongue were fed into an amplifier (Iyodenshikogaku K-1, Nagoya, Japan), monitored on an oscilloscope and audiomonitor. Whole nerve responses were integrated with a time constant of 1.0 s and recorded a computer for later analysis using PowerLab system (PowerLab/sp4; ADInstruments, Australia).

Chemical stimulations to the tongue

The anterior half of the tongue was enclosed in a flow chamber made of silicone rubber (Ninomiya and Funakoshi, 1981). Solutions were delivered into the chamber by gravity flow, and flowed over the tongue for a controlled period. Solutions used as chemical stimuli were: 0.01–1.0 M sucrose (Suc), 0.5 M maltose, 0.5 M fructose (Fru), 0.5 M glucose (Glu), 0.02 M saccharin Na (Sac), 0.3 M D-alanine (D-Ala), 1.0 M glycine (Gly), 0.1 M NH_4Cl, 0.1 M NaCl, 0.01 M HCl and 0.02 M quinine–HCl (quinine) (Wako Pure Chemicals Industries, Osaka, Japan). These chemicals were dissolved in distilled water and used at ~24°C. During chemical stimulation of the tongue, test solution flowed for ~30 s at the same flow rate as the distilled water used for rinsing the tongue (~0.1 ml/s). The tongue was rinsed with distilled water during the interval of ~1 min between successive stimulations. To examine Gur inhibition of the CT responses, the tongue was treated with 30 μg/ml (~7.1 μM) Gur dissolved in 5 mM phosphate buffer (pH 6.8; made with $Na_2HPO_4.12H_2O$ and $NaH_2PO_4.2H_2O$) for 10 min in the same manner as that described by Ninomiya and Imoto (1995). Taste stimuli were applied first without Gur treatment, then the tongue was treated with Gur, and then the same taste stimuli were applied again. The concentration of Gur (30 μg/ml) was chosen because in C57BL mice the suppressive effect of Gur on sucrose responses in the CT nerve had been shown to be nearly maximal at this dose (Ninomiya and Imoto, 1995; Ninomiya *et al.*, 1997, 1998). Application of the solvent without Gur by itself has no effect on the neural measurement. The stability of the preparation was monitored by periodic application of 0.1 M NH_4Cl. A recording was

considered to be stable when magnitudes of NH_4Cl response at the beginning and end of each stimulation series deviated by no more than 15%. Only responses from stable recordings were used in the data analysis.

Data analysis

In the analysis of whole nerve responses, the magnitude of the integrated responses from 5 to 20 s after stimulus onset were measured and averaged. Relative response magnitude (averaged) for each test stimulus was calculated when the response magnitude to 0.1 M NH_4Cl was taken as unity (1.0), and this value was used for statistical analysis. To evaluate the effect of Gur on taste responses statistically, data were analyzed using a two-way repeated-measures analysis of variance (ANOVA) and Student's *t*-test. All calculations were performed using the statistical software package StatView (Abacus Concepts, Inc., Berkeley, CA).

Results

Figure 1 shows sample recordings of the integrated responses of the CT nerve of a 129 mouse to seven taste stimuli before and after lingual application with Gur. The Gur treatment selectively suppressed responses to sweet compounds to ~50% of control, whereas no such suppression by Gur was observed in responses to 0.1 M NaCl, 0.01 M HCl or 0.02 M quinine. Relative magnitudes of responses to 0.1 M NaCl, 0.01 M HCl and 0.02 M quinine after Gur in 129 mice were ~0.75, 1.20 and 0.70, respectively, which were not significantly different from those before (*t*-test, $P > 0.05$, Figure 3), as previously reported in C57BL and BALB mice (Ninomiya and Imoto, 1995; Ninomiya *et al.*, 1997). As seen in Figure 2, responses to Suc after the Gur treatment was significantly different from that before [ANOVA, $F(1,40) = 8.85$, $P < 0.05$]. Student's *t*-tests indicate significant suppression of sucrose responses at 0.1, 0.3, 0.5 and 1.0 M after Gur ($P < 0.05$). Responses to Suc at these concentrations reduced to ~50% of control [i.e. at 0.5 M: 0.69 ± 0.05 (mean ± SD) before and 0.33 ± 0.04 after Gur, $n = 5$], similar to that previously observed in C57BL mice (i.e. at 0.5 M: 1.01 ± 0.05 before and 0.52 ± 0.18 after Gur, $n = 12$; Ninomiya and Imoto, 1995). Responses to all sweet compounds tested, such as 0.5 M Glu, 0.5 M Mal, 0.5 M Fru, 1.0 M Gly, 0.3 M D-Ala and 0.02 M Sac, were also suppressed to ~50% of control by Gur (*t*-test, $P < 0.05$, Figure 3). These results were consistent with those previously obtained from the *Tas1r3* taster, C57BL strain (Ninomiya and Imoto, 1995). This suggests that the *Tas1r3* non-taster strain, 129 mice, unlike another non-taster strain, BALB mice, clearly show Gur-sensitivity in their CT responses to sweet compounds. Thus, Gur-sensitivity in mice appears not to be associated with the *Tas1r3* genotype.

Discussion

It was known that mouse strains possessing different alleles of *Tas1r3* have different Gur sensitivities (Gur-sensitive/taster C57BL versus Gur-insensitive/non-taster BALB) (Ninomiya and Imoto, 1995; Kitagawa *et al.*, 2001; Montmayeur *et al.*, 2001; Reed *et al.*, 2004). Our previous study have shown that congenic mice have at least one same amino acid change (I60T) of the *Tas1r3* allele as BALB mice, but differ from BALB in Gur sensitivity, suggesting the possibility that Gur sensitivity may not be determined by the *Tas1r3* locus (Shigemura *et al.*, 2005). To further investigate this possibility, the present study examined the Gur sensitivity of another non-taster strain (129) possessing non-taster *Tas1r3* allele (e.g. six amino acid changes, including I60T). Our results indicated that Gur (30 μg/ml)

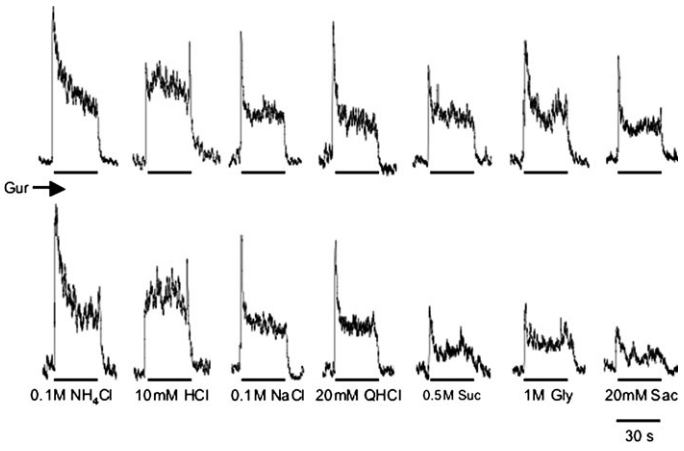

Figure 1 Sample recordings of the integrated responses of the CT nerve to seven taste stimuli before (upper trace) and after (lower trace) lingual treatment with 30 μg/ml gurmarin (Gur) for 10 min in a 129 mouse. Taste stimuli are: 0.1 M NH_4Cl, 10 mM HCl, 0.1 M NaCl, 20 mM quinine–HCl (QHCl), 0.5 M sucrose (Suc), 1.0 M glycine (Gly) and 20 mM saccharin Na (Sac).

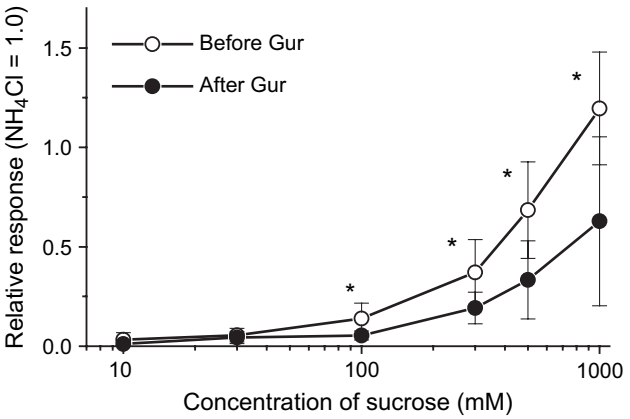

Figure 2 Concentration–response relationships for sucrose before (open circles) and after (filled circles) lingual treatment with 30 μg/ml gurmarin (Gur) for 10 min in 129 mice. Data were obtained from 5–8 mice and expressed as mean values ± SD. *$P < 0.05$ (*t*-test).

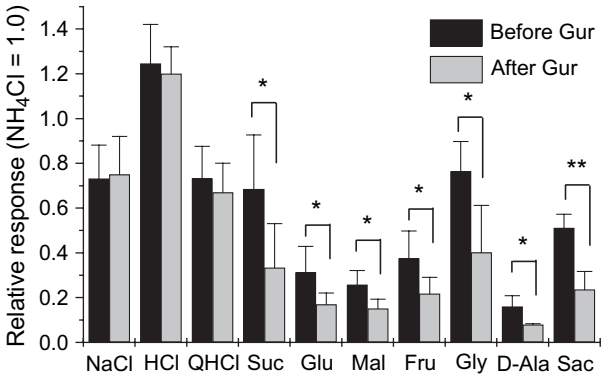

Figure 3 Responses of the CT nerve to seven sweet compounds before (filled columns) and after (gray columns) 30 μg/ml gurmarin (Gur) for 10 min in 129 mice. Taste stimuli are: 0.1 M NaCl, 0.01 M HCl, 0.02 M quinine-HCl (QHCl), 0.5 M sucrose (Suc), 0.5 M glucose (Glu), 0.5 M maltose (Mal), 0.5 M fructose (Fru), 1.0 M glycine (Gly), 0.3 M D-alanine (D-Ala) and 0.02 M saccharin Na (Sac). Data were obtained from 5–12 mice and expressed as mean values ± SD. $*P < 0.05$; $**P < 0.01$ (*t*-test).

suppressed CT responses to sweet compounds in non-taster 129 mice to a similar extent (~50% of control for 0.02 M Sac and 0.1–1.0 M Suc responses) to that found in taster C57BL mice. Since T1R3-knockout mice in the C57BL genetic backgrounds show CT responses to 0.02 M Sac and 0.3 M Suc that are only ~12 and ~20% of those of wild type C57BL mice (Damak *et al.*, 2003), respectively, most of the Gur-sensitive and Gur-insensitive components of the sweet responses in mice are likely to occur through activation of T1R3-containing receptor(s). Taking this into consideration, polymorphic variations in the amino acid sequences of the T1R3 protein (e.g. six amino acid changes, including I60T) in mice may not influence the receptor's interaction with Gur greatly, even though this variation produces clear segregation between taster C57BL and non-taster 129 strains in their sensitivities to sweet compounds (Inoue *et al.*, 2001, 2004; Nelson *et al.*, 2001).

Although the 129 inbred strain is the most widely used strain in gene targeting experiments, numerous substrains exist with differences in their genomic sequences [e.g. simple sequence length polymorphisms (SSLPs)] (Threadgill *et al.*, 1997). In the present study, we used the same 129X1/SvJ strain (formerly 129/SvJ) as used in previous studies (Nelson *et al.*, 2001; Zhao *et al.*, 2003), which differs in 52–54 out of 212 SLPs (~25%) from the other 129 substrains (Threadgill *et al.*, 1997), such as 129P3/J (formerly 129/J) and 129S1/SvImJ or 129T2/SvEmsJ (formerly 129/Sv) used in other taste studies (formerly 129/J: Bachmanov *et al.*, 1997; Li *et al.*, 2001; Inoue *et al.*, 2004; Reed *et al.*, 2004; formerly 129/Sv: Wong *et al.*, 1996; Montmayeur *et al.*, 2001; Damak *et al.*, 2003; He *et al.*, 2004). Our preliminary data indicates that amiloride-sensitivity of NaCl responses differ among 129 substrains. In 129X1/SvJ strain, amiloride suppressed CT responses to 0.1–1.0 M NaCl to ~50% of control similar to C57BL mice (K. Yasumatsu *et al.*, unpublished observation), whereas no such inhibition was evident in 129P3/J mice (Ninomiya *et al.*, 1996; Gannon and Contreras, 1995). Therefore, it is also possible that differences in genetic background may cause differences in the Gur-sensitivity among 129 substrains, although no comparison has been made yet. With regards to CT responses to sweet compounds, 129X1/SvJ mice used in the present study showed similar magnitudes of responses to 1.0 M Suc (~1.1), 0.02 M Sac (~0.6) and 1.0 M Gly (~0.8) as those shown by 129P3/J mice (Inoue *et al.*, 2001). Only responses to 0.5 M Mal appeared slightly larger in 129X1/SvJ (~0.25) than in 129P3/J mice (~0.1; Inoue *et al.*, 2001). Responses to all of these sweet compounds in 129P3/J and 129X1/SvJ mice were smaller than those in C57BL mice (Inoue *et al.*, 2001; Yasumatsu *et al.*, unpublished observation). This similar responses to sweet compounds between two 129 substrains may be due to their same *Tas1r3* non-taster genotype (Bachmanov *et al.*, 2001; Kitagawa *et al.*, 2001; Nelson *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001).

If Gur inhibits CT responses to sweet compounds through its interaction with the T1R2/T1R3 heterodimer, then it is unclear why Gur inhibits CT responses of one *Tas1r3* non-taster (129, this study), but not of another non-taster strain (BALB) (Ninomiya and Imoto, 1995). Furthermore, if T1R2/T1R3 is the site of Gur's action and given that taste buds in BALB, 129 and C57BL mice in the anterior tongue innervated by CT nerve and in the posterior tongue innervated by the IXth nerve express both T1R2 and T1R3 (Kitagawa *et al.*, 2001; Max *et al.*, 2001; Nelson *et al.*, 2001; Kim *et al.*, 2003; Shigemura *et al.*, unpublished observation), it is unclear why Gur inhibits nerve responses to sweet compounds of the CT but not of the IXth nerve (Ninomiya *et al.*, 1997).

It may be that Gur-insensitive components of sweet responses in the CT and IXth nerves in 129 and C57BL mice may be mediated by sweet taste receptors other than T1R3-containing T1R2/T1R3 heterodimers (e.g. T1R2 monomers/homodimers or other hypothetical sweet taste receptors). This may be supported by the fact that T1R3-knockout mice exhibited no significant reduction in Gur-insensitive IXth nerve responses to sucrose, except at a concentration of 1.0 M, and showed diminished, but not abolished, responses to sugars in the Gur-sensitive CT nerve, as mentioned above (Damak *et al.*, 2003); however, CT responses to various sweet compounds were almost abolished in double knockout mice lacking both T1R2 and T1R3 proteins (Zhao *et al.*, 2003).

Gur insensitivity in BALB mice may not relate to the T1R2/T1R3 receptor itself or its components. *dpa*-congenic mice having non-taster BALB genetic backgrounds (including at least one particular site of *Tas1r2* and *Tas1r3)* except for the gene segment (including the *dpa* locus) derived from the donor, taster C57BL strain, showed Gur inhibition of CT responses to sweet compounds (Shigemura *et al.*, 2005).

This observation suggests no major contribution of the *Tas1r3* or *Tas1r2* genotype to the Gur sensitivity in mice. If functional receptors for sweet compounds are limited within possible combinations of monomers and dimers of T1R2 and T1R3, it seems likely that the gene segment including the *dpa* locus in congenic mice may influence the factor involved in downstream signal transduction for the Gur-sensitive response component occurring through the T1R2/T1R3 receptor. To clarify these possibilities, however, future extensive studies are needed.

In summary, the present study examined the effect of Gur in 129 mice carrying *Tas1r3* non-taster genotype. If Gur's site of action is the T1R2/T1R3 sweet receptor, then our results indicate that polymorphic variation in the amino acid sequence of the T1R3 protein does not greatly influence this interaction.

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