Materials and Methods

Generation of transgenic mice

The BAC clone carrying either the mT2R5 or mT1R3 gene was isolated from a mouse (C57BL/6J) BAC library (RPCI23) using the PCR-based screening with primers against the coding region for mT2R5 or mT1R3. The transgene to trace bitter taste neuronal circuitries was generated by connecting 4.8 kb upstream of the starting ATG of mT2R5, mT2R5-GFP, IRES, tWGA-DsRed, and the polyadenylation signal. Briefly, an approximately 14-kb BamHI fragment including mT2R5 was isolated from the BAC clone, and sequenced. This fragment also contains the full coding sequences for mT2R14 1.7 kb upstream of mT2R5, and mT2R4 0.8 kb downstream of mT2R5. From the fragment, a 4-kb XbaI-EcoRI fragment starting from 0.6 kb upstream of mT2R5 was excised and subcloned into pBS-KS. A PacI restriction enzyme site was created just 5’ to the stop codon of the mT2R5 gene to fuse with GFP, using recombinant PCR. On the other hand, the plasmid ETLPa/-LTNL (S1) was modified to generate a GFP/IRES/tWGA-DsRed construct. A PacI-EcoRI fragment of PCR-amplified EGFP was inserted at the 5’ end of the IRES sequence in ETLPa/-LTNL. The tau-lacZ portion of ETLPa/-LTNL was replaced with the BspHI-XbaI fragment of the fusion construct of truncated WGA (S2) and DsRed1 (tWGA-DsRed), which was PCR-amplified by the sense and antisense primers overlapping the two cDNA, and the flanking primers incorporating the compatible BspHI site at the 5’ end of tWGA, and the XbaI site downstream of DsRed1, while the NotI site immediately after the stop codon of DsRed1 in pDsRed1-N1 was included. Then, the XbaI-PacI fragment including mT2R5, the PacI-NotI fragment of GFP/IRES/tWGA-DsRed, and the NotI-XhoI fragment of the PCR-amplified SV40 polyadenylation signal were ligated in pBS-KS. Finally, the upstream region of mT2R5, starting from the BamHI site, was connected as depicted in Fig. 1A. The transgene to trace sweet-umami taste neuronal circuitries was generated by connecting about 12 kb upstream of the starting ATG of mT1R3, mT1R3-GFP, IRES, tWGA-DsRed, and the polyadenylation signal. An approximately 16-kb EcoRI fragment including the six coding exons of mT1R3 was isolated from the BAC clone. A PacI restriction enzyme site was created just 5’ to the stop codon of the mT1R3 gene using recombinant PCR. The EcoRI-PacI fragment including the full sequences of mT1R3 and about 12 kb upstream of the starting ATG, the PacI-NotI fragment of GFP/IRES/tWGA-DsRed, and the PCR-amplified polyadenylation signal were ligated to construct the transgene. Transgenic mice were generated by pronuclear injection of oocytes (BDF1XC57BL/6). The manipulated oocytes were transferred to the oviducts of ICR pseudopregnant recipients. Integration of the transgenes was screened by PCR. For each construct, F1 offspring from three independent founder animals, crossed to C57BL/6, were analyzed and shown to have identical patterns of transgene expression. This study was approved by the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University. The animals
were treated in accordance with the "Guide for the care and use of laboratory animals" (S3), and the "Guide for animal experiments" (S4).

**Direct fluorescence detection**

The expression patterns of the transgenes were examined in coronal cryostat sections, obtained by the film-transfer method, which enabled us to cut the samples including the soft tissues and the hard calcified tissues, as previously reported (S5). In brief, mice (3 to 12 months old) were anesthetized with sodium pentobarbital. The mouse head was isolated, and rapidly frozen in 5% carboxymethyl cellulose (CMC). The frozen CMC block was sectioned from the posterior part of the head using a tungsten carbide blade attached to the cryomicrotome at −25°C. During sectioning, each section surface was covered with a polyvinylidene chloride film (10 µm thick) coated with synthetic rubber cement, and then the sample was cut at a speed not exceeding 4 mm/s. The coronal sections (30 µm) were observed under either a confocal laser microscope or an inverted epifluorescence microscope. The distribution of mT2R5-GFP, mT1R3-GFP, tWGA-DsRed was directly visualized by the GFP and DsRed fluorescence, excited using either laser lines (488, 543, or 633nm) or a Hg-lamp, and collected using the recommended band-pass filter sets.

**In situ hybridization**

Mice were anesthetized by the intraperitoneal injection of sodium pentobarbital (70 µg/g). Mice were perfused with 3.7% paraformaldehyde, and the tongue was isolated, fixed in 3.7% paraformaldehyde for an additional 3 h, soaked in 18% sucrose, and then frozen in 5% CMC. The cryostat sections (20 µm), obtained by the film-transfer method (S5), were hybridized to the DIG-labeled oligo DNA probe against the endogeneous mT2R5 3'-UTR (TTCAGACGCATGTGACCCTGAGAGCTTTTC) and the fluorescein-labeled oligo DNA probe against GFP (GGTGAACAGCTCCTCGCCCTTGCTCACCAT). For double-label fluorescent detection, the cellular distribution of the DIG-labeled probe was determined by TSA detection with the HRP-conjugated anti-DIG antibody and Alexa Fluor 546-labeled tyramides, followed by peroxidase quenching (2% H₂O₂), and further by the second round of TSA detection for the fluorescein-labeled probe using the HRP-conjugated anti-fluorescein antibody and Alexa Fluor 488-labeled tyramides. By confocal microscopy, 0.5-µm optical sections were recorded to ensure that any overlapping signal originated from single cells.

**Immunohistochemistry**

In each mouse, approximately 400 serial coronal sections (30 µm), including the whole brain, were thoroughly obtained from the posterior part of the head using the film-transfer method (S5). The samples were fixed and permeabilized with 3.7% formaldehyde solution (1x PBS, 3.7% formaldehyde, 0.18% Triton X-100) for 10 min. Nonspecific binding sites were blocked with 1% BSA for 30 min. Sections were treated with anti-WGA antibody and then the AP-conjugated secondary antibody. BM purple was used as a substrate for AP to detect tWGA-DsRed distribution. The brain structures were identified microscopically by reference to a mouse brain atlas (S6). The anterior-posterior lengths of brain structures and the locations of the tWGA-DsRed-labeled neurons were calculated from the number of sections multiplied by 30 µm. For each coronal section, the distance to the posterior end of the fasciculus retroflexus (pfr) was calculated and denoted in Figures, while referring to the anterior part as plus (+), and the posterior part as minus (−). Three-dimensional images of
the brain were reconstructed by assembling the brain structures extracted from the serial coronal section images, using the TRI/3D-SRF2 software.

**Anatomical abbreviations used**

2Cb: 2nd cerebellar lobule, 4V: 4th ventricle, 7N: facial nucleus, ACo: anterior cortical amygdaloid nucleus, AHi: amygdalohippocampal transition area, AHiPM: amygdalohippocampal transition area, posteromedial, AI: agranular insular cortex, AP: area postrema, APir: amygdalopiriform transition area, BMA: basomedial amygdaloid nucleus, anterior, BMP: basomedial amygdaloid nucleus, posterior, BLA: basolateral amygdaloid nucleus, anterior, BLB: basolateral amygdaloid nucleus, posterior, CB: cranial bone, CeA: central amygdaloid nucleus, CL: centrolateral thalamic nucleus, CM: central medial thalamic nucleus, Cu: cuneate nucleus, CxA: cortex-amygdala transition zone, DEn: dorsal endopiriform nucleus, DG: dentate gyrus, DI: dysgranular insular cortex, ec: external capsule, DM: dorsomedial hypothalamic nucleus, Ect: ectorhinal cortex, ELPB: external lateral parabrachial nucleus, EMPB: external medial parabrachial nucleus, Ent: entorhinal cortex, fr: fasciculus retroflexus, GI: granular insular cortex, Gi: giantocellular reticular nucleus, GiA: giantocellular reticular nucleus, alpha, Gus: gustatory thalamic nucleus, ic: internal capsule, ICj: islands of Calleja, IRt: intermediate reticular nucleus, IS: inferior salivatory nucleus, KF: Kolliker-Fuse nucleus, La: lateral amygdaloid nucleus, LEnt: lateral entorhinal cortex, LH: lateral hypothalamic area, LPBC: lateral parabrachial nucleus, central, LPBD: lateral parabrachial nucleus, dorsal, mcp: middle cerebellar peduncle, MeA: medial amygdaloid nucleus, anterior, MeAV: medial amygdaloid nucleus, anteromedial part of the trigeminal ganglion were restrictedly labeled by tWGA-DsRed in mT2R5-WGA mice (Fig. S1B), implying that the tWGA-DsRed protein was transferred through the trigeminal ethmoidal nerve from the solitary chemoreceptor cells. However, few labeled neurons were detected in the trigeminal brainstem nuclei in mT2R5-WGA mice although neurons in these areas might contain only a small amount of

**Supporting Online Text**

*Selectivity of tWGA-DsRed transport*

In mT2R5-WGA mice, mT2R5-GFP and tWGA-DsRed were substantially coexpressed in subsets of taste receptor cells located in foliate, circumvallate, and Geschmackstreifen taste buds. mT2R5-GFP and tWGA-DsRed were also coexpressed in small subsets of cells in the nasal epithelium lining the anterior nasal cavity (Fig. S1A), which appear to be solitary chemoreceptor cells as reported to express the T2Rs, but not T1R3 (S7). Subsets of neurons in the anteromedial part of the trigeminal ganglion were restrictedly labeled by tWGA-DsRed in mT2R5-WGA mice (Fig. S1B), implying that the tWGA-DsRed protein was transferred through the trigeminal ethmoidal nerve from the solitary chemoreceptor cells. However, few labeled neurons were detected in the trigeminal brainstem nuclei in mT2R5-WGA mice although neurons in these areas might contain only a small amount of
tracer that is not detected. Instead, tWGA-DsRed was detected in the Kolliker-Fuse nucleus (KF) (Fig. S1C), where direct ethmoidal projections were observed (S8). In sharp contrast, neither mT1R3-GFP nor tWGA-DsRed was expressed in the nasal epithelium in mT1R3-WGA mice. In addition, tWGA-DsRed fluorescence was not detected in the trigeminal ganglion and the trigeminal brainstem nuclei in mT1R3-WGA mice although trigeminal fibers invade fungiform taste buds. Therefore, these results suggest that the tracer transgene, tWGA-DsRed, selectively expressed in taste receptor cells may specifically trace taste neuronal circuitries.

Characteristics of tWGA-DsRed transport and tWGA-DsRed detection

In contrast with active tracers, such as viruses that are self-replicating, the passive tracers get diminished rapidly. However, when tWGA-DsRed, a passive tracer, is expressed in taste receptor cells, tWGA-DsRed is transported through at least 4 synapses to reach the gustatory cortex. The long survival times of the animals might provide the necessary amount of signal to trace the taste neuronal circuitry across the multiple synapses with the aid of the turnover of taste receptor cells.

Most of the tWGA-DsRed–labeled neurons were detected concordantly by direct fluorescence detection of DsRed and by immunohistochemical detection of WGA. However, direct fluorescence detection of DsRed revealed that subsets of neurons in the inferior salivatory nucleus (IS), the facial nucleus (7N), the pontine reticular nucleus (PnC), and KF were slightly labeled by tWGA-DsRed, which was not deduced by immunohistochemical detection of WGA (Fig. S1C and Fig. S3, A-C). It may derive from a small amount of tracer in those neurons, and differences in the signal/noise ratio between direct fluorescence detection and immunohistochemical detection. In comparison with direct fluorescence detection, the immunohistochemical detection enables us to permanently observe the tWGA-DsRed–labeled neuron in the brain, and to detect the labeled neurons in the cortex and the amygdala at the higher signal/noise ratio.

Differences in the tWGA-DsRed–labeled patterns between mT2R5-WGA and mT1R3-WGA mice

To investigate whether there are any differences in the tWGA-DsRed-labeled patterns of the two strains, WGA immunoreactivity was detected in the serial coronal sections collected throughout the brain. Fig. S3A shows the locations of tWGA-DsRed–labeled neurons in the whole brain. tWGA-DsRed–labeled neurons (1–12 cells) were clustered in the spotted regions (Fig. 3, B and C). Our data show the small size of the gustatory relays, which might explain the difficulty and uncertainty in deciphering their exact dimensions (S9, S10). Comparison of the positions of the tWGA-DsRed–labeled neurons in mT2R5-WGA and mT1R3-WGA mice revealed the segregation of inputs into the Sol, PB, and Gus. In those regions, the tWGA-DsRed–labeled neurons in mT2R5-WGA mice, which represent bitter neuronal circuitries, were located in the more posterior part than the labeled neurons in mT1R3-WGA mice, which represent sweet-umami taste relays (Fig. 4 and Fig. S3, A and D-I). One exception was that the tWGA-DsRed–labeled neurons in mT2R5-WGA mice were located rostrally and laterally within the external medial (EMPB) and external lateral (ELPB) subdivisions of the PB, where few inputs from T1R3 were detected (Fig. 1E and Fig. S3). In the amygdala and the gustatory cortex, the dispersed areas of labeled neurons in two strains appeared to partly overlap along the anterior-posterior axis (Fig. 4 and Fig. S3). It is not clear whether inputs from mT2R5 and
mT1R3 overlap at a single neuron level. Although the two partially overlap in the
dysgranular insular cortex (DI), the dispersed area of labeled neurons in mT2R5-WGA
mice was located in the more posterior part than that in mT1R3-WGA mice (Fig. 4 and Fig.
S3, A, D-F, and I), consistent with previous electrophysiological data showing that the
best-stimulus characteristics of cortical gustatory neurons were ordered with sweet-inputs
rostral, and bitter-inputs caudal (SI0). In both mT2R5-WGA and mT1R3-WGA mice,
densely labeled neurons were dispersed in the broad area of the amygdala, which plays an
indispensable role in emotional states and the processing of taste aversion learning
(SI1–SI3), providing the organized patterns of inputs from mT2R5 and mT1R3 (Fig. 3,
Fig. 4 and Fig. S3). Although previous reports indicate that the central nucleus of amygdala
(CeA) receives taste information (SI4, SI5), the CeA was devoid of densely labeled
neurons in both strains. Instead, the labeled neurons were condensed into subsets of
neurons in the basomedial and basolateral amygdalar nuclei (BMP, BMA, BLP, BLA) of
the mT2R5-WGA mice, and into subsets of neurons in the basolateral amygdalar nuclei
(BLP, BLA) of the mT1R3-WGA mice (Fig. S3A). Clusters of tWGA-DsRed–labeled
neurons were ventrally located in the superficial amygdalar nuclei (PMCo, PLCo, APir,
ACo), which receive direct projections from the olfactory bulb, and were ordered
conversely with mT2R5-inputs rostral, and mT1R3-inputs caudal, while the regions
receiving inputs from mT2R5 and mT1R3 partially overlapping. The amygdala (CeA,
BLP, BMA, ACo) receives ascending inputs from the pontine parabrachial nucleus to
process visceral and gustatory information although the precise input patterns for both the
visceral and the gustatory remained elusive (SI4). The amygdaloid output nuclei,
especially the CeA, receive convergent information from several other amygdaloid regions
through the highly organized intra-amygdaloid circuitries, and generate behavioral
responses that reflect the sum of neuronal activities of amygdaloid nuclei (SI3). The lack
of densely labeled neurons in the CeA implies that the gustatory neurons in the pontine
parabrachial nuclei may directly project axons to the specific amygdaloid regions
including BLP, BLA, BMP, BMA, ACo, PMCo, PLCo, and APir, some of which might
send taste information to the CeA; however, tWGA-DsRed might not be transferred to the
CeA. In the medial amygdalar nuclei (MePV, MeA, MeAV), inputs from both mT2R5 and
mT1R3 were observed, and organized with bitter-inputs caudal, and sweet-inputs rostral.
In both mT2R5-WGA and mT1R3-WGA mice, tWGA-DsRed was also transported to the
clusters of neurons in the posterior amygdaloid nuclei corresponding to the
amygdalo-hippocampal transition areas (AHi) (Fig. S3A), which project to the
hypothalamus and the hippocampal sectors, CA3 and CA2 (SI6, SI7).

Few densely labeled neurons were detected in the lateral hypothalamus (LH), which has
been reported to receive taste information (S9, SI5). Perhaps there were
tWGA-DsRed–containing neurons in these areas that were not detected, because they
contained only a small amount of tracer. Another possibility is that the LH receives
gustatory input from the parabrachial nuclei through polysynaptic connections, or that
there are anatomical and functional differences between the axonal systems of the
parabrachial taste neurons projecting to the LH and the other regions such as the amygdala
and the thalamus. In both mT2R5-WGA and mT1R3-WGA mice, the olfactory cortex (Pir,
Ent) was studded with clusters of tWGA-DsRed–labeled neurons (Fig. 3, B and C; Fig.
S3A), implying the interdependent modification between the recognition of taste and
smell. tWGA-DsRed might be anterogradely or retrogradely transported from the pontine
nuclei to Pir and Ent, as suggested by the existence of functional neural circuitries between them (S18, S19). Small subsets of neurons which received tWGA-DsRed from bitter- and sweet-umami–responsive taste cells clustered in the narrow regions of the somatosensory cortex (S1ULp, S1J) in mT2R5-WGA and mT1R3-WGA mice (Fig. 3, B and C; Fig. S3A), implying that those regions also receive taste information.

**Figure legends**

**Fig. S1.** Spatial distribution of mT2R5-GFP and tWGA-DsRed in the mT2R5-WGA mouse strain, additionally detected in the identical mouse representing Fig. 1E. (A) The distribution of mT2R5-GFP– and tWGA-DsRed–expressing cells in the nasal epithelium lining the medial (septum) wall of the anterior nasal cavity in the mT2R5-WGA mouse. (B) tWGA-DsRed fluorescence detected in the anteromedial part of the trigeminal ganglion (bottom). Dark-field images at the same magnification (middle) and at the lower magnification (top) were also shown. (C) Spatial distribution of tWGA-DsRed in coronal sections of the mT2R5-WGA mouse brain, clarified by direct fluorescence detection (left). Dark-field images at the same magnification (middle) and at the lower magnification (right) were also shown. The distance to the posterior end of the fasciculus retroflexus (pfr) was calculated and denoted in each section.

**Fig. S2.** Spatial distribution of tWGA-DsRed in coronal sections of the mT1R3-WGA mouse brain, clarified by direct fluorescence detection (left). Dark-field images at the same magnification (middle) and at the lower magnification (right) were also shown. The distance to pfr was calculated and denoted in each section.

**Fig. S3.** Comparison of the positions of tWGA-DsRed–labeled neurons in the mT2R5-WGA and mT1R3-WGA mouse brains, revealed by immunohistochemical detection of WGA. (A) Locations of tWGA-DsRed–labeled neurons in serial coronal sections (30 µm), thoroughly obtained from the posterior part of the brain of either the mT2R5-WGA or mT1R3-WGA mouse. Dark-field images of serial coronal sections were paneled from the posterior to the anterior, every other section. The positions of tWGA-DsRed–labeled neuronal clusters detected by WGA immunoreactivity were plotted using green and red dots for mT2R5-WGA and mT1R3-WGA mice, respectively, and were numbered. For each coronal section, the distance to pfr was calculated and denoted. Shown are the data obtained from single representative mice of 7 mT2R5-WGA mice and 5 mT1R3-WGA mice analyzed. (B) Larger magnification micrographs of the numbered positions of tWGA-DsRed–labeled neuronal clusters detected by WGA immunoreactivity, which were plotted using green dots for the mT2R5-WGA mouse. (C) Larger magnification micrographs of the numbered positions of tWGA-DsRed–labeled neuronal clusters detected by WGA immunoreactivity, which were plotted using red dots for the mT1R3-WGA mouse. (D-I) Three-dimensional images of the brain reconstructed by assembling the brain structures extracted from the serial coronal section images shown in (A). Three-dimensional images of the brain, viewed from the anterior tip (0º), were rotated at the angles indicated. (D) Three-dimensional images of the whole brain. (E) Three-dimensional images of the left hemisphere of the brain. (F) Three-dimensional images of the right hemisphere of the brain. (G) Three-dimensional images of the posterior part of the brain (the distance to pfr: from –6.66 mm to –2.94 mm). (H) Three-dimensional images of the middle part of the brain (the distance to pfr: from –2.94 mm to +1.26 mm). (I)
Three-dimensional images of the anterior part of the brain (the distance to pfr: from +1.26 mm to +5.04 mm).
References and Notes
S4. "Guide for animal experiments" (Hiroshima University, Hiroshima, Japan, 2004).
### Figure S1

#### A

- **DIC**
- **mT2R5-GFP**
- **tWGA-DsRed**
- **mT2R5-GFP tWGA-DsRed**

#### B

- **MePV** 0.54mm
- **tWGA-DsRed**

#### C

|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|--------|--------|--------|--------|--------|--------|--------|

- **DsRed fluorescence for tWGA-DsRed**
- **DsRed fluorescence for tWGA-DsRed**
Figure S2

1. Sol
2. Sol
3. MPB
4. LPB
5. Gus
6. Gus
7. DI
8. DI

DsRed fluorescence for tWGA-DsRed
Figure S3
A (part 1)
Figure S3

S19

mT1R3-WGA mice

7. LPBD  8. LPBC  9. MPB
10. MPB  11. LEnt  12. LEnt
13. LEnt  14. LEnt  15. APir
16. Ect  17. DG  18. AHiPM
19. Apir, PMCo  20. LEnt  21. PMCo
22. BLP  23. Pir  24. Gus
28. BLV  29. Pir  30. DEn
31. CL  32. BLA  33. Pir
34. DM  35. Ect  36. Ect
37. MeA  38. MeAV  39. BLA
40. Pir  41. ACo  42. DI
43. DI  44. DI  45. S1d
46. ICj  47. DI  48. DI
Figure S3

D  the whole brain

E  the left hemisphere of the brain

F  the right hemisphere of the brain

z: mT2R5-WGA mice
z: mT1R3-WGA mice
Figure S3

G

the posterior part of the brain

0° 20° 40° 60° 80° 100°

120° 140° 160° 180° 200° 220°

240° 260° 280° 300° 320° 340°

H

the middle part of the brain

0° 20° 40° 60° 80° 100°

120° 140° 160° 180° 200° 220°

240° 260° 280° 300° 320° 340°

I

the anterior part of the brain

0° 20° 40° 60° 80° 100°

120° 140° 160° 180° 200° 220°

240° 260° 280° 300° 320° 340°

○: mT2R5-WGA mice
●: mT1R3-WGA mice