Supplementary Material for

Functional divergence of bitter taste receptors in a nectar-feeding bird

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Supplementary Methods:

Functional assays:

All of the *Tas2r* coding sequences for our functional assays were codon-optimized and synthesized. The coding sequences were inserted into the expression vector pCDNA3.1(+), with the first 45 amino acid residues of the rat somatostatin receptor 3 as the signal peptide at the 5'-end of the Tas2r receptors.

The bitter taste chemicals tested in our study included 13 natural and 11 synthetic compounds. Most of the naturally occurring compounds could be isolated from nectars, such as amygdalin, arbutin, caffeine, camphor, taurine and thiamine (Haydak et al. 1942; Lynn 2001; London-Shafir et al. 2003; Jerković and Kuś 2014; Nepi 2014); Some other bitter compounds were synthesized or distributed in plant tissues other than pollen, such as colchicine, salicin, picrotoxinin and yohimbine (Bader et al. 1954; Julkunen-Tiitto 1989; Finkelstein et al. 2010; Gössinger 2010). Additionally, chloramphenicol is a kind of bacteriostatic antibiotic isolated from soil bacterium (Fernandez-Martinez et al. 2014). Detailed information on the chemicals used in this study is presented in supplementary table S1. Our cell-based functional assays were carried out as previously described (Lei et al. 2015; Jiao et al. 2018). The concentrations of bitter compounds were followed according to a previous study (Meyerhof et al. 2010). Calcium mobilization was expressed as the percentage of fluorescence changes (ΔF) relative to the baseline (F); ΔF was quantified as the peak fluorescence minus the baseline. All experiments were run in triplicate.

Literature cited:

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Table S1. Information about 24 bitter compounds and the maximum concentrations used in our assays.

Compounds	Manufacturer	Catalog	(mM)	Source
Acesulfame K	Sigma	4054	10	Synthetic
Amygdalin	Sigma	A6005	30	Occurs naturally in almond nectar, distributed in Asia, Africa, Europe and America
Arbutin	Sigma	A4256	30	Isolated from the nectar of *Arbutus unedo* (Ericaceae), which has a wide distribution
Caffeine	Sigma	C0750	0.3	Occurs naturally in nectar of *Coffea* and *Citrus* species, in Central and South America
Camphor	Sigma	148075	1	Occurs naturally in floral components from *Achillea millefolium*.
Chloramphenicol	Sigma	C0378	1	Chloramphenicol is an antibiotic isolated from *Streptomyces venezuelae*
Chloroquine diphosphate salt	Sigma	C6628	10	Synthetic
Chlorpheniramine maleate	Sigma	C3025	0.1	Synthetic
Colchicine	Sigma	C3915	3	Extracted from two flowering plant: *Colchicum autumnale* and *Gloriosa superba*
Cycloheximide	Sigma	1810	1	Synthetic
Denatonium benzoate	Sigma	D5765	10	Synthetic
Methimazole	Sigma	M8506	5	Synthetic
6-n-propylthiouracil	Sigma	P3755	1	Synthetic
Papaverine hydrochloride	Sigma	P3510	0.01	Mainly naturally exists in the mature capsule shell of *Papaver somniferum*
Phenanthroline	Sigma	131377	1	Synthetic
Phenylthiocarbamide	Sigma	P7629	1	Synthetic
Picrotoxinin	Sigma	P8390	1	Isolated from *Menispermum cocculus* in India and Southeast;
Quinine	Sigma	Q1125	0.01	First isolated from the bark of *cinchona* tree, which are native to western Sourth America.
Ranitidine hydrochloride	Sigma	R101	10	Synthetic
D-Salicin	Sigma	S0625	3	A glucoside for the whole genus of *Salix*, usually exists in flower bud and leaf bud
Sodium thiocyanate	Sigma	S7757	3	Synthetic
Taurine	Sigma	T0625	1	A kind of non-protein amino acid, which exists in animal tissues abundantly, and also found in some insects and nectar

Thiamine	Sigma	T4625	1	Also called vitamin B1, naturally exists in nectar of many native American flowers
Yohimbine	Sigma	Y3125	0.15	An indole alkaloid mainly from African yohimbe tree, but is also found in some other plants, such as *Rauvolfia tetraphylla*, which is native to America

Table S2. Selective pressure analysis of *Tas2r1* genes in the Anna's hummingbird using the improved branch-site model.

Models	np	[a]ω_0	[a]ω_1	[a]ω_2	ln$L$[b]	2Δ(ln L)[c]	*P*-value[d]	Positively selected sites[e]
Data set: *Tas2r1* genes								
Null Model	125	0.200 (50.5%)	1 (49.5%)		-21945.60	23.079	1.55E-06	
Alternative Model	126	0.201 (46.9%)	1 (47.4%)	6.226 (5.7%)	-21934.06			**92I (0.979), 181L (0.912),** 262S (0.819), **266S (0.983), 267L (0.906), 271K (0.845), 274M (0.923)**

[a] The ω values of each site class (ω_0, ω_1 and ω_2) are shown as percentages in parentheses.

[b] The natural logarithm of the likelihood value.

[c] Twice the difference in ln L between the two models compared.

[d] p values were generated by comparing the two models with a chi-square test.

[e] Positively selected sites with the posterior probabilities >0.8 were listed based on bayes empirical bayes (BEB) analysis. Sites in bold were also detected by site models (see the table S3 below)

Table S3. Analysis of positively selected sites of the five hummingbird *Tas2r1* genes using site models.

Site models	np[a]	Ln *L*[b]	Model compared	*p* value[c]	Positively selected sites[d]
M1a	10	-3410.822	M1a vs M2a	3.40E-13	**91K (0.984), 92I (0.986), 94I (0.962), 96S (0.975), 154V (0.971),**
M2a	12	-3382.112			**181L (0.993), 266S (1.000), 267L (0.961), 271K (0.985), 274M (0.989).**
M8a	11	-3410.823	M8a vs M8	6.84E-14	**91K (0.992), 92I (0.993), 94I (0.982), 96S (0.985), 154V (0.985),** 160I (0.967), 162I (0,971),
M8	12	-3382.765			**181L (0.992)**, 187L (0.974), **266S (0.999), 267L (0.984), 271K (0.993), 274M (0.992)**

[a] Numbers of parameters.

[b] The natural logarithm of the likelihood value.

[c] P values were generated by comparing the two models with a chi-square test.

[d] Positively selected sites with the posterior probabilities >0.95 were listed based on bayes empirical bayes (BEB) analysis. Sites in bold were identified by both site models M2a and M8.

Table S4. Response profiles of the nine bird Tas2r1 receptors stimulated by 24 bitter compounds

Bitter Compounds	CalAnn_ Tas2r1a	CalAnn_ Tas2r1b	CalAnn_ Tas2r1c	CalAnn_ Tas2r1d	CalAnn_ Tas2r1e	ChaPel_ Tas2r1a	ChaPel_ Tas2r1b	ChaPel_ Tas2r1c	CapCar_ Tas2r1
Acesulfame K	n	n	n	n	n	n	n	n	n
Amygdalin	n	n	n	n	n	n	n	n	n
Arbutin	n	n	n	n	n	n	n	n	n
Caffeine	n	n	n	n	n	n	n	n	n
Camphor	Y	Y	Y	Y	n	Y	Y	Y	Y
Chloramphenicol	n	n	n	n	n	n	n	n	n
Chloroquine diphosphate salt	n	n	n	n	n	n	n	n	Y
Chlorpheniramine maleate	Y	n	n	n	n	n	n	n	Y
Colchicine	Y	n	n	n	n	n	n	n	Y
Cycloheximide	n	n	n	n	n	n	n	n	n
Denatonium benzoate	Y	n	n	Y	n	n	n	n	n
Methimazole	n	n	n	n	n	n	n	n	n
6-n-propylthiouracil	n	n	n	n	n	n	n	n	n
Papaverine hydrochloride	n	n	n	n	n	n	n	n	n
Phenanthroline	n	n	n	n	n	n	n	n	n
Phenylthiocarbamide	n	n	n	n	n	n	n	n	n
Picrotoxinin	Y	Y	Y	Y	Y	Y	Y	Y	Y
Quinine	n	n	n	n	n	n	n	n	n
Ranitidine hydrochloride	n	n	n	n	n	n	n	n	n
D-Salicin	n	n	n	n	n	n	n	n	n
Sodium thiocyanate	n	n	n	n	n	n	n	n	n
Taurine	n	n	n	n	n	n	n	n	n
Thiamine	n	n	n	n	n	n	n	n	n

Yohimbine	Y	n	n	n	Y	n	n	n	n

Y = Has response, n = Has no response; CalAnn = Anna's hummingbird, ChaPel = chimney swift, CapCar = chuck-will's widow

Data set S1. Tas2r gene sequences used in our cell-based functional assays. We renamed their gene names and provided their previous names in Wang and Zhao 2015 GBE.

>*CalAnn_Tas2r1a* (previously Anna's Hummingbird_Tas2r1)
ATGGAAGCTTGCTCCTCTCAAGGGAAATTTAATGTCACCACGTACAATGCCGTGGCAAT
GGCCATCATCACCCTCCAGACATTTGCTGGCATGTGGATAAATGCTTTCATTGTTTCTGT
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CTCCTGGGATGCTCCCGGGTTGGGTACTTGTGCACTGCATGGGTAGGTTCCTTTATTGA
AAATATTTACTCCTTTTGCGTGTATGTTCACCAGAAAATCCAAATAATTTCAGCTCTTCT
AAGCTTTTTCAACATTTTCAGCCTATGGATCTCTGCCATTCTTTCTGTGTTTTACTGCATC
AAAATTGCAAATTTCCAGCACACCTTCTTCATCTACCTGAAAGTGAGAATTGACAGGAT
CGTGCCATGGCTGATGCTGACTTCAGTCCCTGTATCCCTTGGTGTCAGTGTCTTTGTCTA
CATTGTCATAGATGAAGCACGCTGTGACAACAACAATTGCACCACCTCAGGATATTTGT
GGCATCTGACTGTCAGACCTGAGCTACATTTTTTCCCTCTTTATTTTTTCACTGGTTTTG
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TGAAACATAA

>*CalAnn_Tas2r1b* (previously Anna's Hummingbird_Tas2r6)
ATGGAAGCTTGCTCCTCTCAAGGGAAATTTAATGTCACCACGTACAATGCCATGTCAAT
GGCCATCATCACCCTCCAGACATTTGCTGGCATGTGGATAAATGCTTTCATTGTTTCTGT
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AG

>*CalAnn_Tas2r1c* (previously Anna's Hummingbird_Tas2r3)
ATGGAAGCTTGCACCTCTCAAGGGAAATTTAATGTCACCACGTACAATACTGTGTCAAT
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TCCTGGGATGCTCCCGATTTTGTTATTTGTTCATCACCTGGGTATTTTTCCTTTTTGAATA
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AG

>*CalAnn_Tas2r1d* (previously Anna's Hummingbird_Tas2r5)
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AGACACAAACACAAGATGCAGACAGAATCAGGGAAGAACGTCAGTGTGGATGCCCAT
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TAG

>*CalAnn_Tas2r1e* (previously Anna's Hummingbird_Tas2r2)
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AACTGGAAAAGATATTGCTAAGGACCCTGTCCTGTGCAAAGTGCAAGATCTGCAAGAA
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>*CalAnn_Tas2r3* (previously Anna's Hummingbird_Tas2r4)
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CCAGCATCTTAGGTGCCATTTGAAAATATGA

>*ChaPel_Tas2r1a* (previously Chimney Swift_Tas2r1)
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AACTGGAAAAGACACTGCTTAGGACCCTGCCCTGTGTGAAGTGCAAGATTTGCATGGG
GCAGGAAATGTCATAA

>*ChaPel_Tas2r1b* (previously Chimney Swift_Tas2r3)
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CAGCTTTTCAAACTTCTCCAACCTGTGGGTTTCTGCCTTCCTTTCTGTTTTTTACTGTAT
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TCTGGAAACTGAATATCAAACTTTATCAGCATTTTTTACCTTTTTATTTTATCACTGGATT
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AAACTGAAAAAGACACTGCTGACCCTGTCCTGTGTGAAGTGCAAGATTTGCATGAGG
CAGGAAATGTCATAA

>*ChaPel_Tas2r1c* (previously Chimney Swift_Tas2r2)
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AAAATTGCAAATTTCCAGCACAGCTTCTTCATCCACCTGAAAGTGAAAATTGACAGGA
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CGCATATGCCATTGCATTCACAGCAGTCATCTCTTCTGCCCTTCTCCTCCTCTTTTCTCTC
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CACATCAAAGCTATGAAATCTATTTTGTCCTTTCTCTTCCTGTACATAATTAACTTTATAG
GCTTGGTTTTCAATCTGATTTATTTGACAAAGAAGGGAAATGCTTTGTCGCTTCTCACT
GTAGTATTACAGGATACTTCTCCAACTGTTTATTGCCTCATCCTGATTTTCAGCAATCCC
AAACTCAAAAAGGCACTGCTTAGGACCCTGTCCTGTGTGAAGTGCAAGATTTGCATGA

GGCAGGAAATGTCATAA

>*ChaPel_Tas2r3* (previously Chimney Swift_Tas2r4)
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CAGCATCTTAAGTGCCATTTGGAAAACAAGGCTCTATGCCTCCTGTTATAG

>*CapCar_Tas2r1* (previously Chuck-will's widow_Tas2r2)
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AATGCAAGATGCAAACAAACTCAGAGAAGACCCTCAGCATGGACGTTAACATCAAAG
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>*CapCar_Tas2r2* (previously Chuck-will's widow_Tas2r1)
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GACACTCTATGATCTTAATCTGGAGCAACCCCAAATTTCGAGCTAGGATTTTGCACTAC
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>*CapCar_Tas2r3* (previously Chuck-will’s widow_Tas2r3)
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ACCGTACTGATGCACATTCATAGTCTCTACTTTACAGATGTGCTTAAGTTGGCTTCCGTG
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TGATCCTATACCTCTCCAGTTTTGTAGCTCAAATTCTGTTGATAATGTCGACCTCTCAAA
GTAAAGACGATGTGAAAGTTGCAGTATCCTTAGTTGTATCTGGGGCATATCCTTCTATAC
ACTCTATTATCCTGATCATAGTCAATTCAAAACTGAAATTGGCATTCAGGTTGCTTTGTC
AGCATCTTAAGCACCATTTGGAAAATATGACTCTGTGTCTTATAGCTTAA

Supplementary figure captions

Figure S1. Positive selected sites in hummingbird *Tas2r1* genes. (a) Alignment of the amino acid sequences of five hummingbird Tas2r1s. Positively selected sites identified by both site models were indicated with red asterisks; positively selected sites detected by both site models and the branch-site model were shown in blue. (b) Snake plot of hummingbird Tas2r1s based on the alignment of the amino acid of five hummingbird Tas2r1 receptors. The positively selected sites identified by site models were indicated with an open circle and an amino acid position, while the sites detected by both site models and the branch-site model were indicated by blue circles with amino acid positions. The seven membrane domains were predicted using the TMHMM methods.

Figure S2. Responses of avian Tas2r1 receptors to bitter compounds. HEK293 cells transfected with one bird Tas2r1 construct along with Gα16-gust44 were assayed for their responses to 24 bitter compounds. Black traces, calcium mobilization of the nine Tas2r1 receptors to bitter compounds; grey traces, mock-transfected cells.

Figure S3. Quantitative analysis of responses of bird Tas2r1 receptors to bitter compounds. Responses of HEK293 cells transiently transfected with one bird Tas2r1 receptor and Gα16-gust44 to bitter compounds were quantified by the expression of bar graph. Black bar, responsiveness of Tas2r-transfected cells; grey bar: mock-transfected cells. Data are expressed as mean ± *SE* percent change in fluorescence (ΔF, peak fluorescence-baseline fluorescence) compared with baseline fluorescence (F) from three independent wells. Two-tailed Student's t-test were performed to confirm whether responses from Tas2r-transfected cells were significantly different from that of mock-transfected cells ($p<0.05$). All the bitter compound-elicited responses were significantly higher than mock-transfected cell baseline, with the exception of CalAnn_Tas2r1c toward camphor (c).

Figure S4. Functional responses of bird Tas2r2 and Tas2r3 receptors. Tas2r constructs were expressed along with Gα16-gust44 in HEK293 cells to examine their responses to two bitter compounds. Black bar, responsiveness of Tas2r-transfected cells; grey bar: mock-transfected cells. (a, c) Quantitative analysis of responses of bird Tas2rs to denatonium benzoate (10 mM) and yohimbine (0.15 mM). (b, d) Dose-dependent responses of bird Tas2r receptors to denatonium benzoate and yohimbine. GraphPad Prism 7 was used to fit the curves.

a

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                                                  TM1                                  TM2                           ** * *
CalAnn_Tas2r1a  MEACSSQGKF NVTTYNAVAM AIITLQTFAG MWINAFIVSV LCVSWVKKRS FNTNEKILLL LGCSRVGYLC TAWVGSFIEN IYSFCVYVHQ KIQIISALLS 100
CalAnn_Tas2r1b  MEACSSQGKF NVTTYNAMSM AIITLQTFAG MWINAFIVSV LCVSWVKKRS FNSNEKILLF LGCVRFCYFC ITWVYSFLKI LYQCRFYAHH LAQLFSAIQS
CalAnn_Tas2r1c  MEACTSQGKF NVTTYNTVSM AIVTLQTFAG MWINAFIVSV LCVAWGKKRS FNSNEKIFLF LGCSRFCYLF ITWVFFLFEY IYPFCMDVYP ATQLLQGTQN
CalAnn_Tas2r1d  MEACTFQGKF NVTTYNAVAM AIITLQTFAG MWINAFIVSV LCVAWGKKRS FNSNEKILLF LGCSRSCYLF TTWVFSFFEN IYPLCMYVYS VYLFLVPLQD
CalAnn_Tas2r1e  MEACTSQGKF NVTTYNAVAM AIITLQTFAG MWINAFIVSV LCVSWVKKRS FNTNEKILLL LGCSRFWYSC ITWVFTFLEN IYPLCLYVSP TLQTLFGIQS

                     TM3                                        TM4     *                            *
                FFNIFSLWIS AILSVFYCIK IANFQHTFFI YLKVRIDRIV PWLMLTSVPV SLGVSVFVYI VIDEARCDNN NCTTSGYLWH LTVRPELHFF PLYFFTGFVY 200
                FFSFSNLWVS AFLSVFYCIK IANFQHTFFI YLKMRIDRIV PWLMLASVLV SLVISFLIYG IADEALNNNH NSTAPGNIWK LNIKMDRRFF PIFFISGFEY
                FFSVSSLWVS AILSVFYCVK IANFQHSFFI YLKVKIDRFV PWLMLASVLI SLGSSSFSYK VLSESICNNT NCTSAEYLWK DSNKTHEDLL LFLFLSGIVY
                FFNVSNVCVS AFLSVFYCIK IANFQHSFFI YLKVRIDRMM PWLMLASVLI SLGYSIFVYK IVDESICDNV NCTMLGHIWK YSIRIDQLFS MYVLITGFVF
                LFNFSNLWVS AFLFVFYCIK IANFRNTFFI YLKVRIDKIV PWLMLASVLL SLSYGIYVFE VMDEVVCEKI NCTTPGNFRK QRMGINEYIF TLFFLSGFSY

                     TM5                                                    TM6     **   *  *      TM7
                ATAFAAVISS ALLLLFSLWR HKHRMQTKSG KNVSVDAHIK AMKSILSFFF IDTIQFIILI LSLIYSLKKE KAVMFFITFF LYSFSAAHSL ILIFSNPKLE 300
                VAAFAAVISS ALLLLFSLWR HKHKMQAKSG KNVSVDAHIK AMKSILSFLL IYSIHFICAI MELIYATKKA TAVMIVISLF KHSFPSIYSL ILIFSNPKLE
                ATAFAAVISS ALLLLFSLWR HKHRMQTKSG KNISMDAHIK AMKSIFSFFF VYCINFICLV LSLIYETKRE STMPILILVL LLAFPAVHSL ILIFSNPKLE
                AIAFSAVSFS VLLLLFSLWR HKHKMQTESG KNVSVDAHIK AMKSILSFFF TYTINFVCLV LTLTYPMKNQ KAAKFLIFFF QHSFPAVHSL ILIFSNPKLE
                AIAFIAVICS ALLLLFSLWK HKCKMQTKSA KNVSVDAHIK AVKSIISFLL IYSINFVCLI LSLVYRVKEG SGVPYFISLL QHAFPSVHSI ILVFSNPKLE

                                      320
                KTLLRTLSCL KCKICMRHET
                ETLIRTLSCL KCKICMK---
                KTLLRTLSCL KCNICMK---
                KTLLRTLTCM KCKICKR---
                KILLRTLSCA KCKICKN---
```

b

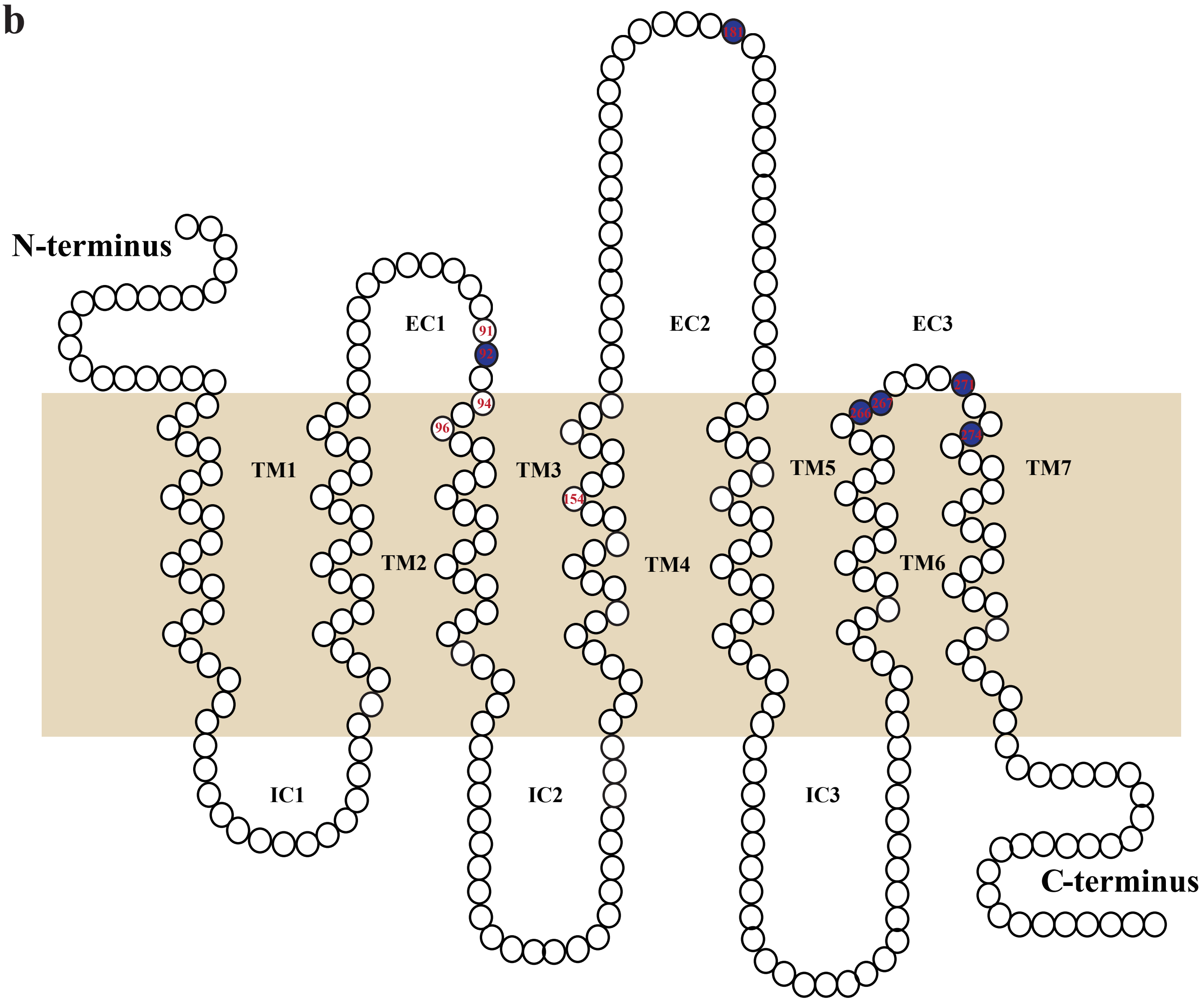
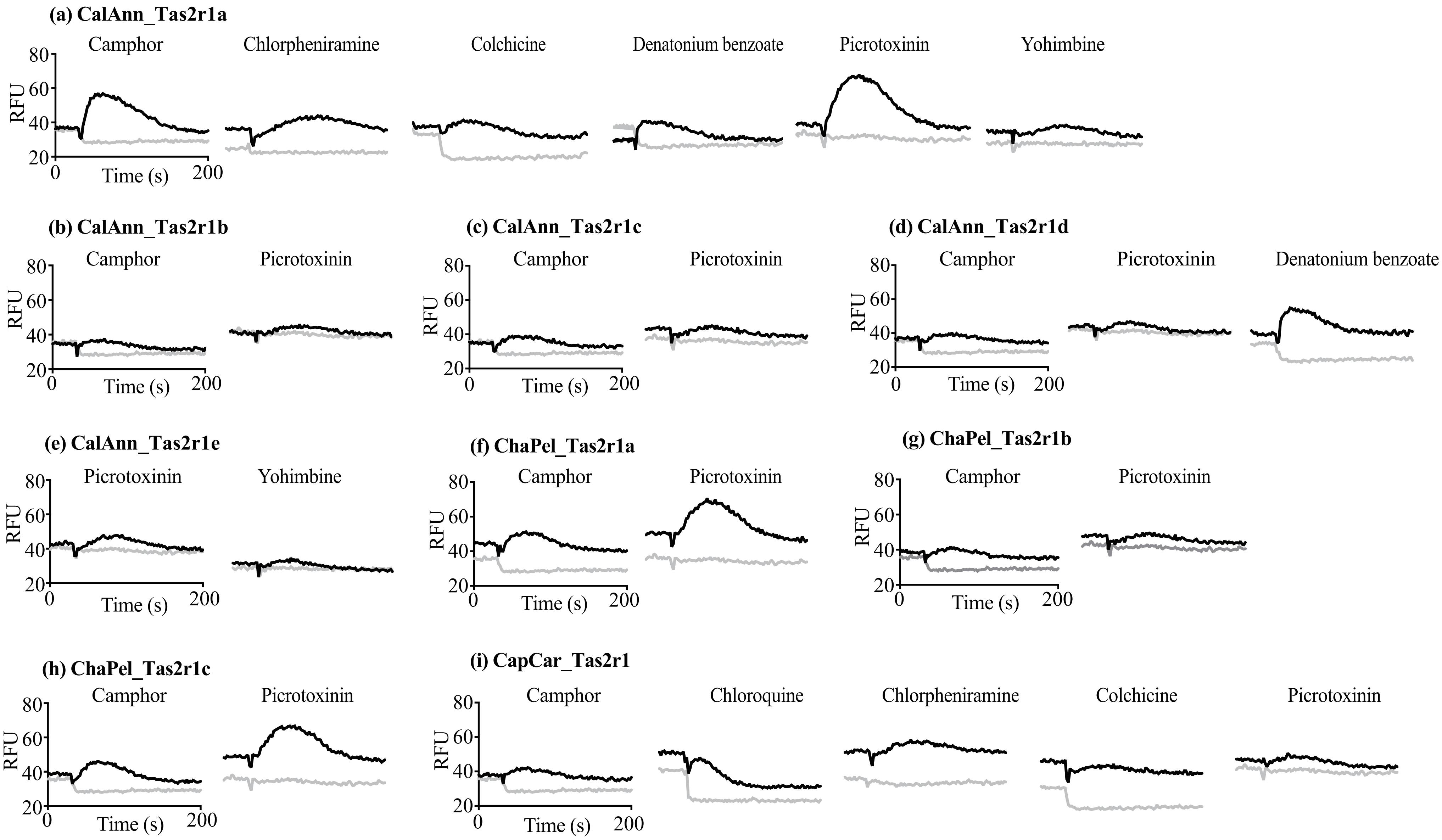


Figure S1



(a) CalAnn_Tas2r1a
Camphor
Chlorpheniramine
Colchicine
Denatonium benzoate
Picrotoxinin
Yohimbine
(b) CalAnn_Tas2r1b
Camphor
Picrotoxinin
(c) CalAnn_Tas2r1c
Camphor
Picrotoxinin
(d) CalAnn_Tas2r1d
Camphor
Picrotoxinin
Denatonium benzoate
(e) CalAnn_Tas2r1e
Picrotoxinin
Yohimbine
(f) ChaPel_Tas2r1a
Camphor
Picrotoxinin
(g) ChaPel_Tas2r1b
Camphor
Picrotoxinin
(h) ChaPel_Tas2r1c
Camphor
Picrotoxinin
(i) CapCar_Tas2r1
Camphor
Chloroquine
Chlorpheniramine
Colchicine
Picrotoxinin
RFU
20
40
60
80
0
Time (s)
200

Figure S2

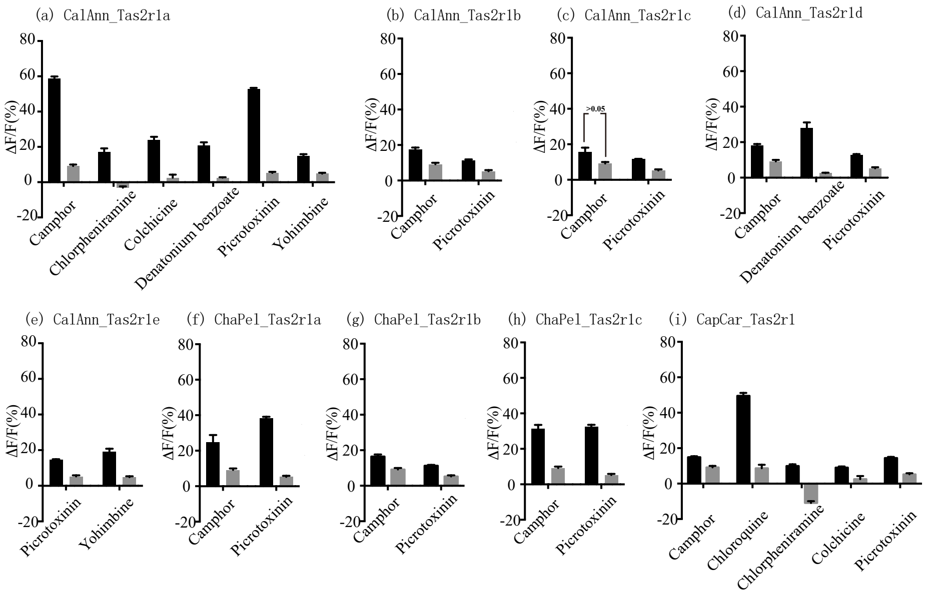

(a) CalAnn_Tas2r1a
(b) CalAnn_Tas2r1b
(c) CalAnn_Tas2r1c
(d) CalAnn_Tas2r1d
(e) CalAnn_Tas2r1e
(f) ChaPel_Tas2r1a
(g) ChaPel_Tas2r1b
(h) ChaPel_Tas2r1c
(i) CapCar_Tas2r1
ΔF/F(%)
80
60
40
20
0
-20
>0.05
Camphor
Chlorpheniramine
Colchicine
Denatonium benzoate
Picrotoxinin
Yohimbine
Chloroquine

Figure S3

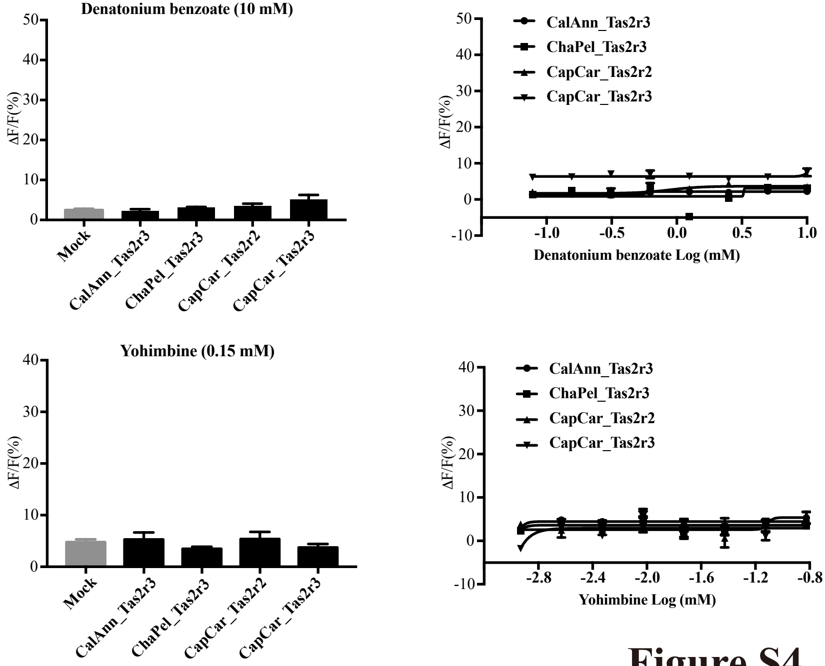

Denatonium benzoate (10 mM)
ΔF/F(%)
Mock
CalAnn_Tas2r3
ChaPel_Tas2r3
CapCar_Tas2r2
CapCar_Tas2r3
CalAnn_Tas2r3
ChaPel_Tas2r3
CapCar_Tas2r2
CapCar_Tas2r3
Denatonium benzoate Log (mM)
Yohimbine (0.15 mM)
Yohimbine Log (mM)

Figure S4