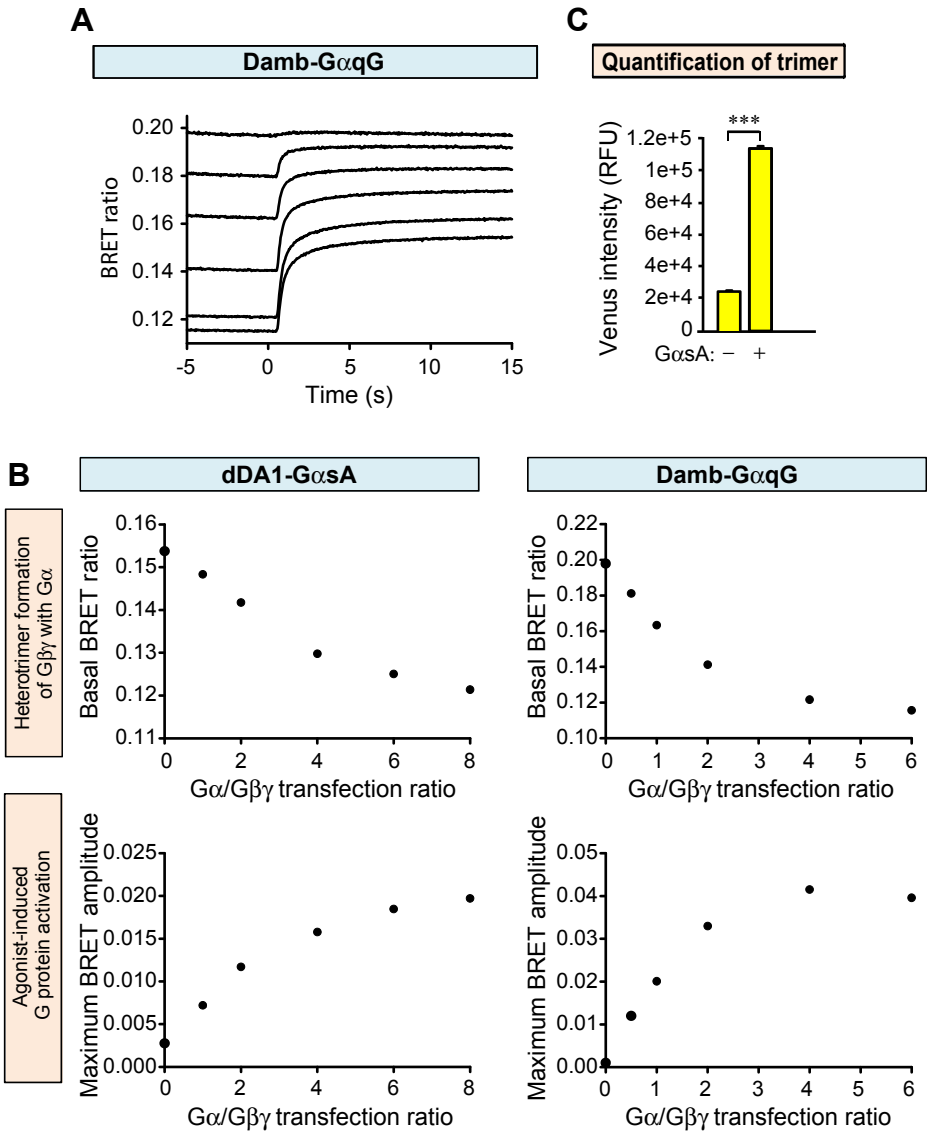

A
Damb-GαqG
BRET ratio
Time (s)
C
Quantification of trimer
Venus intensity (RFU)
GαsA: − +
***
B
dDA1-GαsA
Damb-GαqG
Heterotrimer formation of Gβγ with Gα
Agonist-induced G protein activation
Basal BRET ratio
Maximum BRET amplitude
Gα/Gβγ transfection ratio

Figure S1

Figure S1. Varying the transfection ratio of Gα and Venus-Gβγ subunits for optimal analysis of signaling. Related to Figure 3.

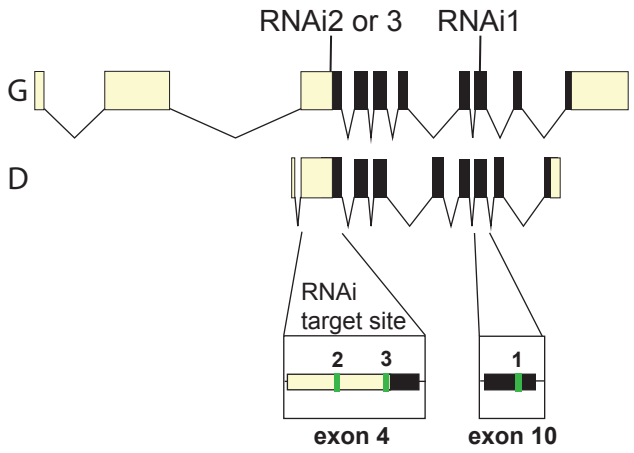
Effect of increasing Gα expression over a constant Venus-Gβγ on basal BRET ratio and agonist-induced BRET amplitude.

A. Cells transfected with Damb/Ric-8A and varying ratios of GαqG/Venus-Gβγ and challenged with 100 μM DA at time zero and G protein activation recorded.

B. Basal BRET ratio and maximum BRET amplitude were plotted as a function of the Gα subunit/Venus-Gβγ plasmid DNA ratio used for transfection. Each data point represents the mean of 6 replicates ± SEM. Error bars are smaller than the size of each point.

C. Quantification of trimeric G proteins. Venus-Gβγ was co-transfected with or without GαsA. Co-expression of GαsA increased Venus intensity, indicating formation of proteolytically stable trimer. *Statistics*: Results are expressed as the mean ± SEM. ANOVA with Tukey *post hoc*, ***$p<0.001$, n=8 per group.

A



Gαq Gene Structure and RNAi Targets

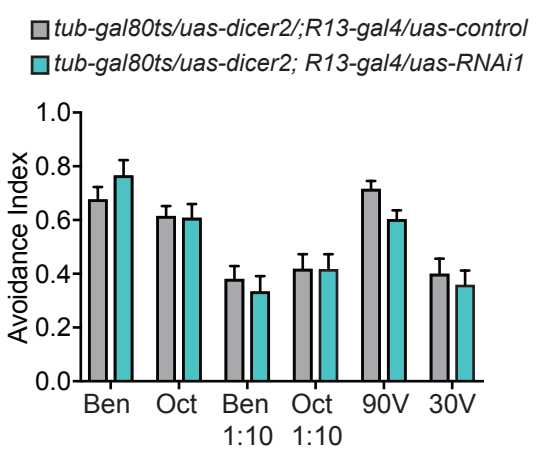
Exon4

ATGTTCTTGGATCGTTCGATGTGGTCTTTGTTCATTAGTAACCATCACTCTCAGCCTCCGAGTCACCGAAATCTGGTCAAGAACT
TAGCGTAGACCCGTAATTAGCAATAGCAATAGCCAAAGAGAGCCTGGATCGTAAGCGCTGGAGGAGGTCTGCAGAGCGAGCAAAG
CCGAAGTACAAATGCACCAATCGAAACTACAACAGAATCTATAACTTGGAACGGGTGAATTCAACGCCCGCCATACAACCAATTA
GCTAGGTGTCCGAGGGTCCAGAGAAGGGAGTCCAGGAGACCATCCCACCATCCAAACAGCATACATTTTATATACGGAACGGTCT
GTCGAGCGTAGTGGCAGCAGCACGCGAAAGCGTCTATAAATCTAGTTAGCATGGAGTGCTGTTTATCGGAGGAGGCCAAGGAACA
AAAGCGCATCAATCAGGAAATCGAGAAGCAGTTGCGCCGGGACAAGAGAGATGCGCGCCGCGAGCTTAAACTGCTACTACTGG

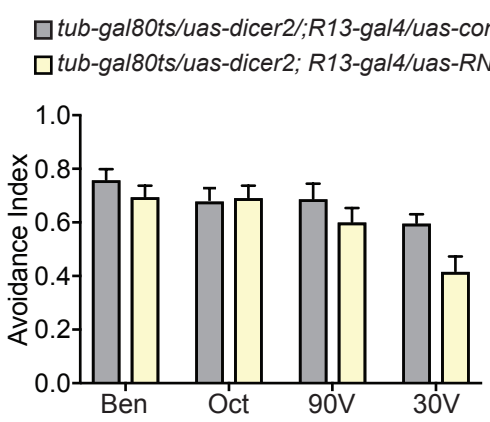
Exon 10

AATCGAATGGAGGAATCTAAAGCTTTATTTCGTACTATAATTACATACCCTTGGTTTCAAAATTCGTCAGTTATTCTTTTCCTGA
ATAAGAAGGACTTGTTGGAAGAGAAAATAATGTATTCGCATTTGGTAGACTATTTTCCTGAATACGATG

B



C



D

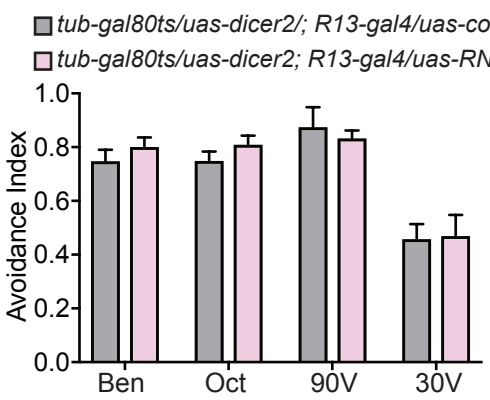


Figure S2

Figure S2. RNAi sequences and olfactory and shock avoidance controls. Related to Figure 4.
A. The graphic illustrates an expanded exon 4 and 10 and the specific sequences targeted by the three RNAi lines. Two different transcriptional units and alternative splicing encode these forms. Sequences shaded with light grey, dark grey and marked with an un-shaded box indicate target sites of Gαq RNAi2, RNAi3, and RNAi1, respectively.
B. Odor and shock avoidance for Gαq RNAi1 flies. The avoidance indices of flies expressing Gαq RNAi1 in MBn were compared to their respective controls. We also tested Gαq RNAi 1 flies with a 10-fold dilution of the experimental odor concentration and at reduced shock intensity of 30V. No significant differences between experimental flies and controls were found. *Statistics*: Results are expressed as the mean ± SEM. Two-tailed, two-sample Student's t-test, *n.s.*, n=6 per group.
C, D. Expressing Gαq RNAi2 and Gαq RNAi3, respectively, in MBn did not alter olfactory avoidance. *Statistics*: Results are expressed as the mean ± SEM. Two-tailed, two-sample Student's t-test, *n.s.*, n=6 per group.

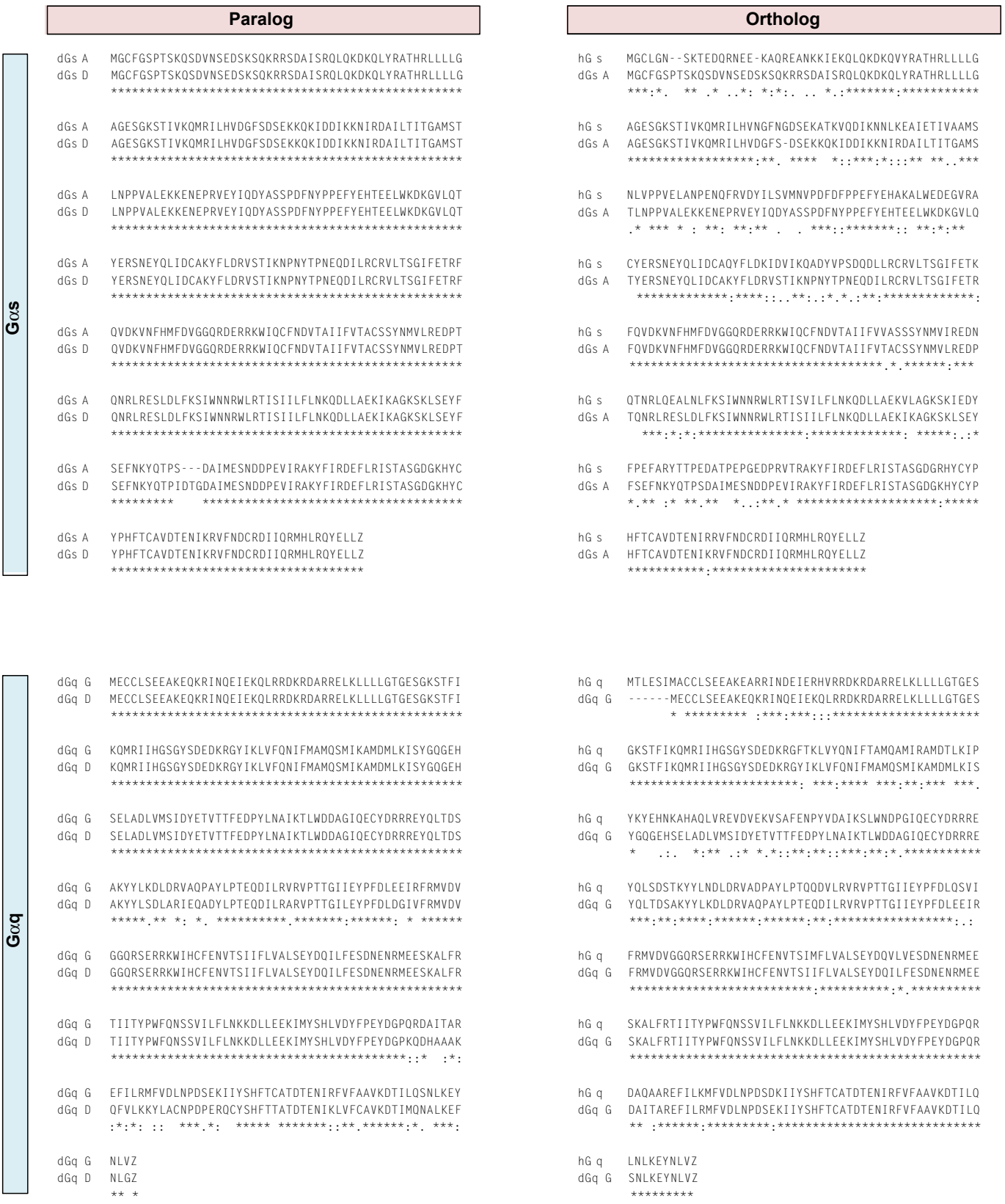

Paralog
Ortholog
Gαs
dGs A MGCFGSPTSKQSDVNSEDSKSQKRRSDAISRQLQKDKQLYRATHRLLLLG
dGs D MGCFGSPTSKQSDVNSEDSKSQKRRSDAISRQLQKDKQLYRATHRLLLLG
dGs A AGESGKSTIVKQMRILHVDGFSDSEKKQKIDDIKKNIRDAILTITGAMST
dGs D AGESGKSTIVKQMRILHVDGFSDSEKKQKIDDIKKNIRDAILTITGAMST
dGs A LNPPVALEKKENEPRVEYIQDYASSPDFNYPPEFYEHTEELWKDKGVLQT
dGs D LNPPVALEKKENEPRVEYIQDYASSPDFNYPPEFYEHTEELWKDKGVLQT
dGs A YERSNEYQLIDCAKYFLDRVSTIKNPNYTPNEQDILRCRVLTSGIFETRF
dGs D YERSNEYQLIDCAKYFLDRVSTIKNPNYTPNEQDILRCRVLTSGIFETRF
dGs A QVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVTACSSYNMVLREDPT
dGs D QVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVTACSSYNMVLREDPT
dGs A QNRLRESLDLFKSIWNNRWLRTISIILFLNKQDLLAEKIKAGKSKLSEYF
dGs D QNRLRESLDLFKSIWNNRWLRTISIILFLNKQDLLAEKIKAGKSKLSEYF
dGs A SEFNKYQTPS---DAIMESNDDPEVIRAKYFIRDEFLRISTASGDGKHYC
dGs D SEFNKYQTPIDTGDAIMESNDDPEVIRAKYFIRDEFLRISTASGDGKHYC
dGs A YPHFTCAVDTENIKRVFNDCRDIIQRMHLRQYELLZ
dGs D YPHFTCAVDTENIKRVFNDCRDIIQRMHLRQYELLZ
hG s MGCLGN--SKTEDQRNEE-KAQREANKKIEKQLQKDKQVYRATHRLLLLG
dGs A MGCFGSPTSKQSDVNSEDSKSQKRRSDAISRQLQKDKQLYRATHRLLLLG
hG s AGESGKSTIVKQMRILHVNGFNGDSEKATKVQDIKNNLKEAIETIVAAMS
dGs A AGESGKSTIVKQMRILHVDGFS-DSEKKQKIDDIKKNIRDAILTITGAMS
hG s NLVPPVELANPENQFRVDYILSVMNVPDFDFPPEFYEHAKALWEDEGVRA
dGs A TLNPPVALEKKENEPRVEYIQDYASSPDFNYPPEFYEHTEELWKDKGVLQ
hG s CYERSNEYQLIDCAQYFLDKIDVIKQADYVPSDQDLLRCRVLTSGIFETK
dGs A TYERSNEYQLIDCAKYFLDRVSTIKNPNYTPNEQDILRCRVLTSGIFETR
hG s FQVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVVASSSYNMVIREDN
dGs A FQVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVTACSSYNMVLREDP
hG s QTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVLAGKSKIEDY
dGs A TQNRLRESLDLFKSIWNNRWLRTISIILFLNKQDLLAEKIKAGKSKLSEY
hG s FPEFARYTTPEDATPEPGEDPRVTRAKYFIRDEFLRISTASGDGRHYCYP
dGs A FSEFNKYQTPSDAIMESNDDPEVIRAKYFIRDEFLRISTASGDGKHYCYP
hG s HFTCAVDTENIRRVFNDCRDIIQRMHLRQYELLZ
dGs A HFTCAVDTENIKRVFNDCRDIIQRMHLRQYELLZ
Gαq
dGq G MECCLSEEAKEQKRINQEIEKQLRRDKRDARRELKLLLLGTGESGKSTFI
dGq D MECCLSEEAKEQKRINQEIEKQLRRDKRDARRELKLLLLGTGESGKSTFI
dGq G KQMRIIHGSGYSDEDKRGYIKLVFQNIFMAMQSMIKAMDMLKISYGQGEH
dGq D KQMRIIHGSGYSDEDKRGYIKLVFQNIFMAMQSMIKAMDMLKISYGQGEH
dGq G SELADLVMSIDYETVTTFEDPYLNAIKTLWDDAGIQECYDRRREYQLTDS
dGq D SELADLVMSIDYETVTTFEDPYLNAIKTLWDDAGIQECYDRRREYQLTDS
dGq G AKYYLKDLDRVAQPAYLPTEQDILRVRVPTTGIIEYPFDLEEIRFRMVDV
dGq D AKYYLSDLARIEQADYLPTEQDILRARVPTTGILEYPFDLDGIVFRMVDV
dGq G GGQRSERRKWIHCFENVTSIIFLVALSEYDQILFESDNENRMEESKALFR
dGq D GGQRSERRKWIHCFENVTSIIFLVALSEYDQILFESDNENRMEESKALFR
dGq G TIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQRDAITAR
dGq D TIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPKQDHAAAK
dGq G EFILRMFVDLNPDSEKIIYSHFTCATDTENIRFVFAAVKDTILQSNLKEY
dGq D QFVLKKYLACNPDPERQCYSHFTTATDTENIKLVFCAVKDTIMQNALKEF
dGq G NLVZ
dGq D NLGZ
hG q MTLESIMACCLSEEAKEARRINDEIERHVRRDKRDARRELKLLLLGTGES
dGq G ------MECCLSEEAKEQKRINQEIEKQLRRDKRDARRELKLLLLGTGES
hG q GKSTFIKQMRIIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMDTLKIP
dGq G GKSTFIKQMRIIHGSGYSDEDKRGYIKLVFQNIFMAMQSMIKAMDMLKIS
hG q YKYEHNKAHAQLVREVDVEKVSAFENPYVDAIKSLWNDPGIQECYDRRRE
dGq G YGQGEHSELADLVMSIDYETVTTFEDPYLNAIKTLWDDAGIQECYDRRRE
hG q YQLSDSTKYYLNDLDRVADPAYLPTQQDVLRVRVPTTGIIEYPFDLQSVI
dGq G YQLTDSAKYYLKDLDRVAQPAYLPTEQDILRVRVPTTGIIEYPFDLEEIR
hG q FRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLVESDNENRMEE
dGq G FRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQILFESDNENRMEE
hG q SKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR
dGq G SKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR
hG q DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVFAAVKDTILQ
dGq G DAITAREFILRMFVDLNPDSEKIIYSHFTCATDTENIRFVFAAVKDTILQ
hG q LNLKEYNLVZ
dGq G SNLKEYNLVZ

Figure S3

Figure S3. Comparison of paralog and ortholog Gαs and Gαq subunits. Related to Figure 3.
Protein sequence alignments of Drosophila GαsA, GαsD, GαqG, GαqD, and human Gαs and Gαq were performed with DNADynamo. An asterisk (*) indicates positions that have an identical amino acid residue. A colon (:) indicates amino acids with highly similar properties. A period (.) indicates amino acids with weakly similar properties.

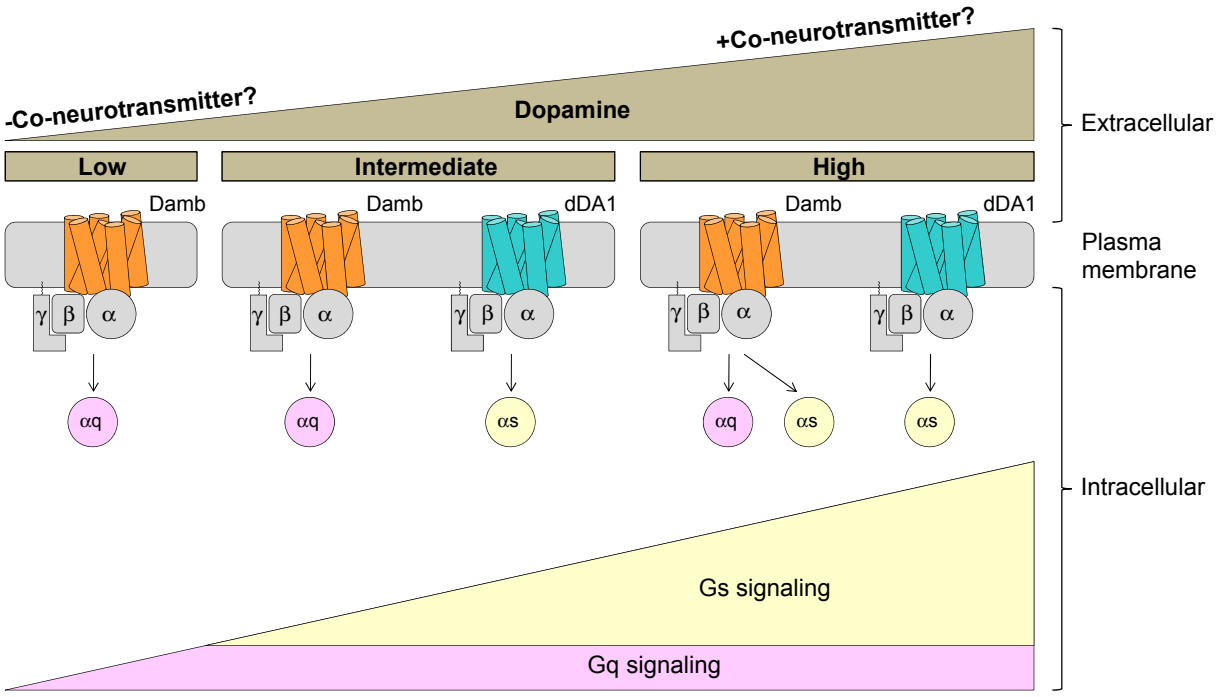

+Co-neurotransmitter?
-Co-neurotransmitter?
Dopamine
Extracellular
Low
Intermediate
High
Damb
Damb
dDA1
Damb
dDA1
Plasma membrane
γ β α
αq
αq
αs
αq
αs
αs
Intracellular
Gs signaling
Gq signaling

Figure S4

Figure S4. Model for the roles of dDA1 and Damb in acquisition and forgetting. Related to Figures 1-4.
The model envisions strong release of DA from DAn due to repetitive electric shock pulses during acquisition of aversive memory. This engages both the dDA1 and the Damb receptors to initiate intracellular cAMP and Ca^{2+} signaling, although the dDA1 receptor has a more prominent role in acquisition. Forgetting occurs from weak DA release after acquisition that engages the Damb receptor and downstream Ca^{2+} signaling.

Supplementary Table 1. Complementary DNA clones of Gα RNA transcripts.

Isoform	PCR Primers	Flybase Accession No.	Clones Identified	Empty Clones	Total Clones Screened*
GαqG	1&2	FBtr0087830	22 (91.7%)	2	24
GαqD	1&3	FBtr0087832	122 (84.7%)	20	144
GαqJ	1&3	FBtr0333101	0	20	144
GαsA	4&5	FBtr0072144	13 (54.1%)	9	24
GαsD	4&5	FBtr0301411	1 (4.1%)	9	24

Complementary DNAs for Gα transcripts produced from randomly primed brain RNA were cloned into plasmid vectors and sequenced. Brain cDNA was prepared as described in Experimental Methods and subsequently amplified using PCR and the DNA primers identified by name, number and sequence in Supplementary Table 2. Three ligations and screens were performed to identify clones: one for GαqG, one for GαqD and J, and one for Gαs. The number of independent clones sequenced representing each transcript is shown in the table followed by the percentage (in parentheses). *Two additional, identical clones were discovered during the GαqD and J screen and one during the Gαs screen that represented non-annotated, alternatively spliced versions of these transcripts.

Supplementary Table 2. DNA primers and sequences.

Primer name		Primer sequence 5' → 3'	Transcript
DAMB FseI FOR	Subcloning primer	GGGAATTCGGCCGGCCAATGGTGGACGACAATG	DAMB
DAMB FseI REV	Subcloning primer	TCCTCTAGGGCCGGCCTCTCACATGAGCGTCCGGTCGATCTG	DAMB
dDA1 Fse1 FOR	Subcloning primer	TGGACAAGGCCGGCCAAATGGATACAATAGTTGG	dDA1
dDA1 Fse1 REV	Subcloning primer	ATTCAGACGGCCGGCCGGTCAAATCGCAGACACCTGCTC	dDA1
Gαq 5'UTR FOR	1	AAGGGAGTCCAGGAGACCA	GαqG, D, J
GαqG 3'UTR REV	2	GTTCCTGATGTAACAAACG	GαqG
GαqJ&D 3'UTR REV	3	AATTCATTTGCATTGCCACT	GαqD or J
Gαs 5'UTR FOR	4	GCAGAACGGAGAAAGCAC	GαsA or D
Gαs 3'UTR REV	5	CATTACGGCGATGGGGAT	GαsA or D
Gαq FseI FOR	Subcloning primer	TCTATAAAGGCCGGCCGCATGGAGTGC	GαsG, D, J
Gαq G FseI REV	Subcloning primer	TTTTCGATGGCCGGCCGTTTAGACCAAATTA	Gαq G
Gαq J&D FseI REV	Subcloning primer	ATCCGCACGGCCGGCCTTAGCCCAAAT	GαqD or J
Gαs FseI FOR	Subcloning primer	TTGGTGGAGGCCGGCCGCTGCGATGGGT	GαsA or D
Gαs FseI REV	Subcloning primer	TTACGGCGGGCCGGCCAACCTATAAC	GαsA or D

Supplementary Table 3. Gαq transcript expression as assayed by real time PCR.

Transcript Assayed	Average Ct Value	Std Dev Ct Value
GαqG	26.92	0.04
GαqD	30.90	0.06
GαqJ	33.87	0.97
Gapdh	27.88	0.04

Taqman real time PCR assays specific for each Gαq transcript were used to assay relative abundance in the 5 day-old *Drosophila* brain. The Gapdh housekeeping gene was also assayed for comparison. The GαqG transcript was much more abundant than GαqD.

Supplemental Experimental Procedures

Genetic constructs for reporters, receptors and G proteins. Human pcDNA3.1(+) plasmids encoding the DA D1 receptor, GαoB, Gαz, Gα11, Gα14, Gα15, Gαs long isoform (GαsL), Gαolf, and Gα13 were purchased from the cDNA Resource Center (www.cDNA.org). The pCMV5 plasmids encoding rat GαoA, rat Gαi1, rat Gαi2, rat Gαi3, human Gαq, and bovine Gαs short isoform (GαsS) were gifts from Dr. Hiroshi Itoh (Nara Institute of Science and Technology, Japan). Plasmids encoding Venus 156-239-Gβ1, and Venus 1-155-Gγ2 were gifts from Dr. Nevin A. Lambert (Hollins et al., 2009). Flag-tagged Ric-8A in pcDNA3 and Flag-tagged Ric-8B in pcDNA3.1 were gifts from Dr. Jean-Pierre Montmayeur (Fenech et al., 2009) and Dr. Bettina Malnic (Von Dannecker et al., 2006), respectively. PTX-S1 in mammalian expression vector was kindly provided by Eitan Reuveny (Raveh et al., 2010). The masGRK3ct-Nluc construct was previously described (Masuho et al., 2015b). The pGloSensor-22F cAMP plasmid was purchased from Promega (Binkowski et al., 2011). CalfluxVTN was synthesized and cloned into pcDNA3.1(+) by GenScript (Yang et al., 2016).

The *Drosophila* Gαq and Gαs mRNA coding regions were isolated from wild type cDNA and cloned into pCAGWBA, a pcDNA3.1-based expression vector A pcDNA3.1-based expression vector (MacMullen et al., 2016). Wild type *Drosophila* RNA was prepared from dissected brains of 10 male and 10 female, 5-day old flies using the Qiagen RNeasy Plus Micro Kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions. The RNA was treated with DNaseI using the TURBO DNA-free kit (Life Technologies, Carlsbad, CA). Fifty nanograms of *Drosophila* brain RNA was used to create randomly primed cDNA using the SuperScript III first-strand synthesis system (Life Technologies). Gαq and Gαs transcripts were amplified from the *Drosophila* brain cDNA using PCR primers located in the 5' and 3' untranslated regions of the transcripts (Table S2). The PCR amplicons were then subcloned into pCRII topo vectors (ThermoFisher Scientific, Waltham, MA) and sequenced. Primers with FseI restriction enzyme site tails were used to amplify the specific transcripts from the subcloned topo vectors for insertion into the pCAGWBA vector backbone via this unique restriction enzyme site (Table S2).

The DAMB transcript was PCR amplified from a *pUAST-damb* overexpression construct (Cervantes-Sandoval et al., 2016) with FseI tailed primers and cloned into the pCAGWBA vector. The dDA1 transcript was PCR amplified from genomic DNA of flies that over-expressed this gene using a *pUAST* vector backbone (flies were a gift from J. Dubnau). See Table S2 for all primer sequences. Transcript and isoform nomenclatures were extracted from NCBI.

Cell culture and transfections. We chose HEK293T/17 cells for expression experiments because of their high efficiency for transfection (Pear et al., 1993). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, MEM non-essential amino acids (Life Technologies), 1 mM sodium pyruvate, and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator equilibrated with 5% CO_2. Six cm culture dishes were coated during incubation for 10 min at 37°C with 2.5 ml of Matrigel solution [~10 μg/ml growth factor-reduced Matrigel (BD Biosciences) in culture medium]. Cells were seeded at a density of 4 x 10^6 cells/dish. After 4 hr, the expression constructs (10 μg/dish) were transfected into the cells using PLUS (10 μl/dish) and Lipofectamine LTX (12 μl/dish). For the cAMP assays, *Drosophila* DA receptor, pGloSensor-22F cAMP, and PTX-S1 constructs were used at a 6:6:1 ratio (ratio 1 = 0.42 μg of plasmid DNA). For the Ca^{2+} assays, *Drosophila* DA receptor, CalfluxVTN, and PTX-S1 constructs were used at a 6:1:1 ratio. Given the promiscuous nature of G protein-coupling of GPCRs (Masuho et al., 2015b), a construct expressing the catalytic subunit of pertussis toxin, PTX-S1, was co-transfected to inhibit the possible coupling of endogenous Gi/o to DA receptors. This ensures that all signal recorded in these assays is generated exclusively by the activation of Gs or Gq. Empty vector pcDNA3.1(+) was used to normalize the total amount of transfected plasmid DNA. For real-time monitoring of G protein activity with BRET sensors, the transfections were performed as previously reported (Masuho et al., 2015a; Masuho et al., 2015b) with minor modifications.

GloSensor F-22 cAMP assay. Sixteen to 24 hr post-transfection, transfected cells were detached with 1 ml of CO_2 independent medium (Invitrogen) containing 10% FBS. Twenty-five μl of cell suspension was transferred to the each well of a 96-well plate containing 25 μl of 2X GloSensor cAMP Reagent prepared according to the manufacturer's directions. Following incubation for 2 hr at room temperature, luminescence was monitored continuously on a POLARstar Omega microplate reader at room temperature. Fifty μl of DA dissolved in PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose was then applied to cells.

CalfluxVTN Ca^{2+} assay. Sixteen to 24 hr post-transfection, transfected cells were washed once with PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose and detached by gentle, repeated pipetting in the same solution. Cells were harvested by centrifugation at 500xg for 5 min and resuspended in PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose. Approximately 50,000 to 100,000 cells per well were distributed in 96-well flat-bottomed white microplates (Greiner Bio-One). The Nluc substrate, furimazine, was purchased from Promega and used according to the manufacturer's instructions. BRET measurements were made using a POLARstar Omega microplate reader at room temperature equipped with two emission photomultiplier tubes. The BRET signal was calculated by measuring the ratio of the light emitted by the Venus reporter (535 with 30 nm band pass filter) relative to light emitted by the Nluc reporter (475 nm with 30 nm band pass filter). The average baseline value (basal BRET ratio) recorded prior to agonist stimulation was subtracted from the experimental BRET signal values to obtain the ΔBRET ratio. The largest agonist-induced ΔBRET ratio was plotted as the maximum BRET amplitude.

Real-time monitoring of G protein activity with BRET sensors. Cellular measurements of BRET between Venus-Gβγ and masGRK3ct-Nluc were performed as previously reported with minor modifications (Masuho et al., 2015a; Masuho et al., 2015b).

Fly Stocks and Behavioral Experiments. All flies were raised on standard food at room temperature. Gαq RNAi flies and the respective control line were developed within the Transgenic-RNAi-Project (TRiP; Ni et al., 2009) at Harvard Medical School and were obtained from Bloomington stock center. The control (parental) flies of the Gαq RNAi lines contain a P{*attP2, y+*} docking site in a *vermillion (v^-)* and *yellow (y^-)* genetic background. This line controls for possible non-specific effects of the inserted docking site, and is identified in the text and figures as *"uas-control."*

We used 6 day-old flies for all behavioral experiments. Flies were collected in vials with food ~16 hr before behavioral experiments. They were transferred into new vials with food 30 min prior to training and kept under the conditions for which behavioral experiments were conducted: dim red light, 25°C and ~75% relative humidity. Behavioral experiments utilized the standard aversive olfactory conditioning paradigm as previously described (Beck et al., 2000). A group of ~60 flies were transferred into a training tube where they received the following sequence of stimuli: 30 sec of fresh air, 1 min of an odor paired with 12 pulses of 90 V electric shock (CS+), 30 sec of air, 1 min of a second odor with no electric shock pulses (CS-), and finally 30 sec of air. We used 3-octanol (OCT) and benzaldehyde (BEN) as standard odorants in our experiments. Odors were diluted between 0.12% and 0.07% in mineral oil and different concentrations were tested using naïve flies to empirically determine the concentrations that led to equal avoidance of the two odors. After conditioning, the flies were tested in T-mazes in which they were allowed to choose for 2 min between the CS+ and CS- presented in two different arms of the T-maze. Memory was quantified by calculating the Performance Index (PI) as the (number of flies in the CS- arm)-(number of flies in the CS+ arm)/(number of flies in the CS- arm)+(number of flies in the CS+ arm). Memory retention was tested at 1, 3, 4.5 and 6 hr after conditioning. Memory acquisition was tested by shocking flies at 90V with 1, 3, 6 or 12 shocks that were evenly distributed during the one min of CS+ odor presentation.

We performed shock and odor avoidance tests on naïve flies with odor concentrations that were used during olfactory conditioning to control for any possible change in odor/shock perception and avoidance. We used the same odor and shock conditions as for classical conditioning as well as a 10-fold dilution of odor concentration and 30V shock to exclude the possibility of behavioral ceiling effects.

The TARGET system (McGuire et al., 2003) for all behavioral experiments. This provided for temporal control over the expression of the *uas-gal4* system due to the constitutive expression of the temperature-sensitive Gal4 inhibitor $Gal80^{ts}$. A temperature shift from 18°C to 30°C destabilizes $Gal80^{ts}$ and allows Gal4 to drive the expression of a *uas-transgene* in the fly's genome.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism 7. Memory scores follow a normal distribution (Walkinshaw et al., 2015) and were analyzed using the parametric two-tailed, two-sample Student's t-test. ANOVA with post hoc tests were utilized for multiple group comparisons**.**

Supplemental References

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