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Supplemental Information

Cell Type-Specific Transcriptome Analysis in the *Drosophila* Mushroom Body Reveals Memory-Related Changes in Gene Expression

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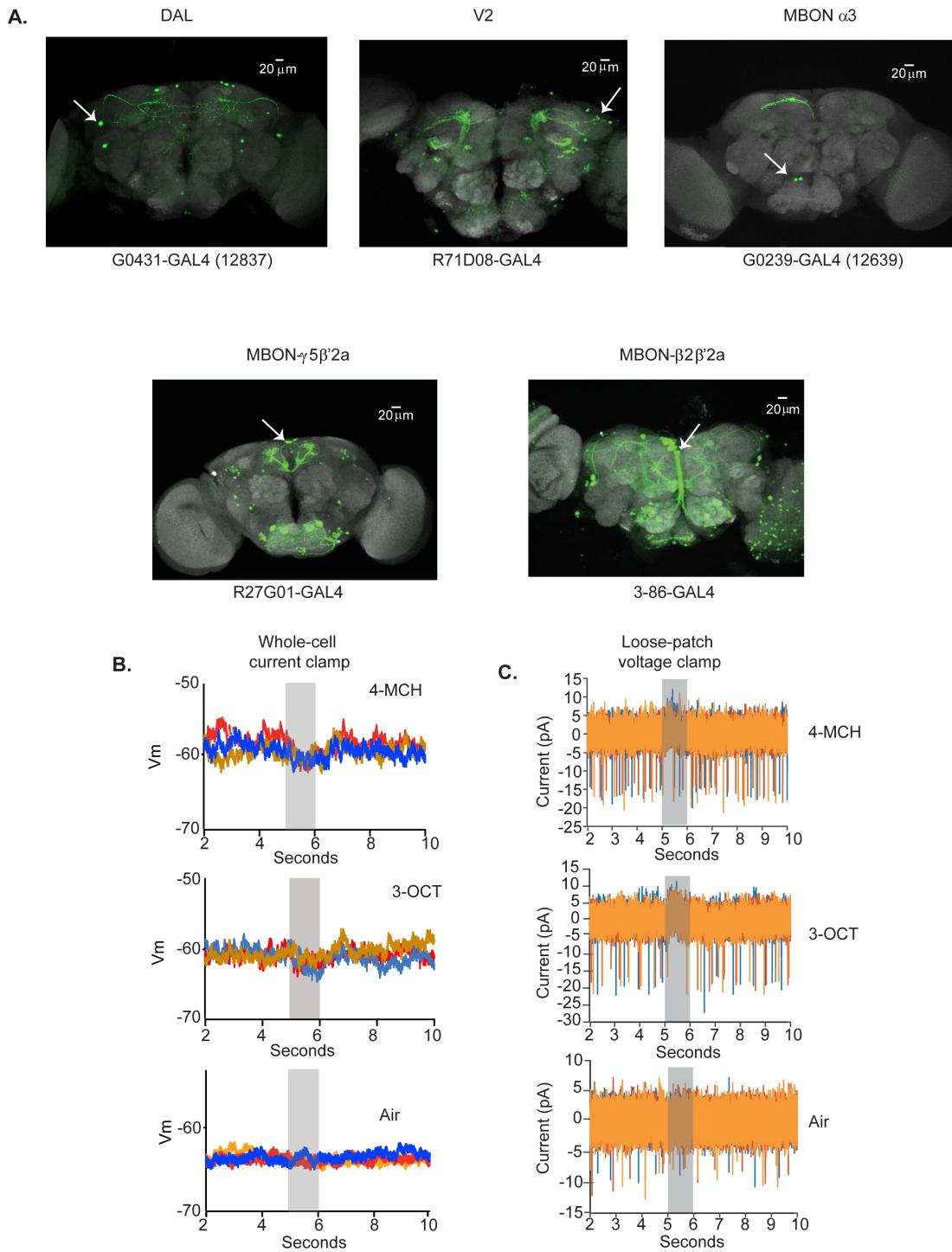


Figure S1. Related to Figure 1.

A Confocal max projection images of each GAL4 line used in the study. Expression pattern is shown using green fluorescent protein (GFP), with a counter-stain for neuropil (nc82) in gray. White arrows indicate cell body locations.

B Individual traces of odor responses from a DAL neuron recorded via whole-cell patch in current clamp mode. Three consecutive trials for each odor are overlaid to show the consistency of the odor response. Odor was delivered for 1 second (gray box), and each trial lasted for 20 seconds. 3-Octonal (3-OCT) and 4-methylcyclohexanol (4-MCH) were used at 1:100 dilution.

C Recording from a DAL neuron in the loose cell-attached configuration in voltage clamp mode. Odor-driven inhibition is observed in both recordings.

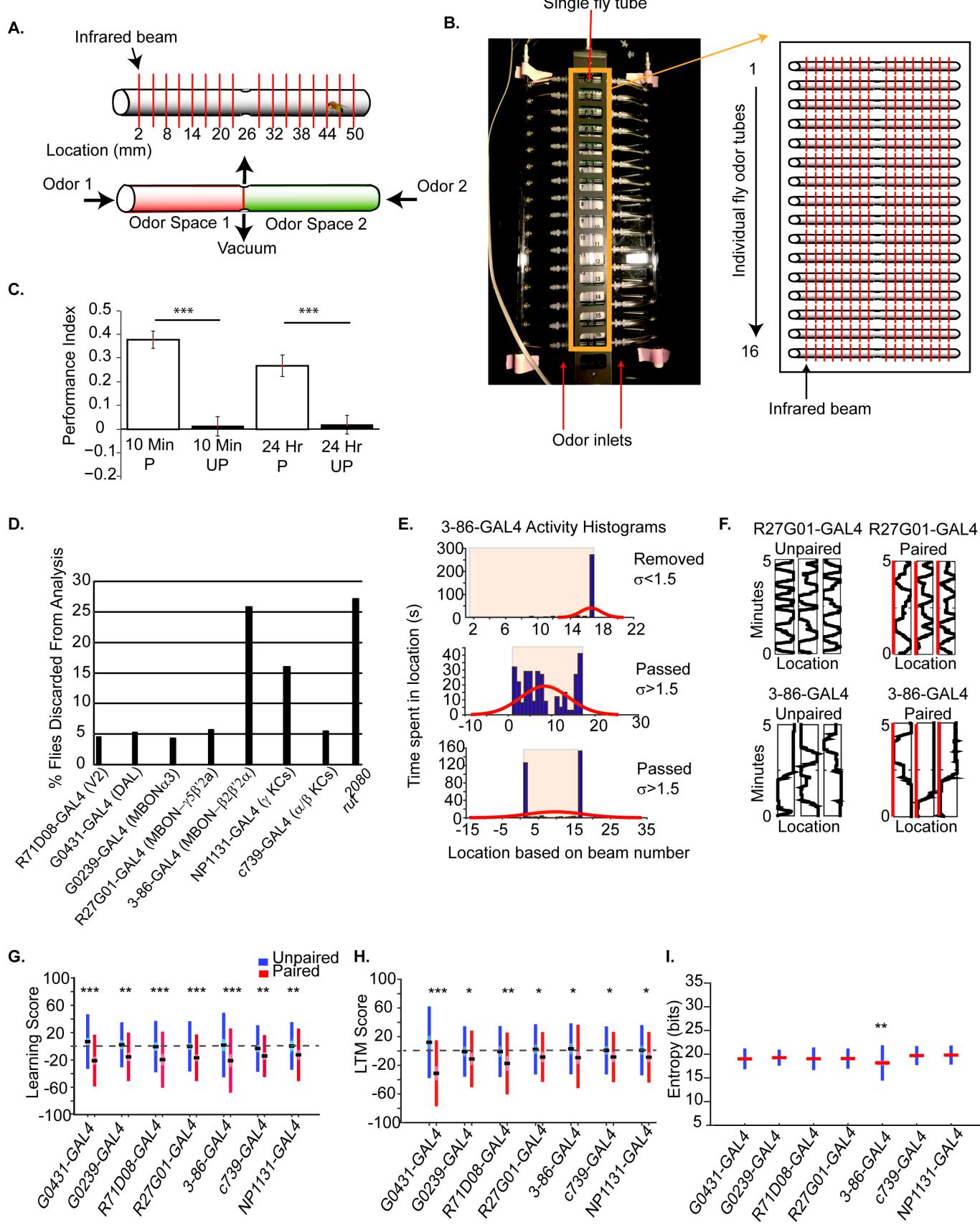


Figure S2. Related to Figure 1.

A Schematic of an individual behavior tube. The single fly learning and memory assay uses the Trikinetics multibeam DAMS monitoring system (Trikinetics Waltham, MA). There are 17 infrared beams spanning each 53 mm tube. Due to the vacuum line connected at the center of the tube, crossings of beam number nine are omitted from analysis.

B Image of the behavioral setup using the multi-beam monitors and manifolds. Each individual fly tube slides through a manifold tube, to create a central uniform vacuum. Manifolds with 16 ports are located on each side and are connected to each odor. The entire behavioral assay sits in a black box that prevents UV and room light from entering. 16 animals can be run at a time. The beam crossing sampling rate is 1 Hz and behavior is assayed for a total of 5 minutes at a time.

C Performance Indices (PIs) calculated similar to those for population T-maze assays (see Experimental Procedures). PI was calculated as the number of flies biasing towards the untrained odor side (over 5 minutes) minus the number of flies biasing towards the trained odor side (over 5 minutes), divided by the total number of flies. This was calculated for every trial run (16 flies at a time) in our single fly assay. Learning scores were collected 10min after training (10min P (Paired) N=46, 10min UP (Unpaired) N=54), and memory scores were collected 24hr after training (24Hr P(Paired) N=36, 24Hr UP(Unpaired) N=38). Mean and SE are shown. Students T-test ***p<0.005.

D Percent of flies excluded from analysis.

E Flies failing to show adequate movement in the tubes were discarded. Adequate movement was determined by fitting a curve to the histogram of time spent (by each fly) at each location in the behavioral tube. The sigma level of 1.5 was used as the cut-off. Shaded area on plot represents the length of the tube. 3 examples are shown.

F Single fly trajectories for 2 different GAL4 lines at the 10 minute learning time point. Red bar denotes trained odor side.

G,H 10 minute learning scores (**G**) and 24 memory scores (**H**) for each GAL4 line. Blue are Unpaired and Red are Paired samples for each genotype. Box plot contains mean, SEM and Standard Deviation. Student's T-test, *** p<0.001, ** p<0.01, *p<0.05. Sample sizes: G0431-GAL4 N= 213 for 10 minute Unpaired, N=145 10 minute Paired, N= 102 Unpaired 24 hours, N=78 Paired 24 Hours. G0239-GAL4 N=81 10 minute unpaired, N=104 10 minute Paired, N=89 24 hour unpaired, N=75 24 hour Paired. R71D08 N=130 10 minute Unpaired, N=82 10 minute paired, N=76 24 hour Unpaired, N=60 24 hour Paired. R27G01 N=195 10 Minute Unpaired, N=156 10 minute paired, N= 93 24 hour Unpaired, N=76 24 hour Paired. 3-86 N=101 10 minute Unpaired, N=87 10 minute Paired, N=72 24 hour Unpaired, N=79 24 Hour Paired. c739-GAL4 N=143 10 minute Unpaired, N=106 10 minute Paired, N=99 24 Hour Unpaired, N=80 24 Hour Paired. NP1131-GAL4 N=108 10 minute Unpaired, N=90 10 minute Paired, N=99 24 Hour Unpaired, N=87 24 hour Paired.

I Shannon Entropy scores were calculated to reflect the inability to predict the fly's location across the 10 minute learning time point. This was done for all GAL4 lines. Lower values reflect a less distributed location histogram (see **E**), and thus a more predictable location and less entropy. Anova with Tukey HSD **p<0.01.

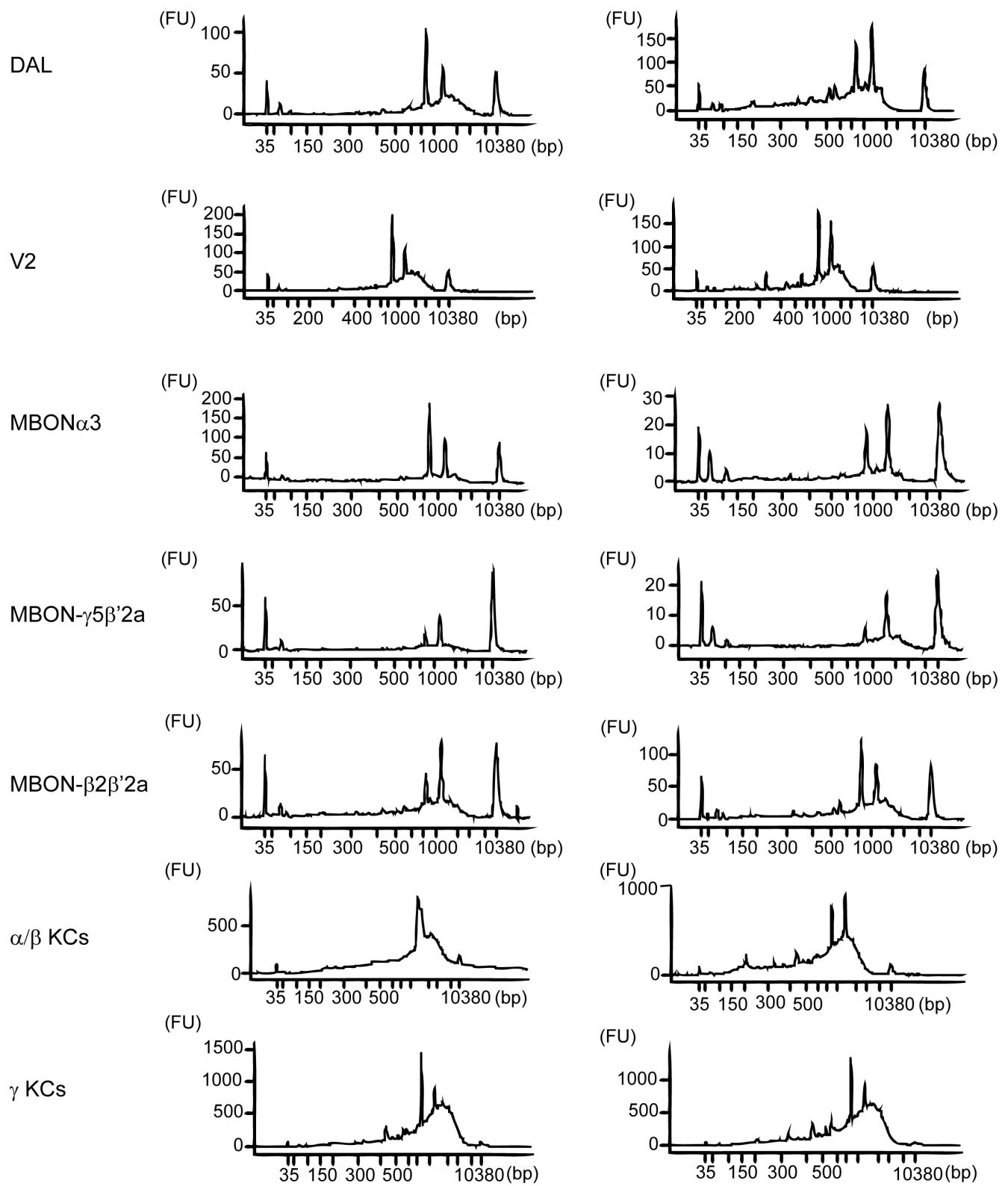


Figure S3. Related to Figure 1.

Two BioAnalyzer traces for each cell type are shown. Amount of product amplified for each sample was determined from these traces by taking the concentration between 4-10 Kb.

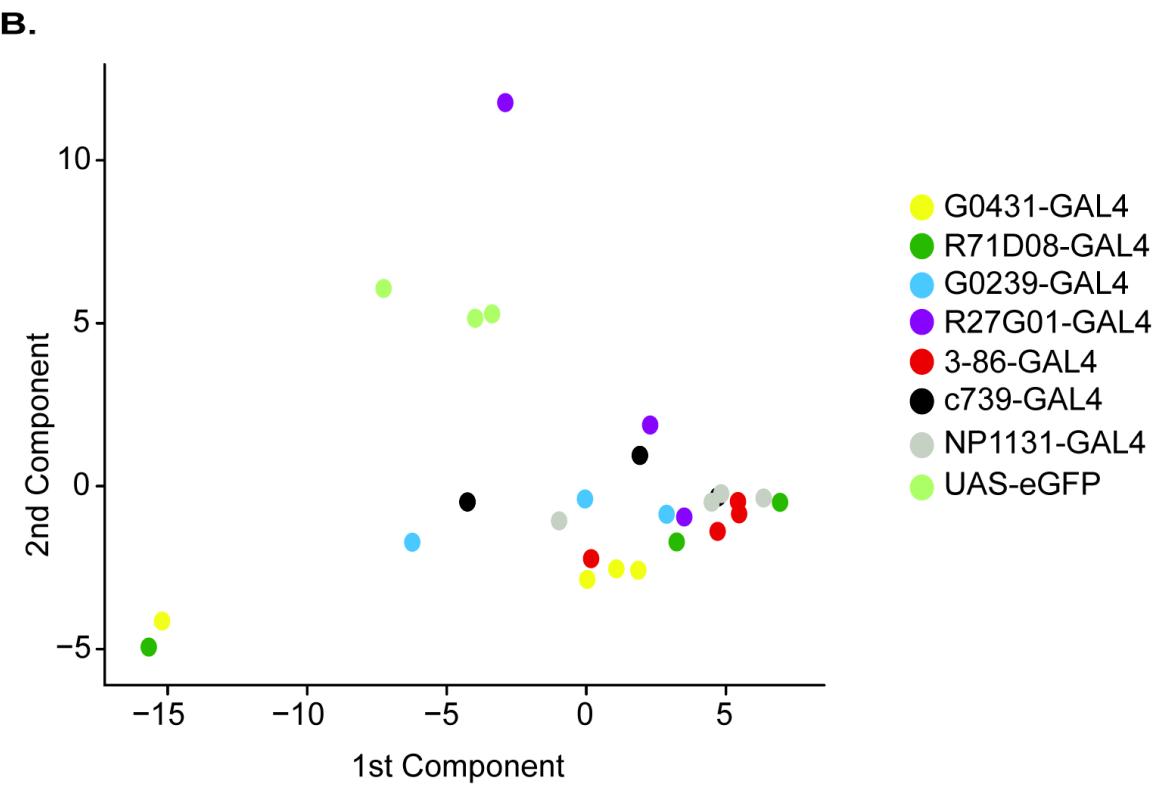
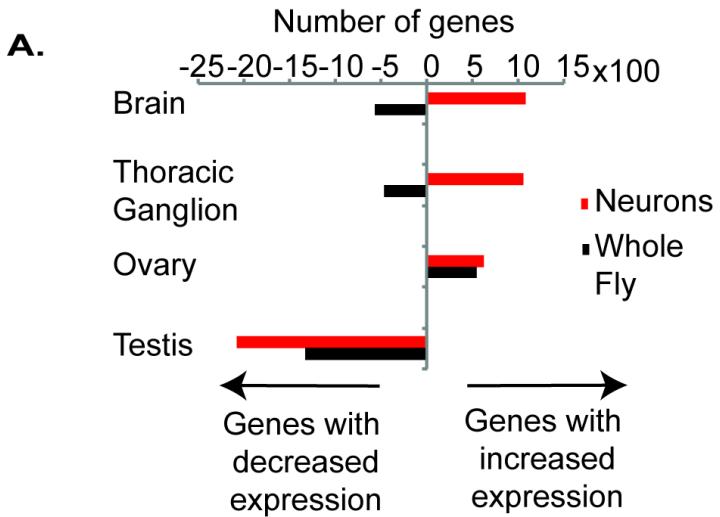


Figure S4. Related to Figure 3.

A Flymine (www.flymine.org) was used to determine tissue-specific gene expression enrichment, based on data from FlyAtlas. The more positive the number, the more genes from our samples (neuronal (red) or whole fly (black)) that are also known to be enriched in that tissue. Negative numbers indicate our samples contained more genes absent or with reduced expression in that tissue. All samples (both neuronal and whole fly) came from female flies.

B PCA using 434 genes found in the Cell Surface Receptor Signaling category (GO:0007166) from whole fly samples (N=27). Whole fly samples came from the same GAL4/UAS-eGFP flies used in single cell-type harvesting and other assays (as indicated by the color scheme).

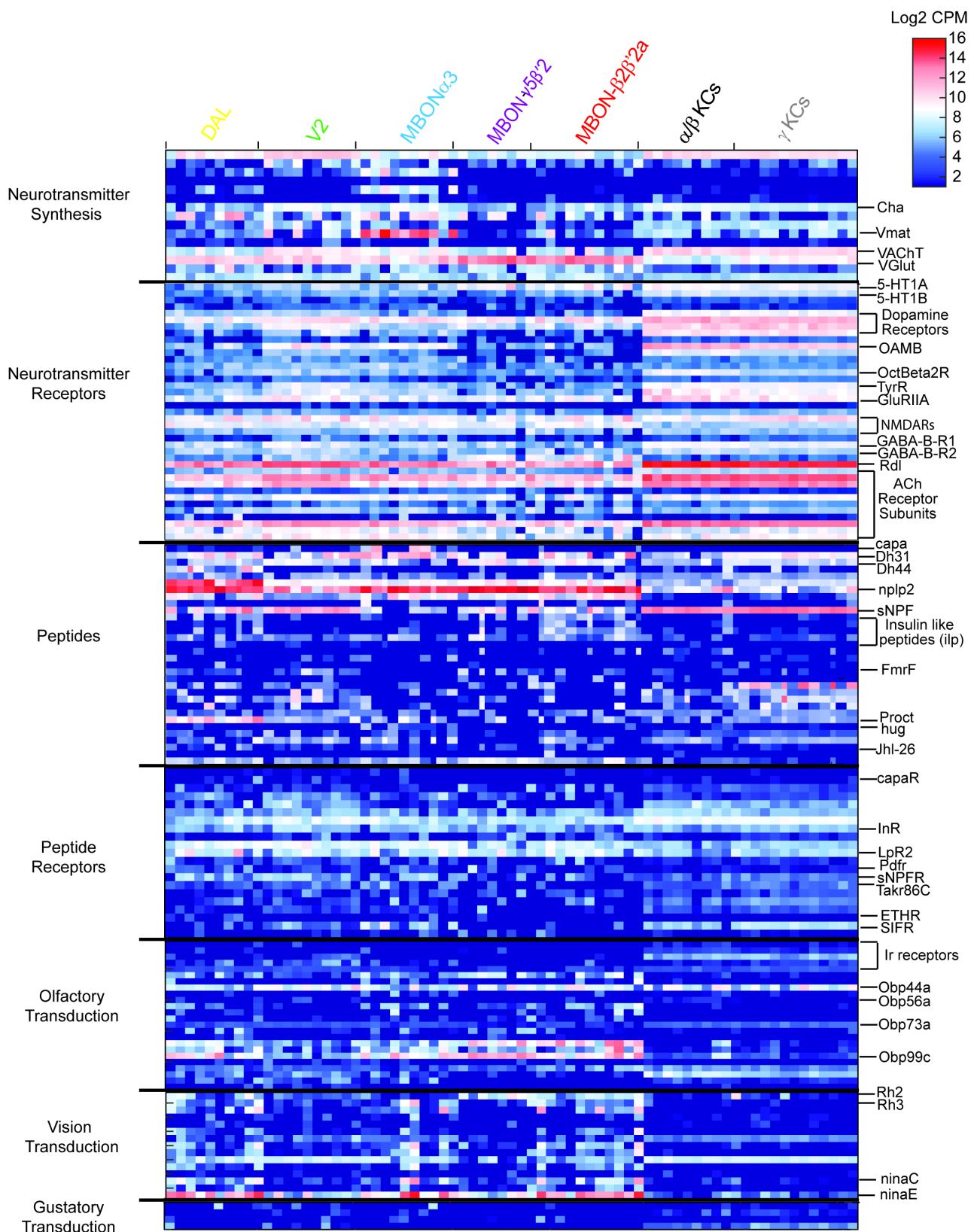


Figure S5. Related to Figure 4.

Raw counts from HTseq-count were converted to counts per million for each sample and then log transformed. The gene list for each section is the same as in Figure 4. Each column represents one sample of the cell type denoted above the column.

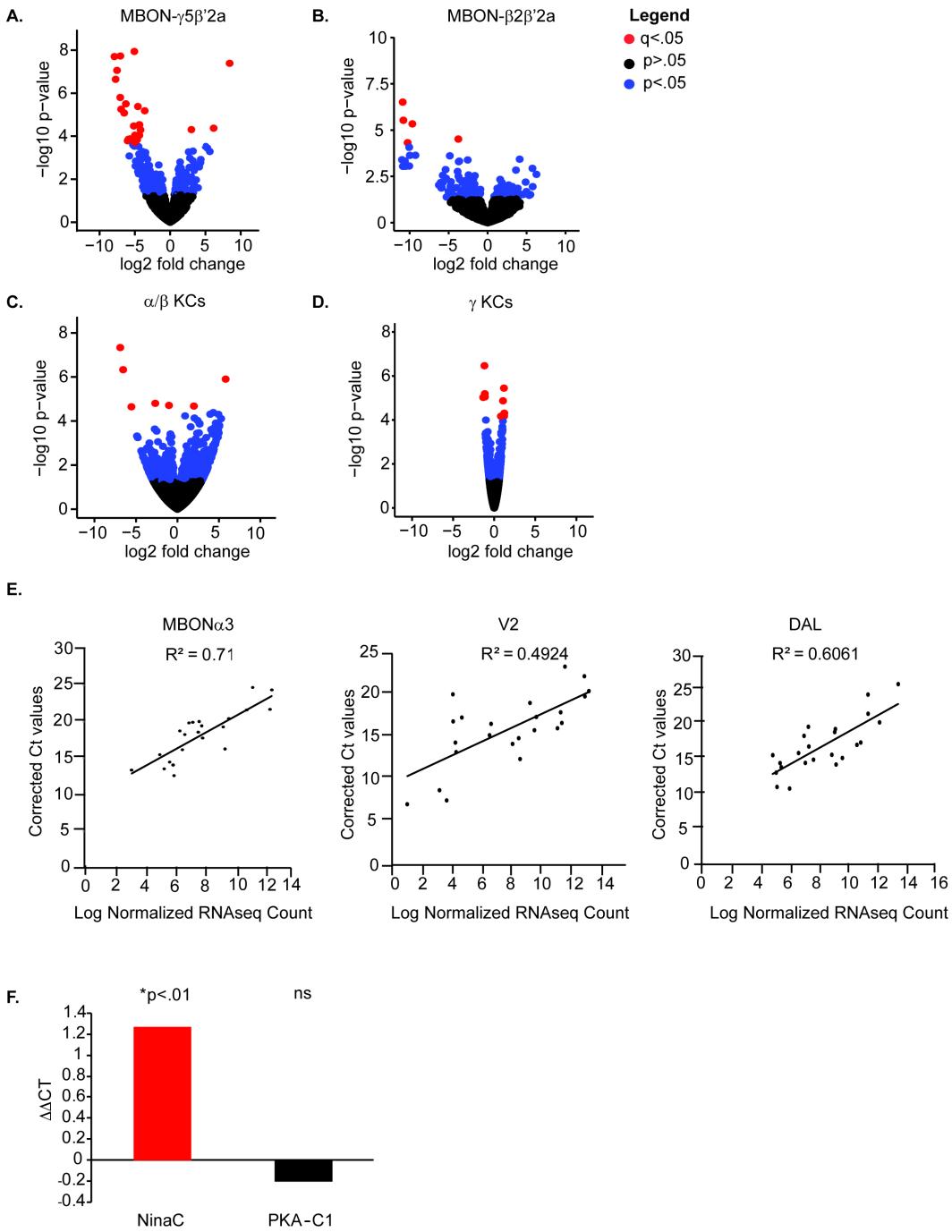


Figure S6. Related to Figure 5.

Differential expression as determined by DESeq2 for (A) MBON- γ 5 β' 2a, (B) MBON- β 2 β' 2mp, (C) α/β KCs, and (D) γ KCS. red dots = q-value < 0.05, blue dots = p-value < 0.05, black dots = p-value > 0.05. Genes differentially expressed are listed in Table S6.

E We found differential gene expression (following LTM induction) in three cell types – here we compare base expression levels of a subset of genes in these three cell types by qPCR versus RNAseq. Mean corrected Ct values are plotted against the mean count value (from HTseq-counts) for a subset of 23 genes. Genes for MBONα3 are: Gpdh, sNPF, Neos, Cpn, CG6254, ninaC, Rh3, rut, Creb-A, ninaE, PKA-C1, orb2, obp83a, ninaA, sNPFR, Rh4, Eaat1, Gaba-B-R3, Tdc2, Gad1, DopR, DopR2, toy. Genes for V2 are: Gpdh, ey, Pinta, Ube3a, Ir68a, Mlc1, toy, rut, Creb-A, ninaE, PKA-C1, orb2, sNPFR, obp83a, Rh3, ninaC, Tdc2, sNPFR, Gad1, Eaat1, DopR, Gaba-B-R3, DopR2. Genes for DAL are: Gpdh, Iris, EndoGI, Brf, Lea, Zfh1, Rh4, obp83a, rut, Creb-A, ninaE, PKA-C1, orb2, sNPFR, ninaC, Pinta, sNPFR, Eaat1, Gaba-B-R3, Tdc2, Gad1, DopR, DopR2.

F We confirmed that the ninaC transcript is differentially expressed in MBONα3 neurons by qPCR. PKA-C1 is shown as a control, as it was not found to be differentially expressed following learning in the RNAseq experiment in this cell type. Genes are normalized to GAPDH, as previously described (Perrat et al., 2013).

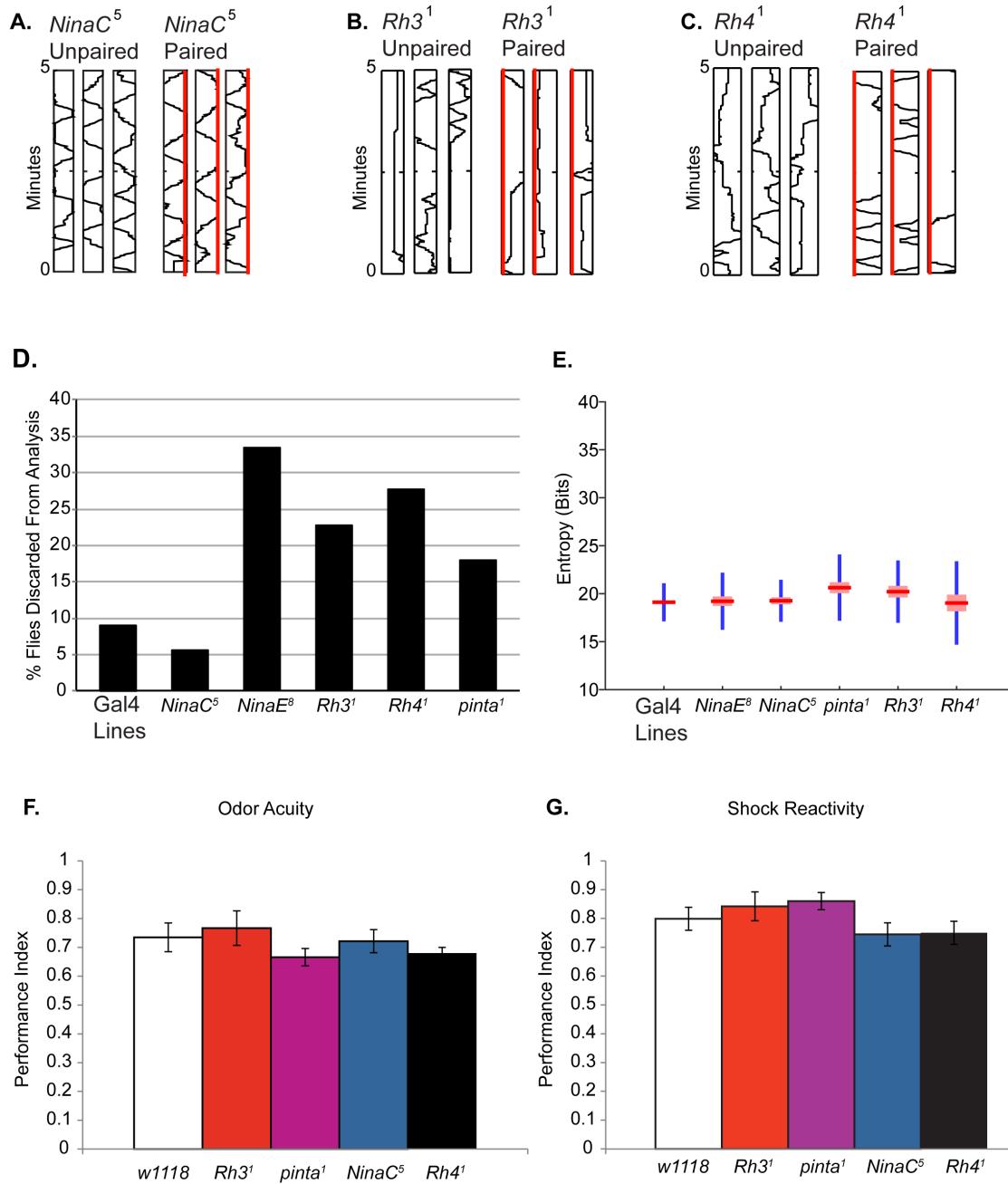


Figure S7. Related to Figure 5.

A-C Single fly behavioral trajectories for 3 Unpaired and Paired flies of the indicated genotype. Red line denotes trained odor side. *Rh3¹* and *Rh4¹* did not move well in the tubes particularly after training.

D Percentage of flies excluded from analysis due to failing to meet the three criteria (see Experimental Procedures). All mutants with the exception of *NinaC⁵* had a high exclusion rate compared to the GAL4 line exclusion rate (see Fig. S2).

E Entropy scores for each mutant line. *Pinta¹* and *Rh3¹* were significantly different from all other lines. Anova and Tukey HSD post-hoc analysis **p<.01.

F Odor acuity for each mutant line displaying a memory defect. All testing was done on populations (see Experimental Procedures), with N=8 trials of either 3-OCT or 4-MCH for each line.

G Shock reactivity for each mutant line displaying a memory defect. All testing was done on populations (see Experimental Procedures), with N=6 trials for each line.

Supplemental Tables

Table S1- PCA Loadings From Figure 2G and 2H

Table S2- Top 50 Go Terms From Neuron and Whole Fly Samples

Table S3- Information Values for the 476 GO Terms in Figure 3C

Table S4- List of Genes Found in Only One Cell Type

Table S5- PCA Loadings From Figure 3E and 3D

Table S6- DESeq2 Results for Each Cell Type

Tables S2, S3, S4 and S6 are too large to reproduce here and are presented as separate Excel Spreadsheets.

Table S1**A. Top 25 Gene loadings driving PCA clustering in Figure 2G**

Genes	PC1	Genes	PC2
yip7	0.063133	CG34402	-0.05385
bcd	0.043814	ey	-0.05382
Mlp84B	0.038986	prt	-0.05095
CG2982	0.038323	CG30158	-0.048
wupA	0.038241	CG32204	-0.047
Nop60B	0.037594	Lgr1	-0.04672
Ag5r	0.037262	CG17639	-0.04617
Cp7Fb	0.036829	DopR2	-0.04591
ball	0.036676	CG15539	-0.0448
dup	0.035781	toy	-0.04458
yin	0.034964	CG9975	-0.04444
CG9135	0.034755	CG7918	-0.04427
Tm2	0.03459	elk	-0.04287
Aph-4	0.03414	CG13055	-0.04237
CG30172	-0.03431	CG6154	-0.04209
CG43222	-0.03405	rad	-0.04199
CG5599	0.033723	Hr51	-0.04182
muskelin	0.033709	PH4alphaNE1	-0.0415
CG5535	0.033368	CG34219	-0.04141
sls	0.033326	Sytalpha	-0.04097
I(2)gl	0.032946	Npc2g	0.040576
I(2)09851	0.032928	CG18067	0.040243
VGlut	-0.03275	CG16836	0.040167
jdp	-0.03261	Or45a	-0.04011
CG7182	0.032586	CG42629	-0.04008

B. Top 25 Gene loadings driving PCA clustering in Figure 2H

Genes	PC1	Genes	PC2
Ubx	-0.255	CG6020	-0.085
Lgr1	-0.08884	RpL8	-0.08317
VGlut	0.086459	sta	-0.08295
CG17838	-0.08519	RpL3	-0.08256
Cda5	-0.08347	RpLPO	-0.08105
qm	-0.08324	RpL7A	-0.08104
crb	-0.08272	CG2469	-0.0788
Eip93F	-0.08046	RpS7	-0.07484
CG2469	-0.07747	RpS18	-0.07194
CG32581	0.077469	awd	-0.07104
CG6300	-0.07728	RpL11	-0.07063
trn	-0.07658	RpL4	-0.06988
CG42392	0.075823	Gycalpha99B	-0.06922
Lmpt	-0.07495	RpS6	-0.06885
Gprk1	0.071323	RpS2	-0.06848
CG43759	-0.07114	RpL17	-0.06812
fok	-0.06848	RpL6	-0.068
Atg8a	0.06746	RpS26	-0.06786
CG34362	-0.06527	RpS4	-0.06733
CG32982	-0.06509	RpL23A	-0.06729
CG42784	-0.06455	Rack1	-0.06696
swi2	-0.06453	RpL18A	-0.06681
zfh2	0.06284	RpS5a	-0.06652
miple	0.062779	RpL19	-0.06647
Pde8	-0.06086	RpS16	-0.06619

Table S5**A. Top 25 Gene loadings driving PCA clustering in Figure 3D**

Genes	PC1	Genes	PC2
Ilp5	-0.04855	Trissin	-0.07473
CG43236	-0.04519	npf	-0.06142
Ilp2	-0.04414	Act57B	-0.05886
Dro	-0.04367	ltd	-0.05825
CG34402	0.042015	CG17109	0.05711
Pbprp2	-0.04073	CG40485	-0.05721
CG42807	-0.03962	Rh3	-0.05538
Sodh-1	-0.03921	Rh4	-0.05507
SPE	-0.03835	trpl	-0.05435
prt	0.038045	CG2444	-0.05348
Mtk	-0.03763	trp	-0.05344
ey	0.037428	Tsp42Ea	-0.05287
DptB	-0.03718	chp	-0.05189
CG13422	-0.03717	DptB	0.050598
CG17639	0.036662	CG18598	-0.05044
Npc2h	-0.03601	Cpn	-0.04852
CG43114	-0.03576	CG43402	-0.0474
CG9396	-0.03571	inaD	-0.04584
bru-2	0.035424	Pbprp3	-0.04497
CG16926	-0.0352	CG32532	0.044959
fon	-0.03515	CG4766	0.044797
CG13055	0.034891	Ast-C	0.044698
CG43103	-0.03488	Tdc2	-0.04369
CG9975	0.034867	retn	-0.04322
CG9336	-0.03483	CG13565	-0.04252

B. Top 25 Gene loadings driving PCA clustering in Figure 3E

Genes	PC1	Genes	PC2
Ilp2	-0.17121	Trissin	0.573928
SPE	-0.16723	npf	0.391733
IM23	-0.14892	itp	0.231478
Ilp5	-0.14781	Proct	0.157937
Lgr1	0.136181	Ilp5	-0.1487
Ast-C	0.132386	Rh4	0.11153
ninaC	-0.13004	CG7918	0.110697
Gbeta76C	-0.12964	Rh3	0.108085
DopR2	0.129238	trp	0.104372
tsl	-0.12571	mtt	0.099625
daw	-0.12185	Lgr1	0.095737
CG7918	0.119371	Spn27A	-0.09526
inaC	-0.11924	Rh6	0.092384
mtt	0.114448	trr	-0.08831
GRHR	-0.11442	Nplp1	0.086418
Dh44-R1	0.114349	DmsR-1	0.083299
Gbp	-0.11429	p38b	0.080684
CG32547	0.104902	D2R	0.079347
Nplp3	-0.10935	mthl8	0.078642
fus	-0.10551	AICR2	0.078272
PGRP-SA	-0.10511	CG33696	0.078007
nec	-0.10395	brk	-0.07731
Oamb	0.103719	Dredd	-0.07667
D2R	0.102506	ninaC	0.076223
dlp	0.100341	Proc-R	-0.07511

Supplemental Experimental Procedures

Fly Lines:

GAL4 lines used in this study: c739 (α/β KCs), NP1131 (γ KCs), 3-86 (MBON- $\beta 2\beta' 2mp$), R27G01 (MBON- $\gamma 5\beta' 2a$), R71D08 (V2), G0431 (DAL), G0239 (MBON $\alpha 3$), R27G01 (49233), G0239 (12639), and G0431 (12837) were ordered from Bloomington. NP1131 was ordered from DGRC. R71D08 was a gift from Dr. H. Tanimoto. 3-86 was a gift from Dr. U. Heberlein. c739 was a gift from Dr. A. Sehgal. UAS-2xeGFP (6874) was ordered from Bloomington. NinaC⁵ (1996), NinaE⁸ (2001) and pinta¹ (24860) mutants were ordered from Bloomington. Rh3¹ and Rh4¹ mutants were a gift from Dr. C. Desplan. All GAL4 lines and the UAS-eGFP line were backcrossed into a *white* (specifically *w1118*) background (which is not a Canton-S derivative). GAL4 lines were crossed to the UAS-eGFP line and progeny (heterozygous for both the GAL4 and UAS-eGFP) used for each experiment. Flies were all raised at 25C in a 12 hour: 12 hour light cycle. Behavioral training was done 3 hours after lights on. Only female flies were used in all experiments.

Anatomical Characterization of MB extrinsic neurons:

Confocal images were acquired on a Zeiss LSM 710 microscope, with an APO 40X objective. All GFP images were counterstained with anti-nc82 (Iowa DSHB), which was diluted to 1:20.

Single Fly Olfactory Learning and Memory Assay:

Flies were first trained as a group. Flies were loaded into a tube with a printed circuit board as described previously (Krashes and Waddell, 2011; Tully and Quinn, 1985); the tube was connected to air and controllable odor flow (0.2 L/minute). A custom-built shock device was used to deliver a 1 minute shock (12 60V shocks, each 5 seconds in duration). To train flies, odor was paired with the shock for one minute using a custom-built olfactometer. The “paired protocol” was as follows: Each session consisted of in sequence: i) 1min shock with odor 1 (12 60V shocks, each lasting 5sec), ii) 1min clean air (rest period), and iii) 1min odor2 without shock. We repeated this protocol for 8 sessions, with 10 min clean air between sessions, for a total training time of 104min. This protocol is widely used for inducing long-term memories (e.g., see (Krashes and Waddell, 2011) and (Tully et al., 1994; Yin et al., 1995)). The “unpaired protocol” was as follows: Each session consisted of in sequence: i) 1min shock (12 60V shocks, each lasting 5sec), ii) 1min clean air (rest period), iii) 1min odor 1 without shock, iv) 1min clean air (rest period), and v) 1min odor 2 without shock. We repeated this protocol for 8 sessions, with 10 min clean air between sessions, for a total training time of 120min.

To assay learning or memory in single flies, we used the DAMS multibeam activity monitor (Fig. S2) (Trikinetics, Waltham MA) (Hamblen et al., 1986). Each tube is 81 mm long and flies could move about in the central 53 mm of the tube. This distance was covered by 17 infrared beams, to track the location of the fly throughout the duration of the experiment (Fig. S2). The use of beam crossings as a measure of fly position within the tube eliminated the need to video record the movements of individual flies (Claridge-Chang et al., 2009). Each tube had a 1 mm vacuum hole in the center. At the ends of the tubes, air entered; flow rate was modulated depending on the fly strain used (0.01-.028 L/min). The 3-OCT odor side was always 0.002 L/min less than the 4-MCH side, because we found that this balanced the exploration of both sides of the tube by WT flies, while still allowing us to use the concentration of 1:100 of each odor in paraffin oil. Behavioral testing was performed in the dark at 23C and 40% relative humidity. All flies were tested 10 minutes after the end of the spaced training sessions to score immediate learning. This test lasted 5 minutes. A subset of flies that were not tested for immediate learning were kept and placed back on food to test the next day for 24 hour memory formation. Entropy calculations in Figure S3 and Figure S10 were calculated in MATLAB using the *ksdensity* function to fit a line to a histogram (for amount of time spent at each location in the behavior tube). We then calculated Shannon’s entropy from this function. Anova with Tukey HSD was used to compare entropy values across lines.

For cyclohexamide experiments, flies (R71D08, UAS-eGFP) were either given 35mM cyclohexamide, 5% sucrose and 3% ETOH solution for 12-16 hours prior to training or only 5% sucrose and 3% ETOH, as previously described (Tully et al., 1994). These flies were either immediately tested for learning, or returned to normal food following the spaced training protocol, to be tested 24 hours later for memory formation.

Odor acuity and shock reactivity testing was performed as previously described (Skoulakis and Davis, 1996; Tully et al., 1994). A population T-maze was used to test odor acuity: clean air was run through one arm, while the other arm contained either 3-OCT or 4-MCH using the same air flow settings described above. Performance indices were calculated as the number of flies on the clean air side minus the number of flies on the odor side, divided by the total number of flies (Skoulakis and Davis, 1996). Results were pooled across both odors and plotted in Fig. S10 (N=8 for each genotype). For shock reactivity, a population T-Maze was set up with a shock grid in either arm, with one of the two administering the shock protocol for 120 seconds. The number of flies in the non-shocked arm minus those in the shocked arm over the total population was used to calculate the performance indices (Tully et al., 1994). An Anova with Tukey HSD was used to compare odor acuity and shock reactivity across mutant fly lines.

Learning and Memory Score Calculations:

Individual fly movement trajectories were calculated for each fly over 5 minutes using a custom MATLAB (Mathworks, Inc.) script. Odor preference (Learning or Memory score) was calculated as the percent of time over the 5 minutes spent in odor space 1 versus odor space 2. The center beam location as well as the two flanking it (beams 8-10) were removed from analysis because the vacuum manifold was over these locations, and so this space could not be categorized as odor 1 or odor 2. Flies that only spent time on one side of the tube or did not cross beams at least 30 times were removed from analysis. Individual flies were trained with either 3-OCT or 4-MCH and odors were switched between sides of the tubes on a given trial to correct for any location effects. All analysis was done in MATLAB or R(RCoreTeam, 2013). All learning and memory scores were normally distributed as determined by Kolmogorov-Smirnov test and the students t-test was used to determine significance. A more traditional performance index (similar to the 2min performance index used for flies in a standard T-maze apparatus) was also calculated as the number of flies biasing towards the unpaired odor stream minus the number of flies biasing toward the paired odor stream, divided by the total number of flies tested (see Fig. S2). A students t-test was also used to determine significance in the traditional performance index scoring.

Electrophysiology:

Animals were anesthetized in a glass vial on ice for a minute and then fixed to a custom cut plastic holder with wax. They were mounted so their antennae, proboscis and underside were open under the chamber and accessible to odor delivery. The proboscis was stabilized with a small amount of wax. A small opening was cut in the back of the head to allow access to the brain. Perineural sheath was carefully removed with forceps (no collagenase was used). Some head muscles were removed in order to stabilize the movement of the brain. Oxygenated extracellular saline was perfused over the brain during dissection and during recordings (see (Murthy and Turner, 2013) for more details). Patch clamp recordings were performed as previously described. Loose cell pipets were pulled with a resistance of 4-5 MΩ, whole cell pipets were pulled with a higher resistance ~5-6MΩ. All physiology was performed under visual control using IR-DIC optics and GFP fluorescence on a Olympus BX51WI using a 40X water immersion lens. Loose cell recordings were done in voltage clamp mode while whole cell recordings were done in current clamp mode using MultiClamp-700B amplifier. Signals were high-pass filtered at 2KHz, and acquired in IGOR Pro (Wavernetrics, Inc). The head stage resistor was set to 50 MΩ. Odor delivery was performed as described previously (Murthy and Turner, 2013) and all odors were used at 1:100 dilutions in Paraffin oil (Sigma-Aldrich).

Harvesting Individual Neuron Types for RNA Sequencing:

Flies showing the strongest learning scores (< -50) were selected for dissection within 30 minutes of spaced training (see above). This delay includes the 5 minutes to score learning and roughly 15 minutes for dissecting the head cuticle for cell harvesting. Care was taken to minimize the opening in the cuticle necessary to harvest cells. Using an unpolished patch pipet (size varied due to cell size, but each tip was matched to the size of the cell), GFP labeled cells were hand picked via suction. In the case of MBON- β 2 β' 2mp and MBON- γ 5 β' 2a neurons, the relevant GAL4 line labels nearby non-MBONs. We were able to fill these neurons with a dye (followed by imaging) to practice identifying the anatomical location of the soma relative to nearby neurons and structures. The MBON- β 2 β' 2mp is located anterior to the Pars Intracerebralis (PI) neurons and when looking at the PI region with the brain slightly tipped back, the processes can be visualized, making them distinguishable from nearby cells. The MBON- γ 5 β' 2a is distinguishable because it is the larger of the two visible cells in the PI region of the brain. Cells designated for sequencing were harvested into 0.5ul nuclease free water in the pipet tip and then the tip was broken into a 96 well PCR tube containing RNase inhibitors and buffer as described in Clontech's HV SMARTer Ultra Low RNAseq kit (Catalog# 634823). This caused the cell to lyse without mechanical means. Amplification was performed as described in the Clontech Ultra-Low high volume SMARTer RNAseq Protocol. qPCR experiments were performed on separate cell isolates. If cells were being pulled for qPCR then the tips were broken into tubes containing the buffer described by Life Technologies single-cell to CT kit (Catalog# 4458237). For the DAL neuron, the MBON α 3 neurons, the MBON- γ 5 β' 2a neurons, and MBON- β 2 β' 2a neurons, 4 cells were pooled into each tube; thus, these samples contained cells from more than one fly. For the V2 neurons, α/β KCs and γ KCs, all cells were taken from one animal per sample. V2 samples contained 14 cells and the α/β KC and γ KC samples each contained about 100 cells. 15 rounds of PCR amplification were performed using the Clontech SMARTer Ultra low RNAseq Kit and 18 rounds for qPCR.

cDNA Library Preparation and Sequencing:

Following amplification, samples were run on an Agilent BioAnalyzer using a high sensitivity DNA chip. Histograms of base pair (bp) length at each fluorescent unit were used to remove samples of poor quality. We looked for a peak around 7 kb and then looked for .4-2ng/ul of product between the range 400bp-10kb. Samples lacking this peak or samples that were biased toward short length products or had many small peaks were not used. Samples were then sheared using a Covaris LE220 sonicator to 200bp fragments. The libraries were made using IntegenX's Apollo 324 automated library prep system. It used the PrepX Illumina DNA library prep kit/PrepX CHIPseq kit (WaferGen Biosystems Inc) and ran for 17-22 PCR cycles. Samples were then barcoded (Bio Scientific). Samples were checked again on the Bioanalyzer and cleaned up using the IntegenX PCR cleanup kit. Libraries were run on the Illumina HiSeq2500, 12 samples per lane, and each sample run across two lanes. This resulted in a sequencing depth of 30 million reads. Preliminary sequencing runs on DAL samples (not included in analysis)

determined 30 million reads was sufficient for depth and increasing depth did not increase complexity. Sequencing was single-ended. In total 143 samples were sequenced (19 DAL samples, 12 V2 Samples, 12 MBON α 3 samples, 21 MBON- β 2 β' 2a samples, 12 MBON- γ 5 β' 2a, 16 α/β KC samples (two technical replicates included), and 12 γ KC samples, 2 were whole brain samples and 34 control whole fly samples). Whole fly samples came from flies of the same genetic background (GAL4 and UAS-GFP), and whole brain samples came from flies containing only the UAS-GFP transgene. To collect cells for the technical replicate, approximately 200 cells were collected and lysed, then the sample was split and cDNA and PCR amplification was performed in parallel.

Analysis of Sequencing Reads:

Initial quality control of sequencing runs was performed to look for excess primer sequence presence (through FastQC (Andrews, 2012) on the raw illumina reads). Samples all contained a bias for polyA and T sequences. This was uniform across all samples and was removed from sequences prior to mapping using the Cutadapt program in Galaxy. We also examined the number of hexamer sequences over represented in the samples (due to primers and barcode sequences used in the library preparation), and removed these prior to mapping. After passing initial quality control, in Galaxy, sequences were mapped to the fly genome using TopHat2 with Bowtie2 (Kim et al., 2013; Langmead and Salzberg, 2012). The reference sequence and the gene transfer file (GTF) formatted files were taken from Ensembl (Flicek et al., 2013). BDGP 5.74 was the Fly GTF file used and the reference genome was BDGP 5.25. Tophat2 settings can be found in the Galaxy Settings Section at the end of the Supplemental Experimental Procedures.. The aligned SAM/BAM file (SAM stands for Sequence Alignment/Map file and BAM is the binary form of the SAM file) was processed using HTseq-count (Anders et al., 2015) to map exons and determine gene counts. Intersection mode (strict) was used to map exon junctions and gene overlaps. SAM-stats were also exported to look for 3' prime biases and other confounds. Raw sequencing data is in submission to GEO (Main accession number is GSE74989, and individual accession numbers found at the end of the main text)..

Gene Expression Analysis:

Samples needed to contain more than 4 million counts following HTseq-count mapping to be included in analysis. This 4 million counts cutoff also sets a minimum count per gene for it to be considered present. This removed 4 DAL samples, 1 V2 sample, 2 α/β KC samples, 1 MBON- γ 5 β' 2a, 3 MBON- β 2 β' 2a. All genes with counts less than 2 counts per million (8 counts) were considered noise and removed from analysis. Pairwise distance matrices were generated in MATLAB using the *pdist* function: from this analysis we removed additional neuronal samples that were more closely matched to whole fly samples or were very different from all other samples. We also removed the preliminary DAL samples used to look at sequencing depth. This removed 3 MBON- γ 5 β' 2a, 5 DAL samples, 1 V2 sample, 2 α/β KC samples, 2 MBON α 3 samples and 9 MBON- β 2 β' 2a. We were left with 10 DAL samples, 10 V2 samples, 10 α/β KC samples, 12 γ KC samples, 10 MBON α 3 samples, 8 MBON- γ 5 β' 2a, 9 MBON- β 2 β' 2mp for further analysis. The DAL samples contained 5 Paired and 5 Unpaired conditions, V2 contained 5 Paired and 5 Unpaired, the α/β KC samples contained 5 Paired and 5 Unpaired, the γ KC samples contained 6 Paired and 6 Unpaired, the MBON α 3 samples contained 5 Paired and 5 Unpaired, the MBON- γ 5 β' 2a contained 4 Paired and 4 Unpaired and the MBON- β 2 β' 2a contained 4 Paired and 5 Unpaired. In all analyses on high expressing genes, we defined high expressing genes as any gene with a count of at least 50 counts per million (if the minimum for including a sample was 4 million mapped counts, then the threshold was increased to counts of 200 or higher).

Principal Components Analysis:

Principal components analysis (PCA) was performed on normalized gene counts using DESeq2 (Love et al., 2014)(regularized log transformation of normalized data) after removing genes with less than 2 CPM in the α/β KC samples. The R function *prcomp* was used to generate the principle components. For Figure 2A-H, 7234 genes were used for analysis. In Figure 3D-E and Fig. S4, the 434 genes in GOTerm Cell Surface Receptor Signaling (GO:0007166) were used to create the plot.

Gene Ontology Analysis:

The list of genes was generated by determining genes present at values >50 CPM in neuronal samples (4233 genes) and whole fly samples (2097 genes). We also determined a list of all unique genes -- present in only one cell type (576 genes), and a list of all up-regulated genes at a q-value <0.1 in the DAL, MBON α 3, V2 following learning (235 genes). Flymine (<http://www.flymine.org/>) and Princeton University's GOTermFinder (<http://go.princeton.edu/cgi-bin/GOTermFinder>) was used to analyze each list (Boyle et al., 2004; Lyne et al., 2007). Flymine generated the list of GOTerms for the neuronal samples, whole fly samples and unique gene list. P-values represent the probability that we see that number of genes in a given GO term when accounting for the size of the list and the number of genes present in that GO term in the entire genome. A Holm-Bonferroni correction was used to calculate adjusted p-values <0.05 for the neuronal and whole fly samples. Group identity was determined using the Lin measure in Revigo (<http://revigo.irb.hr/>). There were 475 terms with adjusted p-values <0.05 and these each contained more than 100 genes. We removed terms with less than 100 genes because we found these terms were redundant with other larger lists. Only the terms with p-value < 0.2 are shown in Figure 3C (we were only looking for GOTerms that contained the most genes from all the cells not enrichment). Thus we did not do any correction for p-value. Princeton University's GOTermFinder (<http://go.princeton.edu/cgi-bin/GOTermFinder>) was used to identify GOTerm

enrichment for the 235 gene list following learning. A Bonferroni correction was used and all p-values in Table 2 are shown as adjusted p-values <0.05.

Classification of Cell Types:

All counts were transformed using a regularized log normalization (using DESeq2) taking into account cell type. All genes with no counts in any sample type were removed from analysis. Any gene determined not present based on expression in less than half the samples of that cell type, was recoded as zero for that cell type. The counts (of all genes or of subsets defined by GO terms) were used to infer cell type from the expression data. To that end, the dissimilarity of expression profiles was quantified using the city block metric. This yielded a distance matrix, holding the distance between all pairs of samples (Fig. 3A). Then, a nearest neighbor classifier was used to assign each sample's gene expression profile to a cell type. That is, a single sample of each cell type was used as a template and then the remaining, non-template samples were assigned to the cell type their nearest template belonged to (see Clemens et al., 2011). This was iterated 1000 times, each time with random sets of templates. The results of the nearest neighbor classifier were tabulated in a confusion matrix $p(x,x')$, which contains the probability that a sample coming from cell type x was assigned to cell type x' . The diagonal entries of the confusion matrix correspond to correctly assigned samples; off diagonal elements represent falsely assigned samples. The mutual information of this confusion matrix yields a lower bound of the mutual information between expression profiles and cell type: $I(x,x') = \sum_{x,x'} p(x,x') \log_2[p(x,x') / p(x) / p(x')]$ (see Clemens et al. 2011). An upper bound is given by the entropy of samples $H(x) = -\sum p(x) \log_2(p(x)) = 2.81$ bits and is achieved by e.g. perfect classification. The minimal information is 0 bits and corresponds to a uniformly distributed confusion matrix.

Heatmap and Z-score of Gene Counts

Z-scored normalized count means per sample type were calculated in R using the *scale* function and maintaining the center and scale as TRUE. This was done after turning HTseq-count values for every gene to CPM value. Heatmap of average z-score for each cell group was plotted using the *gplot heatmap* function in R.

Differential Expression

Due to confounds introduced by having very different sample sizes (e.g., 100 cells/sample for KCs but only 4 cells/sample for some MBONs) and different soma sizes for different cell types, we only examined differential expression within the same cell type. Differential expression was determined using DESeq2 (version 1.1.3) with the setting *betaPrior = "FALSE"* in R. The selection of DESeq2 was based on the ability of the program to handle batched data. We found batch effects based on parents (which vials flies were taken from) and amplification day. To account for this, trained samples were only compared to control (unpaired) samples amplified on the same day or from the same parents. Instead of using the adjusted p-value built into DESeq2 we determined the q-value for all our p-values. We removed extreme outliers or highly variable genes using Cooks distance, used trimmed means to replace a single outlier, and removed data with no information from the analysis, minimizing unnecessary statistical tests (Love et al., 2014).

qPCR Analysis

The single cell to CT kit from Life Technologies in combination with the Taqman assays (Life Technologies) was used to quantify gene abundance. New cell isolates were collected for each cell type tested (V2 n=7 (all unpaired); MBON α 3 n= 4 paired and n=4 control; DAL n=4 (all unpaired)). A custom GAL4 taqman primer was developed and the sequence is included in Supplemental Experimental Procedures. The genes examined were: (for MBON α 3) Gpdh, sNPF, Neos, Cpn, CG6254, ninaC, Rh3, rut, Creb-A, ninaE, PKA-C1, orb2, obp83a, ninaA, sNPF, Rh4, Eaat1, Gaba-B-R3, Tdc2, Gad1, DopR, DopR2, toy, V2 are: Gpdh, ey, Pinta, Ube3a, Ir68a, Mlc1, toy, rut, Creb-A, ninaE, PKA-C1, orb2, sNPF, obp83a, Rh3, ninaC, Tdc2, sNPF, Gad1, Eaat1, DopR, Gaba-B-R3, DopR2. (for DAL): Gpdh, Iris, EndoGI, Brf, Lea, Zfh1, Rh4, obp83a, rut, Creb-A, ninaE, PKA-C1, orb2, sNPF, ninaC, Pinta, sNPF, Eaat1, Gaba-B-R3, Tdc2, Gad1, DopR, DopR2. All reactions were run in 10ul reaction volumes in 384 well plates. Plates were run on an ABI 7900. Gpdh was used as the control gene for all ddCT calculations.

Galaxy settings for TopHat2 and HTseq-counts:

TopHat2 Settings:

Is this library mate-paired?

Single-end

RNA-Seq FASTQ file

Output dataset 'output' from cutadapt

Use a built in reference genome or own from your history

Use a built-in genome

Select a reference genome

ensembl_dmel_bdgp5.25

TopHat settings to use

Full parameter list

Max realign edit distance
1000
Max edit distance
2
Library Type
FR Unstranded
Final read mismatches
2
Use bowtie -n mode
No
Anchor length (at least 3)
8
Maximum number of mismatches that can appear in the anchor region of spliced alignment
0
The minimum intron length
70
The maximum intron length
500000
Allow indel search
No
Maximum number of alignments to be allowed
1
Minimum intron length that may be found during split-segment (default) search
50
Maximum intron length that may be found during split-segment (default) search
500000
Number of mismatches allowed in each segment alignment for reads mapped independently
2
Minimum length of read segments
25
Use Own Junctions
Yes
Use Gene Annotation Model
Yes
Gene Model Annotations
select at runtime
Use Raw Junctions
No
Only look for supplied junctions
Yes
Use Coverage Search
No
Use Microexon Search
No
Do Fusion Search
No
Set Bowtie2 settings
No
Specify read group?
No

HTseq-Counts Settings:

Aligned SAM/BAM File
Output dataset 'accepted_hits' from TopHat
GFF File
select at runtime
Mode
Intersection (strict)
Stranded
No

Minimum alignment quality

10

Feature type

exon

ID Attribute

gene_name

Additional BAM Output

False

qPCR Taqman Primers from Life Technologies:

Gene	AssayID
ey	Dm01820714_m1
Pinta	Dm02143224_g1
Ube3a	Dm01802270_g1
Ir68a	Dm01802284_g1
Mlc1	Dm02134518_g1
toy	Dm01825584_m1
rut	Dm01813542_g1
CrebA	Dm01798688_m1
ninaE	Dm02136091_g1
PKA-C1	Dm01803876_m1
orb2	Dm02372502_s1
sNPF	Dm01811108_g1
obp83a	Dm02135386_g1
Rh4	Dm01841465_m1
Rh3	Dm02136102_s1
ninaC	Dm01803394_g1
ninaA	Dm01841365_g1
sNPFR	Dm01823178_m1
Eaat1	Dm01803826_g1
Gaba-B-R3	Dm01814156_g1
Tdc2	Dm01811652_m1
Gad1	Dm01822042_m1
DopR	Dm02134812_m1
DopR2	Dm02151743_g1
Iris	Dm01800461_s1
EndoG1	Dm01791741_g1
Brf	Dm02141511_m1
Lea	Dm01843958_m1
Zfh1	Dm02134683_m1
Neos	Dm01822286_g1
cpn	Dm02332164_g1
CG6254	Dm02138635_g1
Gpdh	Dm01841185_m1

The GAL4 Taqman (Life Technologies) primer was custom designed with the following sequences:

Forward: TTATGCCAGGGATGCTCTT

Reverse: GCCATCCGACATGTCATCCT

Supplemental References:

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