# Figure S1

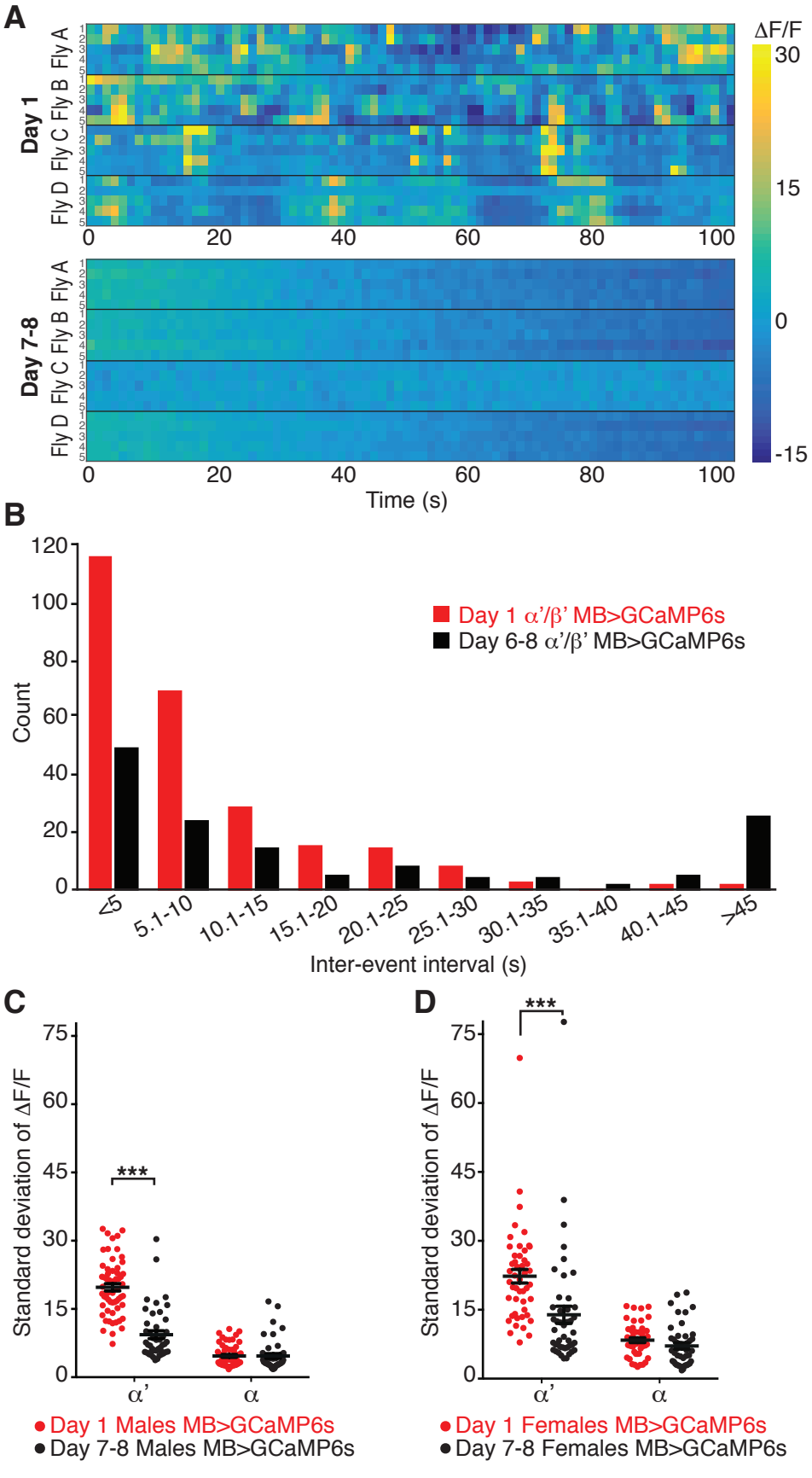
A
Day 1
Day 7-8
Fly A
Fly B
Fly C
Fly D
ΔF/F
30
15
0
-15
0
20
40
60
80
100
Time (s)
B
Day 1 α'/β' MB>GCaMP6s
Day 6-8 α'/β' MB>GCaMP6s
Count
120
100
80
60
40
20
0
<5
5.1-10
10.1-15
15.1-20
20.1-25
25.1-30
30.1-35
35.1-40
40.1-45
>45
Inter-event interval (s)
C
D
Standard deviation of ΔF/F
75
60
45
30
15
0
***
α'
α
Day 1 Males MB>GCaMP6s
Day 7-8 Males MB>GCaMP6s
Day 1 Females MB>GCaMP6s
Day 7-8 Females MB>GCaMP6s

**Figure S1. Males and females have age-dependent decreases in α'/β' KC neural activity. Related to figure 1.**

**(A)** Heatmaps of ΔF/F of GCaMP activity in single KC claw ROIs of day 1 (top) or day 7-8 (bottom) flies over time with warmest and coolest colors representing the largest changes in activity from the average over the entire recording. 5 ROIs from most variable claws shown (1 row is one ROI) for each of 4 representative flies per age.

**(B)** Binned counts of all inter-event intervals in one representative α'/β' KC ROI per fly with more frequent shorter interval events occurring in young day 1 flies (n=44 flies per age).

**(C and D)** Standard deviation of ΔF/F reflects baseline activity levels in ROIs containing α' or α KCs in day 1 or day 7-8 (**C**) male flies or (**D**) mated females flies. Each point represents median ROI from 1 fly; error bars are mean and SEM. ***P<0.001, 2-way ANOVA with significant lobe and age effects and Sidak's multiple comparisons test.

# Figure S2

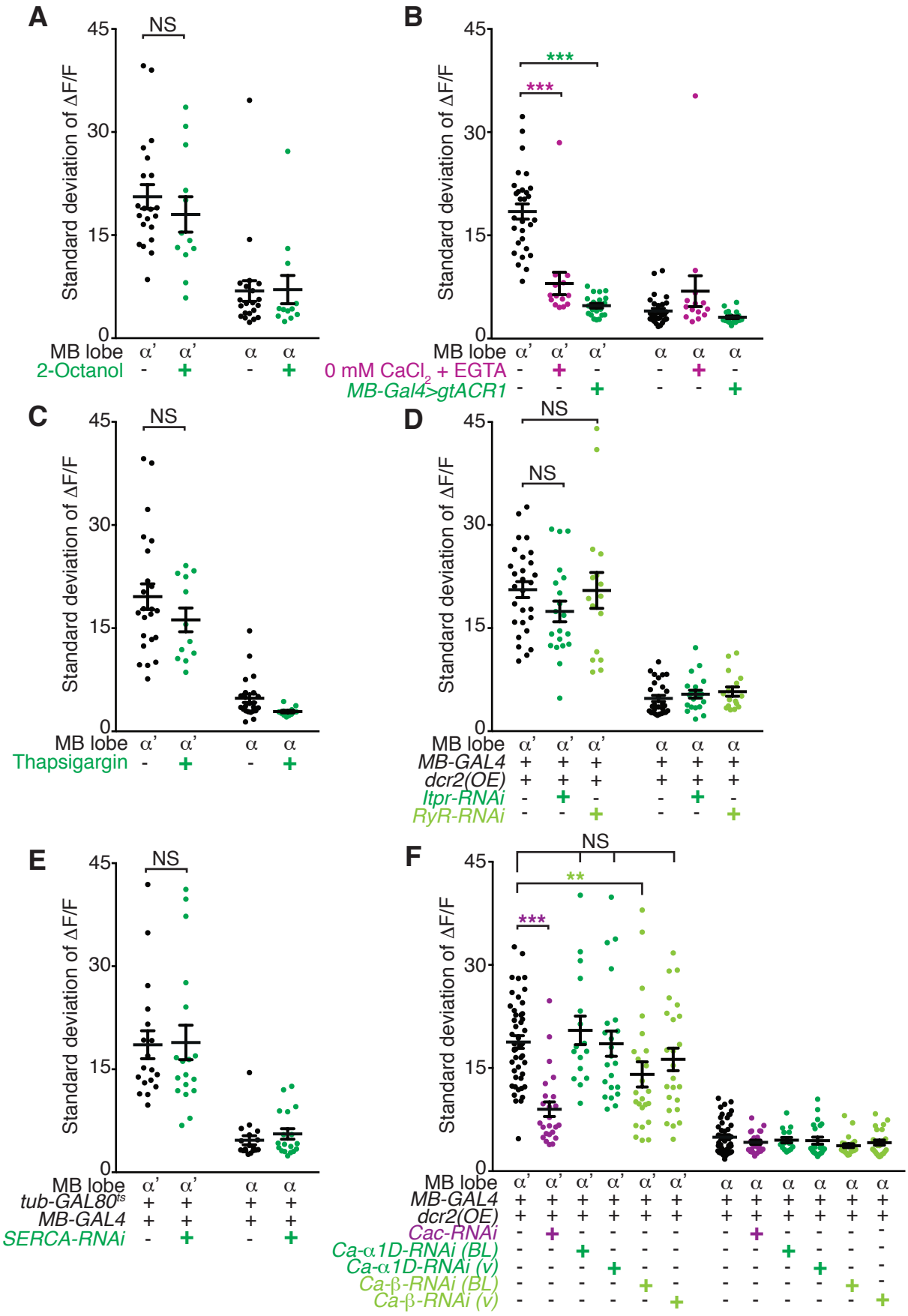
A
B
C
D
E
F
Standard deviation of ΔF/F
NS
***
MB lobe
2-Octanol
0 mM CaCl2 + EGTA
MB-Gal4>gtACR1
Thapsigargin
MB-GAL4
dcr2(OE)
Itpr-RNAi
RyR-RNAi
tub-GAL80ts
SERCA-RNAi
Cac-RNAi
Ca-α1D-RNAi (BL)
Ca-α1D-RNAi (v)
Ca-β-RNAi (BL)
Ca-β-RNAi (v)

**Figure S2. Spontaneous α'/β' KC activity requires voltage-gated calcium channels, not calcium release from internal stores. Related to figure 2.**

**(A)** KC GCaMP activity with or without 2-octanol perfusion (10 mM, beginning 10 minutes prior to assay) to inhibit gap junctions.

**(B)** KC activity imaged with normal artificial hemolymph solution or in artificial hemolymph solution lacking calcium chloride with added EGTA (2 mM) or upon continuous hyperpolarization of KCs with gtACR1 and light from the imagining 488 laser.

**(C)** KC GCaMP activity with or without thapsigargin perfusion (20 μM, beginning 15 minutes prior to assay) to inhibit SERCA pump for calcium release from internal stores.

**(D)** Activity of KCs expressing *UAS-RNAi* and *UAS-dcr2* transgenes to target the intracellular calcium channels in the endoplasmic reticulum, *inositol 1,4,5-tris-phosphate receptor* (*Itpr*) and *ryanodine receptor* (*Ryr*).

**(E)** Activity of KCs expressing *UAS-SERCA-RNAi* with *tub-GAL80$^{ts}$* and shifted to 30 degrees 48 hours prior to imaging to temporally restrict the knockdown to the peri-eclosion time period.

**(F)** Activity of KCs expressing *UAS-RNAi* and *UAS-dcr2* transgenes to target *Cac, Ca-α1D* and *Ca-β*. RNAi labeled (v) from Vienna *Drosophila* Resource Center and (BL) from Bloomington *Drosophila* Stock Center.

**(A-F)** Day 1 male flies imaged *in vivo*. Each point is median ROI for indicated lobe from 1 fly; error bars are mean and SEM. N=6-23 flies with each hemisphere of the brain shown as one point. NS, P>0.05 and ***P<0.001,**P<0.01, 2-way ANOVA with Sidak's multiple comparisons test. All comparisons for α lobe are NS.

# Figure S3

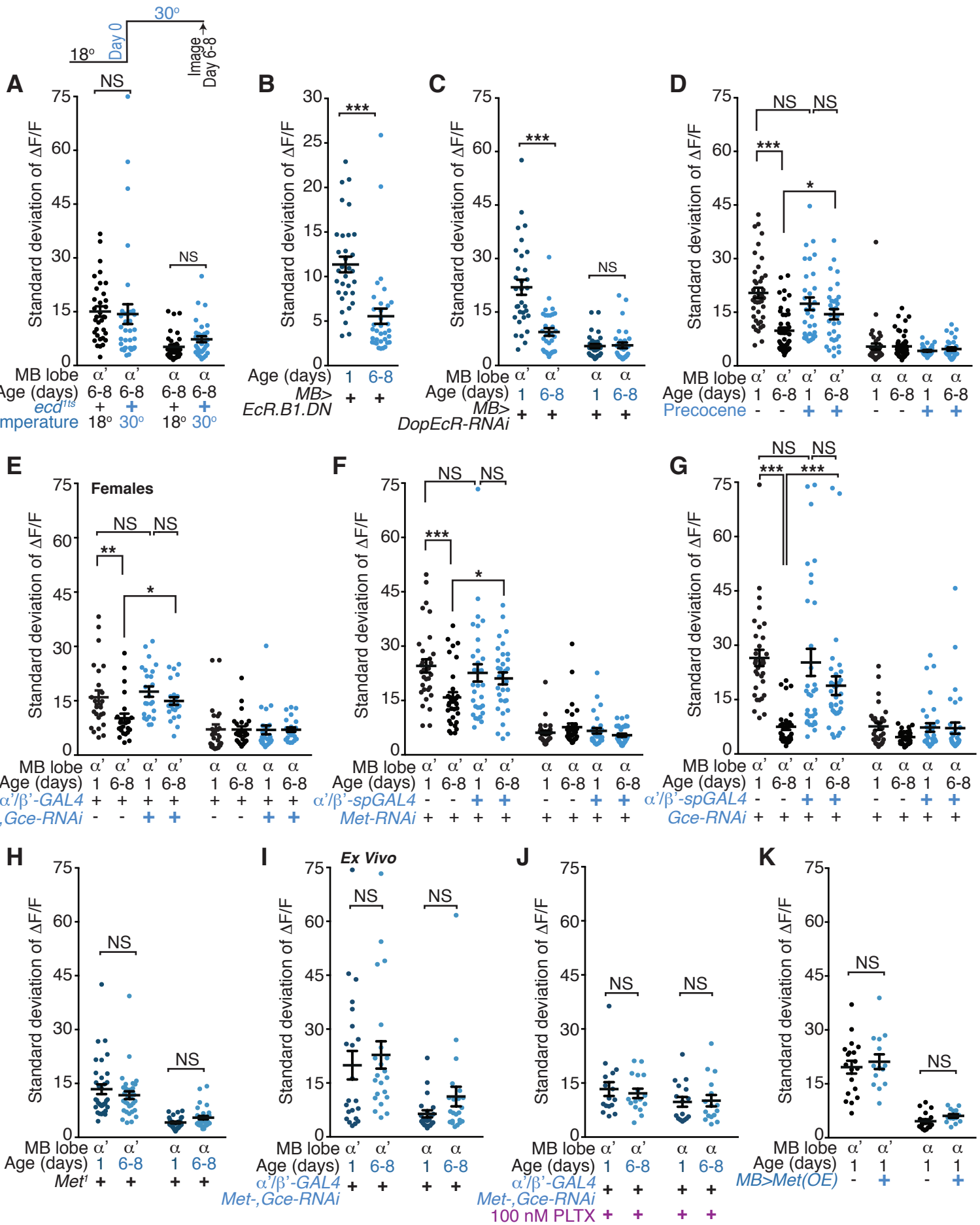
30°
18°
Day 0
Image Day 6-8
A
B
C
D
E
F
G
H
I
J
K
Standard deviation of ΔF/F
NS
***
**
*
Females
Ex Vivo
MB lobe
Age (days)
ecd1ts
Temperature
MB>
EcR.B1.DN
DopEcR-RNAi
Precocene
α'/β'-GAL4
Met-,Gce-RNAi
α'/β'-spGAL4
Met-RNAi
Gce-RNAi
Met1
MB>Met(OE)
100 nM PLTX

**Figure S3. JH, not ecdysone, signaling sculpts spontaneous α'/β' KC activity in early adulthood. Related to figure 3.**

**(A)** Ecdysoneless (*ecd*$^{1ts}$) mutants, whose ecdysone production is abolished, were raised at 18 degrees (control, permissive temperature) or shifted to 30 degrees at eclosion (mutant, restrictive temperature) to induce the mutation. KC activity of the temperature controls and the mutants was compared at day 6-8.

**(B)** Activity of KCs expressing a dominant negative ecdysone receptor (*EcR-DN*), which induced MB morphological abnormalities as expected, at day 1 or 6-8. Activity quantified in the MB vertical lobe, ***P<0.001, two-tailed unpaired t-test.

**(C)** Activity of KCs expressing RNAi to knockdown the ecdysone receptor *DopEcR* at day 1 or 6-8.

**(D)** KC activity of flies fed Precocene or solvent from eclosion until imaging on day 1 or day 6-8.

**(E)** KC activity in mated female flies with α'/β' expression of *Met* and *Gce* RNAi transgenes at day 1 or 6-8 or genetic controls.

**(F and G)** KC activity in genetic controls and in flies with α'/β' expression of (**F**) *Met-RNAi* or (**G**) *Gce-RNAi* transgenes at day 1 or 6-8.

**(H)** KC activity in *Met*$^{1}$ mutant day 1 or 6-8 flies.

**(I)** *Ex vivo* KC activity in brains from day 1 or 6-8 animals with α'/β'-specific knockdown of *Met* and *Gce*.

**(J)** KC activity in day 1 or 6-8 animals with α'/β'-specific knockdown of *Met* and *Gce* upon acute perfusion of the voltage-gated calcium channel antagonist Plectreurys toxin (PLTX, 100nM).

**(K)** KC activity in day 1 genetic controls and flies overexpressing Met in KCs.

**(A-K)** Each point is activity in median ROI for indicated lobe from 1 male fly (or female fly as indicated in **E**). N=7-18 flies with each hemisphere of the brain shown as one point. Error bars are mean and SEM. NS, P>0.05 and ***P<0.001, 2-way ANOVA with Sidak's multiple comparisons test. All comparisons for α lobe are NS.

## Figure S4

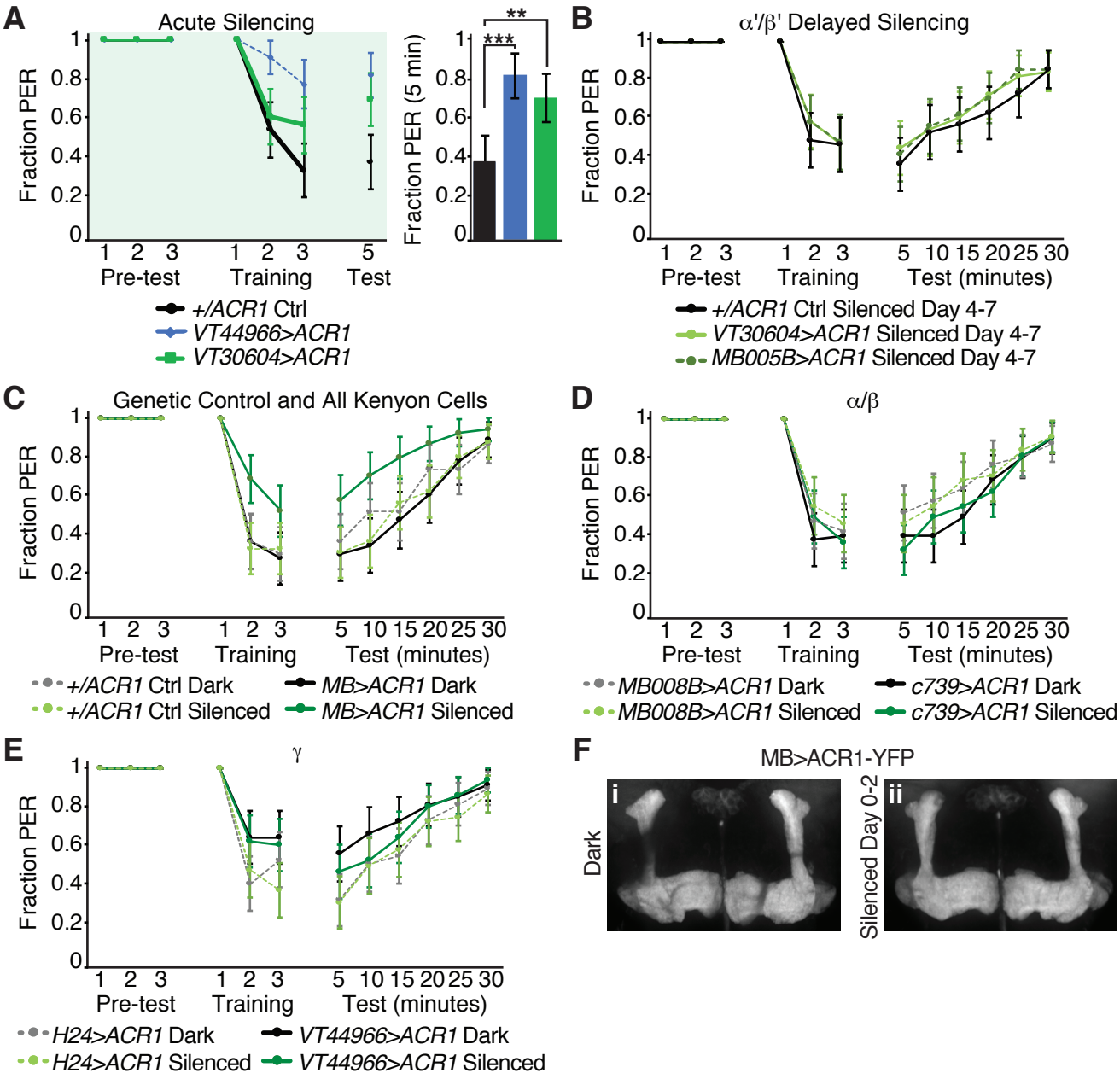
A
Acute Silencing
Fraction PER
Pre-test
Training
Test
Fraction PER (5 min)
**
***
+/ACR1 Ctrl
VT44966>ACR1
VT30604>ACR1
B
α'/β' Delayed Silencing
Test (minutes)
+/ACR1 Ctrl Silenced Day 4-7
VT30604>ACR1 Silenced Day 4-7
MB005B>ACR1 Silenced Day 4-7
C
Genetic Control and All Kenyon Cells
+/ACR1 Ctrl Dark
+/ACR1 Ctrl Silenced
MB>ACR1 Dark
MB>ACR1 Silenced
D
α/β
MB008B>ACR1 Dark
MB008B>ACR1 Silenced
c739>ACR1 Dark
c739>ACR1 Silenced
E
γ
H24>ACR1 Dark
H24>ACR1 Silenced
VT44966>ACR1 Dark
VT44966>ACR1 Silenced
F
MB>ACR1-YFP
Dark
Silenced Day 0-2
i
ii

**Figure S4. Specificity of KC cellular and temporal requirements for mature aversive taste memory behavior. Related to figure 4.**

**(A)** Aversive taste memory of mature day 7 flies with γ KCs (blue dotted line and bar) or α'/β' KCs (green line and bar) acutely silenced with a 532nm green laser throughout the entire assay (from 30 s prior to pre-test through the 5 minute post-pairing test, as indicated by light green shading) and genetic controls (black line and bar). ***P<0.001, **P<0.01, Fisher's Exact Test with Benjamini-Hochberg False Discovery Rate adjustment.

**(B)** Aversive taste memory of mature day 7 flies after delayed silencing from day 4-7 (flies reared in green light day 4-7 only; no silencing during assay). Genetic controls compared to flies expressing gtACR1 in α'/β' KCs with two independent drivers.

**(C-E)** Aversive taste memory of mature day 7-8 flies; comparison of sibling controls reared in the dark only (no silencing, black or gray lines) and flies reared in green light from eclosion to day 2 to induce early adult developmental silencing of KCs with gtACR1. Silencing is from day 0-2, *not* during the behavioral assay: (**C**) genetic controls (+/UAS-gtACR1 without Gal4 driver) and all KCs silenced, (**D**) α/β KCs silenced with 2 independent drivers, and (**E**) γ KCs silenced with 2 independent drivers from day 0-2. Data from the 5 minute test point only is represented in Figure 4F bar graph.

**(F)** Representative images of the morphology of MB, visualized with MB-Gal4>UAS-gtACR1-eYFP at day 7. Gross morphology is unaffected by silencing of all KCs with gtACR1 from day 0-2, as compared to dark reared (no silencing) siblings.

**(A-E)** N=45-71 flies, data shown as mean with 95% confidence interval error bars.

**Table S1. List of *Drosophila* Stocks. Related to STAR Methods.**

| |
|---|
| (v) indicates stock from Vienna Drosophila Resource Center and (BL) indicates stock from Bloomington Drosophila Stock Center. |
| UAS-PLTX (Brain McCabe) |
| UAS-gtACR1-eYFP(attP2) (Adam Claridge-Chang) |
| UAS-gtACR1-mCherry (Vivek Jayaraman) |
| |
| ***Full genotypes:*** |
| *MB-Gal4 or MB> indicates the OK107-Gal4.* |
| |
| **Figure 1:** |
| w*; UAS-GCaMP6s/CyO; UAS-GCaMP6s/TM2; OK107-Gal4/cID |
| w*; UAS-GCaMP6s/MB005B-AD; UAS-GCaMP6s/MB005B-DBD |
| w*; UAS-GCaMP6s/MB008B-AD; UAS-GCaMP6s/MB008B-DBD |
| w*; UAS-GCaMP6s/+; UAS-GCaMP6s/H24-Gal4 |
| w*; UAS-CaMPARI (BL58761)/+; +; OK107-Gal4/+ |
| |
| **Figure 2:** |
| w*; UAS-GCaMP6s/CyO; UAS-GCaMP6s/TM2; OK107-Gal4/cID |
| w*; UAS-GCaMP6s/+; UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-PLTX/UAS-GCaMP6s; UAS-PLTX/UAS-GCaMP6s; OK107-Gal4/+ |
| $cac^{MI02836\text{-}FlpStop.ND}$ (BL67681)/Y; UAS-GCaMP6s/+; tub-$GAL80^{ts}$/UAS-FLPD5 (BL55805); OK107-Gal4/+ (raised at 19 degrees and shifted to 30 degrees for 48-60 hrs pre-imaging) |
| $cac^{MI02836\text{-}FlpStop.ND}$ (BL67681)/Y; UAS-GCaMP6s/+; tub-$GAL80^{ts}$/+; OK107-Gal4/+ (raised at 19 degrees and shifted to 30 degrees for 48-60 hrs pre-imaging) |
| $cac^{MI02836\text{-}FlpStop.ND}$ (BL67681)/Y; LexAop-GCaMP6s/UAS-FLPL (BL62141); MB247-lexA/VT30604-Gal4 |
| $cac^{MI02836\text{-}FlpStop.ND}$ (BL67681)/Y; LexAop-GCaMP6s/CyO; MB247-lexA/VT30604-Gal4 |
| |
| **Figure 3:** |
| w*; LexAop-GCaMP6s/+; MB247-lexA, VT30604-Gal4/+ |
| w*; LexAop-GCaMP6s/UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852); MB247-lexA, VT30604-Gal4/+ |
| w*; UAS-CaMPARI (BL58761)/+; +; OK107-Gal4/+ |
| w*; UAS-CaMPARI (BL58761)/UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852); +; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-GCaMP6s/tub-$GAL80^{ts}$; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852); UAS-GCaMP6s/tub-$GAL80^{ts}$; OK107-Gal4/+ |
| |
| **Figure 4:** |
| CantonS |
| w*; +; VT30604-Gal4/UAS-gtACR1 |
| w*; MB005B-AD/+; MB005B-DBD/UAS-gtACR1 |
| w*; +; UAS-gtACR1(attP2)/+ |
| w*; +; UAS-gtACR1(attP2)/+; OK107-Gal4/+ |
| w*; c739-Gal4/+; UAS-gtACR1(attP2)/+ |
| w*; MB008B-AD/+; MB008B-DBD/UAS-gtACR1 |
| w*; +; VT44966-Gal4/UAS-gtACR1 |
| w*; +; H24-Gal4/UAS-gtACR1 |
| |
| **Figure 5:** |
| w*; +; VT30604-Gal4/+ |
| w*; UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852)/+; + |

| |
|---|
| w*; UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852)/+; VT30604-Gal4/+ |
| w*; UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852)/+; tub-GAL80$^{ts}$/+ (raised at 19 degrees and shifted to 30 degrees from day 0-2 or day 4-7) |
| w*; UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852)/+; VT30604-Gal4/tub-GAL80$^{ts}$ (raised at 19 degrees and shifted to 30 degrees from day 0-2 or day 4-7) |
| |
| **Figure S1:** |
| w*; UAS-GCaMP6s/CyO; UAS-GCaMP6s/TM2; OK107-Gal4/cID |
| |
| **Figure S2:** |
| w*; UAS-GCaMP6s/CyO; UAS-GCaMP6s/TM2; OK107-Gal4/cID |
| w*; UAS-GCaMP6s/+; UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/UAS-gtACR1-mCherry(su(Hw)attP5); UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-dcr2/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-dcr2/UAS-Itpr-RNAi (v6484); OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-dcr2/UAS-RyR-RNAi (BL29445); OK107-Gal4/+ |
| w*; UAS-GCaMP6s/UAS-Cac-RNAi (v104168); UAS-dcr2/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-dcr2/UAS-Ca-α1D-RNAi (BL33413); OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-dcr2/UAS-Ca-α1D-RNAi (v51491); OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-dcr2/UAS-Ca-β-RNAi (BL29575); OK107-Gal4/+ |
| w*; UAS-GCaMP6s/UAS- Ca-β-RNAi (v102188); UAS-dcr2/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/tub-GAL80$^{ts}$; UAS-GCaMP6s/+; OK107-Gal4/+ (raised at 19 degrees and shifted to 30 degrees for 48-60 hrs pre-imaging) |
| w*; UAS-GCaMP6s/tub-GAL80$^{ts}$; UAS-GCaMP6s/UAS-SERCA-RNAi (BL44581); OK107-Gal4/+ (raised at 19 degrees and shifted to 30 degrees for 48-60 hrs pre-imaging) |
| |
| **Figure S3:** |
| w*; UAS-GCaMP6s/+; ecd$^{1ts}$ (BL218); OK107-Gal4/+ (raised at 19 degrees only or shifted to 30 degrees at eclosion) |
| w*; UAS-GCaMP6s/UAS-EcR.B1-DN (BL6842); UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/UAS-DopEcR-RNAi (v103494); UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/CyO; UAS-GCaMP6s/TM2; OK107-Gal4/cID |
| w*; LexAop-GCaMP6s/+; MB247-lexA, VT30604-Gal4/+ |
| w*; LexAop-GCaMP6s/UAS-Met-RNAi (v100638), UAS-Gce-RNAi (BL61852); MB247-lexA, VT30604-Gal4/+ |
| w*; LexAop-GCaMP6s, UAS-Met-RNAi (v100638)/+; MB247-lexA/+ |
| w*; LexAop-GCaMP6s, UAS-Met-RNAi (v100638)/MB005B-AD; MB247-lexA/MB005B-DBD |
| w*; LexAop-GCaMP6s, UAS-Gce-RNAi (BL61852)/+; MB247-lexA/+ |
| w*; LexAop-GCaMP6s, UAS-Gce-RNAi (BL61852)/MB005B-AD; MB247-lexA/MB005B-DBD |
| Met$^{1}$(BL3472)/Y; UAS-GCaMP6s/+; UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-GCaMP6s/+; OK107-Gal4/+ |
| w*; UAS-GCaMP6s/+; UAS-GCaMP6s/UAS-Met.ORF (FlyORF_F000644); OK107-Gal4/+ |
| |
| **Figure S4:** |
| w*; +; UAS-gtACR1(attP2)/+ |
| w*; +; VT44966-Gal4/UAS-gtACR1 |
| w*; +; VT30604-Gal4/UAS-gtACR1 |
| w*; MB005B-AD/+; MB005B-DBD/UAS-gtACR1 |
| w*; +; UAS-gtACR1(attP2)/+; OK107-Gal4/+ |
| w*; c739-Gal4/+; UAS-gtACR1(attP2)/+ |
| w*; MB008B-AD/+; MB008B-DBD/UAS-gtACR1 |
| w*; +; H24-Gal4/UAS-gtACR1 |