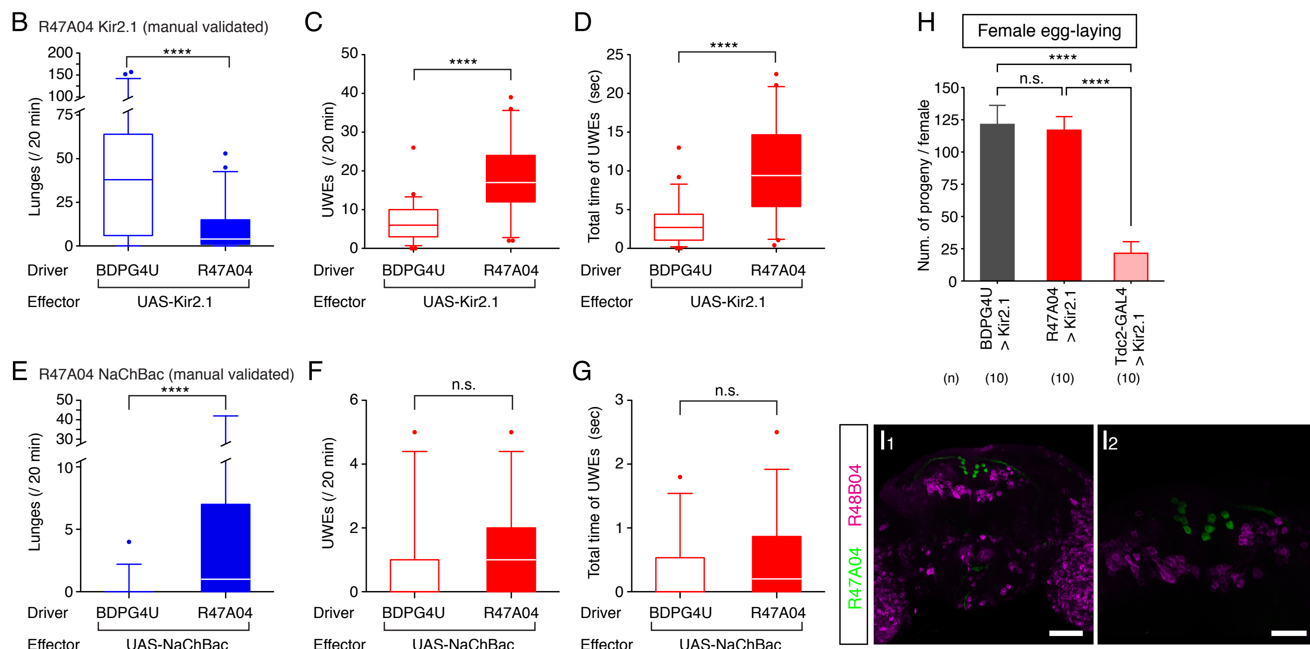

Figure S1. R47A04 Neurons Are Responsible for Male-male Aggressive Behavior, Related to Figure 1

(A) Schematic representation of 4 octopamine receptor genes (Han et al., 1998; Balfanz et al., 2005; Evans and Maqueira, 2005; Maqueira et al., 2005): *Oamb (octopamine α1 receptor)*, *Octβ1R (oa2)*, *Octβ2R (octopamine β2 receptor)*, *Octβ3R (octopamine β3 receptor)*, and the genomic regions (blue boxes) for generating the GAL4 lines used in this study. RA-RK indicate alternative splicing variants with pink boxes showing coding regions. Two genomic regions used for GAL4 lines, *R47A04* and *R48B04* identified by 1st and 2nd screening are indicated with red boxes. *R50A06*, a CRM linking these two fragments, showed a significant decrease in aggression driving Kir2.1 expression, but no significant increase when driving NaChBac expression (Fig. 1E, F). Genomic maps were adapted from FlyBase (flybase.org).

(B-D) Number of lunges (B), unilateral wing extensions (UWEs) (C) and total time of UWEs (D) during silencing of R47A04 neurons after manual validation.

(E-G) Number of lunges (E), UWEs (F) and total time of UWEs (G) during activation of R47A04 neurons after manual validation of CADABRA results.

(H) Number of progeny from single mated females of the indicated genotypes. (I$_{1\text{-}2}$) Confocal images of *R47A04-LexA>mCD8::GFP* (green) and *R48B04-GAL4>mCD8::RFP* (magenta) in the male brain (I$_1$) and the superior medial protocerebrum (SMP) region (I$_2$, partial Z-stack image). Central neurons in the SMP region labeled by *R47A04* were distinct from those labeled by *R48B04*.

Scale bars in panel I$_1$ is 50 µm and I$_2$ is 20 µm.

For H, error bars denote ± S.D. For B-D and E-G, Mann-Whitney U tests were performed. For H, Kruskal-Wallis one-way ANOVA and post hoc Mann-Whitney U tests were performed.

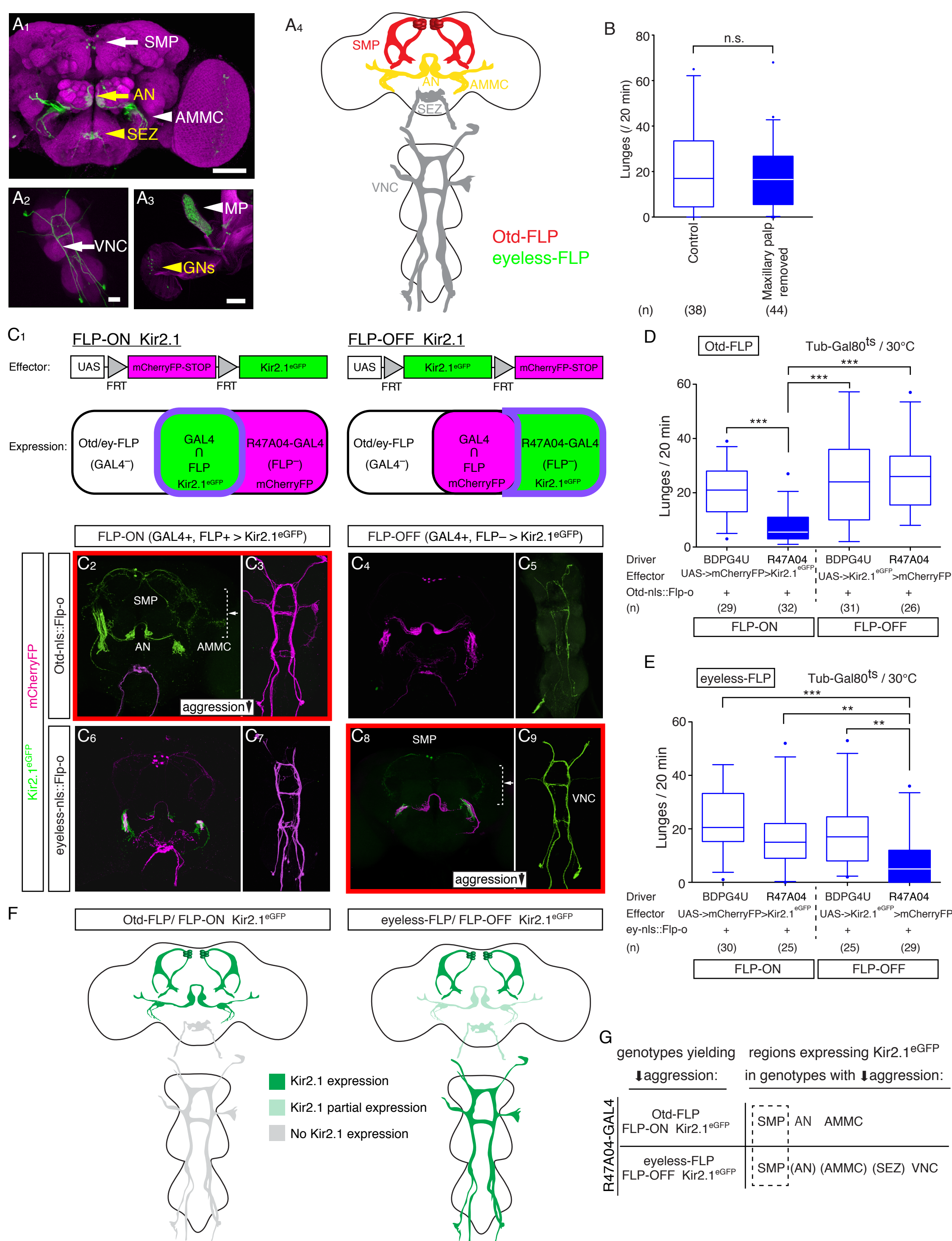

Figure S2. SMP Neurons in *R47A04-GAL4* Control Aggression, Related to Figure 3

($A_{1\text{-}3}$) Confocal images showing mCD8::GFP expression (green) ($A_{1\text{-}3}$) and the neuropil marker nc82 (magenta) ($A_{1\text{-}2}$) or autofluorescence (magenta) (A_3). Image for the brain (A_1) is reprinted from Fig. 1G to facilitate comparison. (A_4) Schematic illustrating overlap of Otd-nls::FLPo (red) or eyeless-nls::FLPo (green) recombination with *R47A04* expression. Otd-nls::FLPo promotes recombination in the central brain, while eyeless-nls::FLPo promotes in the peripheral neurons.

(B) Number of lunges in control and maxillary pulp removed male flies.

(C_1) Schematic of positive and negative intersectional strategies using *R47A04-GAL4* to drive a FLP-ON Kir2.1^{eGFP} (*UAS>mCherryFP-stop>Kir2.1^{eGFP}*) or a FLP-OFF Kir2.1^{eGFP} (*UAS>Kir2.1^{eGFP}-stop>mCherryFP*), combined with *Otd-nls::FLPo* or *eyeless-nls::FLPo*. Green areas in the Venn diagrams show genetic combinations that express Kir2.1. ($C_{2\text{-}9}$) Confocal images showing immunostaining of Kir2.1^{eGFP} (green) and mCherryFP (magenta) expression in the brain ($C_{2, 4, 6, 8}$) and the ventral nerve cord ($C_{3, 5, 7, 9}$). The expression patterns showing suppression of aggressive behavior were indicated with red boxes. (C_2, C_3) *Otd-nls::FLPo* with FLP-ON Kir.2.1. Kir2.1 expression was observed in the SMP, AN and AMMC but not in the SEG or VNC. (C_8, C_9) *eyeless-nls::FLPo* with FLP-OFF Kir2.1. Kir2.1 expression was observed in the SMP and VNC, and weak/partial expression was observed in the AN, AMMC and SEZ.

(D) Number of lunges during silencing with *R47A04-GAL4* driving FLP-ON or FLP-OFF Kir2.1^{eGFP} with *Otd-nls::FLPo*.

(E) Number of lunges during silencing with *R47A04-GAL4* driving FLP-ON or FLP-OFF Kir2.1^{eGFP} with *eyeless-nls::FLPo*.

(F) Summary illustrating Kir2.1 expression in the genetic intersectional silencing experiments.

(G) Table indicating genetic intersections that yielded reduced aggression and expression sites. Intersectional experiments using *Otd-nls::FLPo*, which promotes recombination in the central brain but not in the VNC, and FLP-ON or FLP-OFF responder cassettes that do or do not express eGFP::Kir2.1, respectively, in the presence of FLPo, indicated that decreased aggression was observed only when eGFP::Kir2.1 was expressed in the central brain. Similar experiments using *eyeless-nls::FLPo* fractionate central R47A04 neurons involved in aggression. These results indicated that aggression was reduced when eGFP::Kir2.1 was expressed in patterns containing the SMP cluster, but did not completely exclude a contribution from neurons in the AMMC or AN, due to incomplete recombination by eyeless-nls::FLPo. () indicates the area with partial/weak Kir2.1 expression.

SMP, superior medial protocerebrum, AL, antennal lobe, AN, antennal nerve; AMMC, antennal mechanosensory and motor center; SEZ, subesophageal zone ; VNC, ventral nerve cord, MP, maxillary palp, GNs, gustatory neurons.

Scale bars in A_1 is 50 μm and $A_{2\text{-}3}$ are 20 μm.

For B, D and E, Kruskal-Wallis one-way ANOVA and/or post hoc Mann-Whitney U tests were performed.

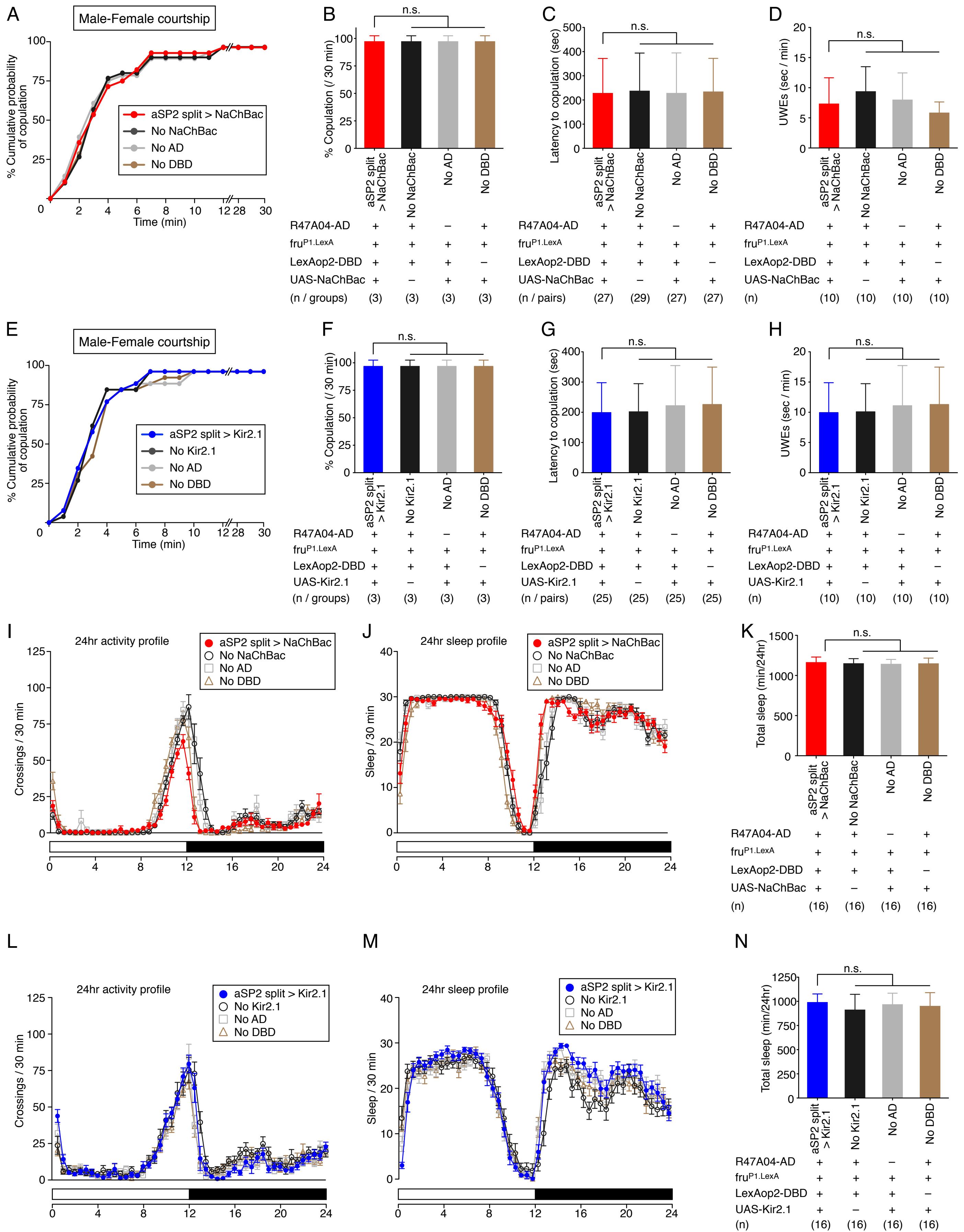

Figure S3. R47A04^{aSP2} Neurons Do Not Affect Other Behaviors, Related to Figure 3

(A-H) Male-female courtship behavior for males with activation or silencing of R47A04^{aSP2} neurons. Cumulative probability curve (A), copulation rate (B), copulation latency (C) and unilateral wing extension frequency (D) during activating of R47A04^{aSP2} neurons with NaChBac. Cumulative probability curve (E), copulation rate (F), copulation latency (G) and unilateral wing extension frequency (H) during silencing of R47A04^{aSP2} neurons with Kir2.1.

(I-N) Locomotor activity of males with activation or silencing of R47A04^{aSP2} neurons. Activity profiles measured by the number of beam crossing (I), 24 hr sleep profiles of single flies indicated with the number of sleep bouts in 30 min time window (J) and total sleep amounts during activation (K) of R47A04^{aSP2} neurons with NaChBac. Activity profiles measured by the number of beam crossing (L), 24 hr sleep profiles of single flies indicated with the number of sleep bouts in 30 min time window (M) and total sleep amounts (N) during silencing of R47A04^{aSP2} neurons with Kir2.1. Day and night are indicated by the white and black lines.

For B, C, D, F, G, H, K and N, Kruskal-Wallis one-way ANOVA and post hoc Mann-Whitney U tests were performed. Error bars denote ± S.D.

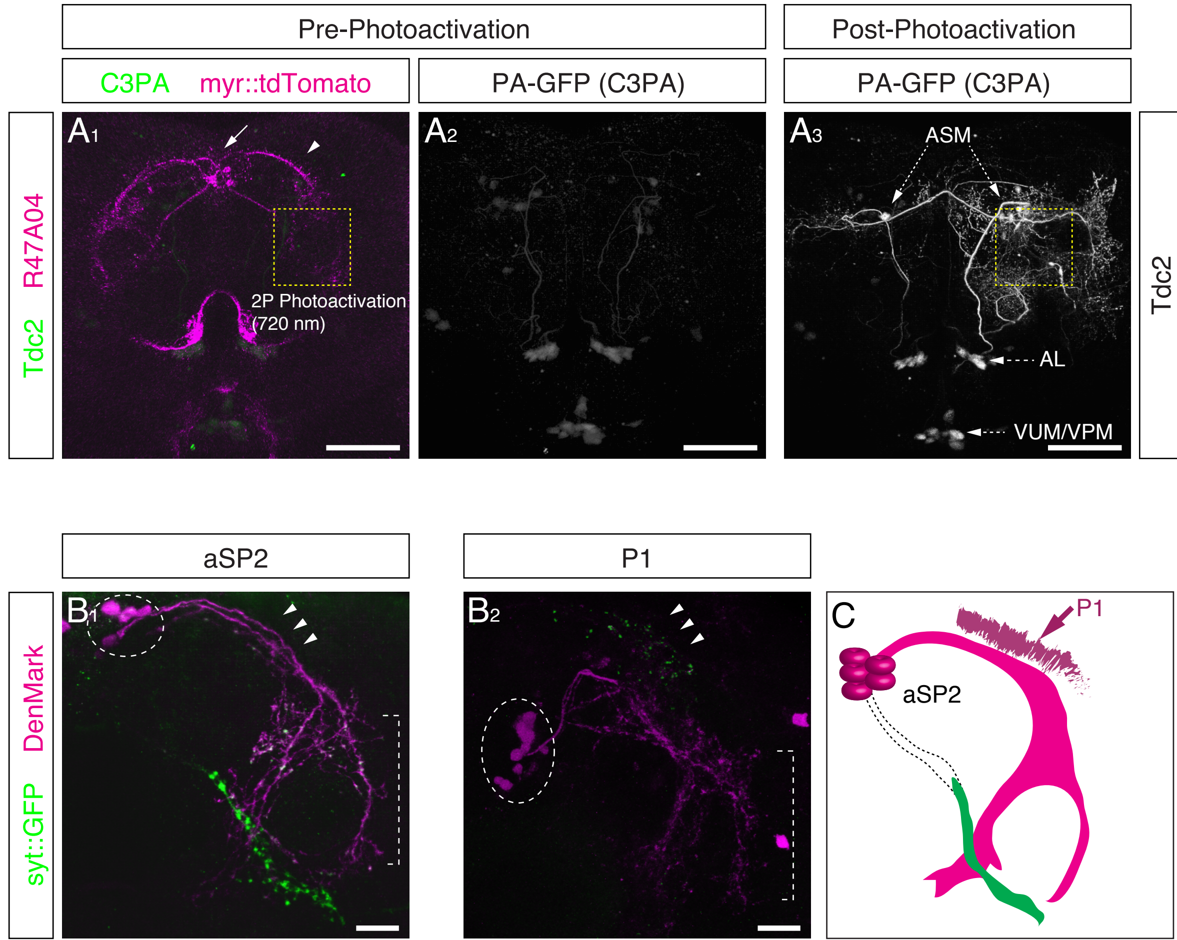

Figure S4. Anatomic Analysis of R47A04^{aSP2} Neurons, OANs and P1 Neurons, Related to Figure 4

($A_{1\text{-}3}$) Subsets of Tdc2-GAL4 neurons were labeled by photoactivation of PA-GFP (C3PA) in Tdc2-GAL4 neurons. R47A04 neurons were labeled by myr::tdTomato with *R47A04-LexA* (A_1). Pre-Photoactivation image of PA-GFP (green,A_1 and A_2) and Post-Photoactivation image of PA-GFP (A_3). The region of photoactivation is delineated by a yellow dashed rectangle, surrounding the ring region. White arrow: cell bodies of R47A04^{aSP2} neurons, white arrowhead: the arch region of R47A04^{aSP2} neurons. The anterior superior medial protocerebral cluster (OA-ASM), the ventrolateral protocerebral cluster (OA-VL), ventral paired median neurons (OA-VPM) and ventral unpaired median neurons (OA-VUM) are indicated. For clarity, nonspecific background fluorescence has been masked in A (Ruta et al., 2010).

(B_1 and B_2) Confocal images of R47A04^{aSP2} (*R47A04-GAL4*, B_1) and P1 neurons (*R15A01-GAL4*, B_2) expressing syt::GFP (green, presynaptic) and DenMark (magenta, postsynaptic). Dashed circle: cell bodies of R47A04^{aSP2} neurons (B_1) and P1 neurons (B_2), dashed bracket: ring region ($B_{1,2}$).

(C) Schematic of R47A04^{aSP2} neurons with inferred input site from P1 neurons (see also Fig. 5A, B). Regions labeled with syt::GFP (green) and DenMark (magenta) are illustrated.

Scale bars in $A_{1\text{-}3}$ are 50 µm and $B_{1\text{-}3}$ are 20 μm.

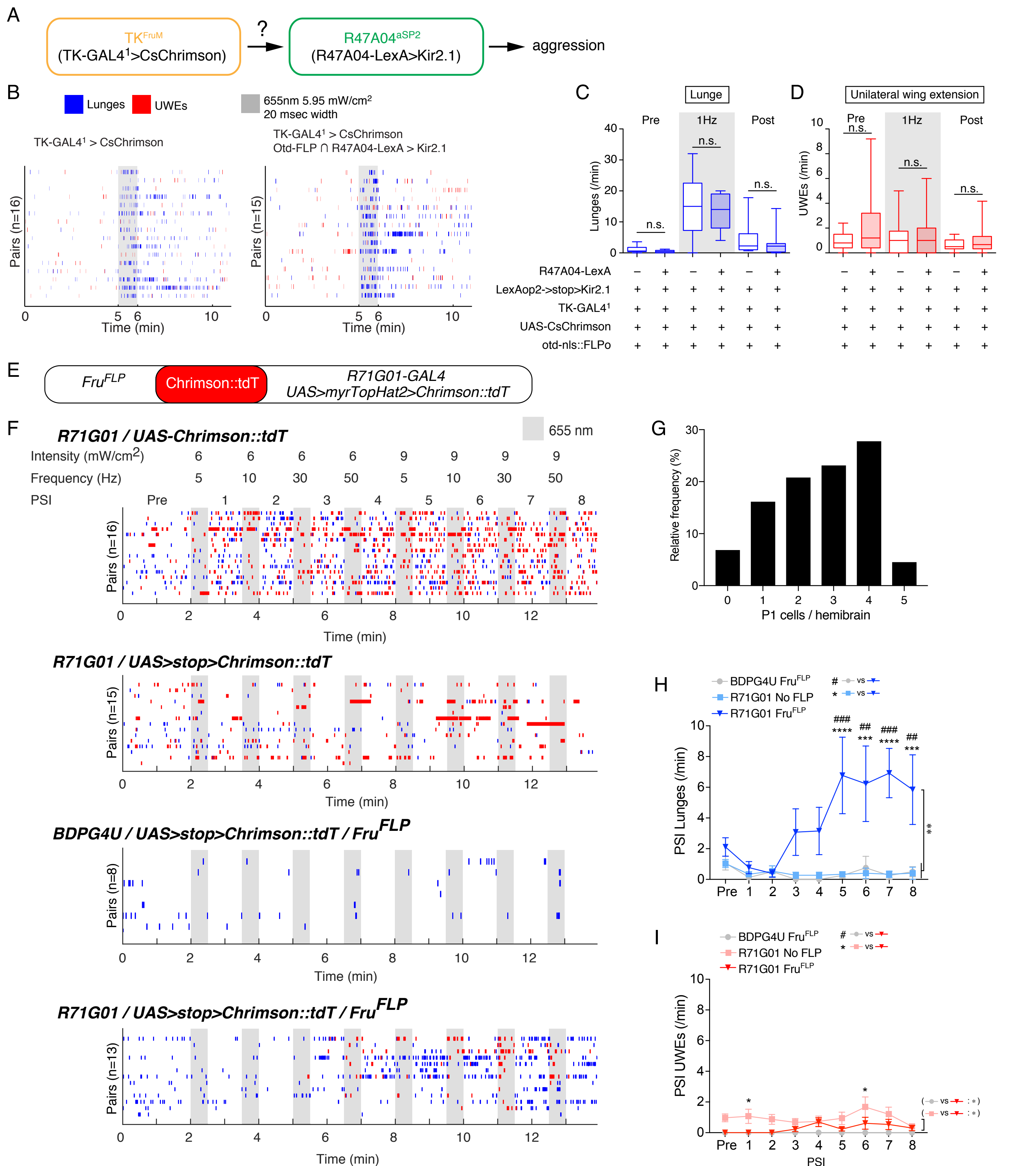

Figure S5. Interaction of R47A04 Neurons and Other Aggression Neurons, Related to Figure 5

(A-D) Epistasis experiment between TK^{FruM} and R47A04^{aSP2} neurons. (A) Schematic of experimental genotypes. R47A04^{aSP2} neurons were silenced using *R47A04-LexA*>FLP ON Kir2.1 with *Otd-nls::FLPo* while TK^{FruM} neurons were activated using *TK-GAL4^{1}>CsChrimson*. (B) Raster plots illustrating bouts of lunges (blue) and UWEs (red) for each genotype. 1 min of single photostimulation (grey bar, 655 nm, 5.95 mW/cm^2) was delivered after 5 min pre-stimulation period. Frequency of lunges (C) and UWEs (D).

(E) Intersectional strategy for targeting FruM$^+$ neurons in *R71G01-GAL4* line. The intersection labeled an average of 3 ± 1 neurons per hemisphere in 5-6 day old males.

(F) Raster plots illustrating bouts of lunges (blue) and UWEs (red) for each genotype. Blocks of frequency and intensity-titrated 30 s photostimulation trials (grey bars, 655 nm) with 1 min inter-trial intervals were delivered as indicated.

(G) Relative number of P1 cells labeled with *R71G01-GAL4*, *Fru^{FLP}* and *UAS>myr::TopHat2>Chrimson::tdTomato*.

(H and I) Frequency of lunges (H) and UWEs (I) during pre-stimulation period (Pre) and each post stimulation interval (PSI). Data points indicate mean ± S.E.M. To facilitate comparison, the data for *R71G01/UAS-Chrimson::tdTomato* were omitted from H and I.

For C and D, Kruskal-Wallis one-way ANOVA and post hoc Mann-Whitney U tests were performed. For H and I, two-way ANOVA and Tukey's multiple comparisons test were performed.

Figure S6. Attenuation of OAergic Modulation Suppresses Male-male Aggression Induced by P1 Stimulation, and Is Rescued by aSP2 Activation, Related to Figure 6

(A-D) Combined pharmacologic and genetic behavioral epistasis experiment to investigate the interaction of OA signaling and P1 stimulation on R47A04^{aSP2} neurons in male social behavior. (A-C) Blocking of OA signaling using mianserin was combined with the artificial activation of R47A04^{aSP2} neurons using NaChBac, while P1 neurons were activated by CsChrimson. Frequency of lunges (A) or UWEs (B) and the SBPI (C) during indicated conditions. For C, error bars denote ± S.D. (D) Baseline locomotor activity before P1 activation (Pre-stimulation period) measured by average velocity of indicated genotypes.

For A-D, Kruskal-Wallis one-way ANOVA and post hoc Mann-Whitney U tests were performed.

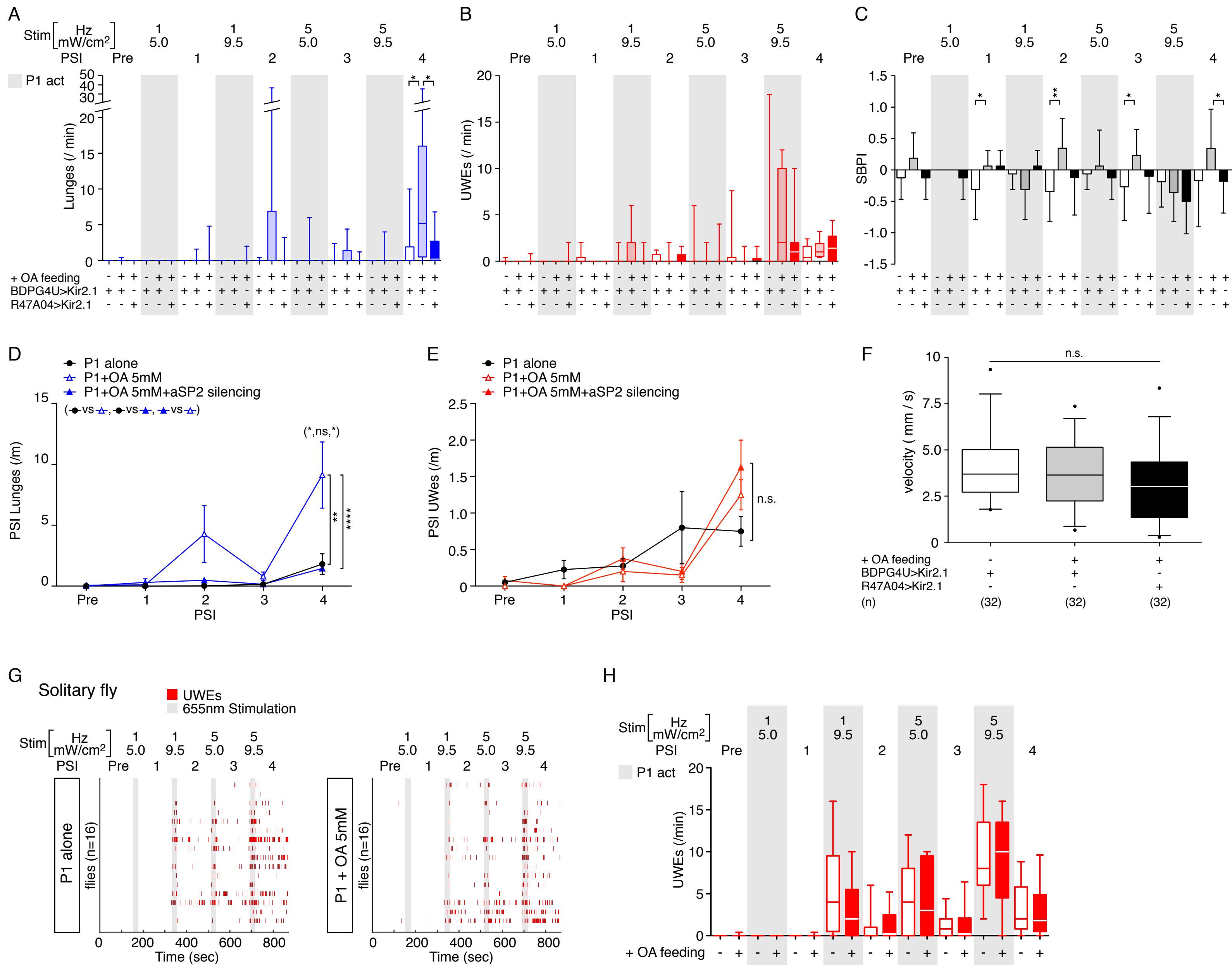

Figure S7. OAergic Modulation Enhances Male-male Aggression Induced by P1 Stimulation via R47A04 Neurons, Related to Figure 7

Two-way behavioral epistasis experiment to investigate the interaction of OA signaling and P1 stimulation on R47A04^{aSP2} neurons in male social behavior.

(A-E) 5 mM of OA feeding was combined with silencing of R47A04^{aSP2} neurons using Kir2.1 while P1 neurons were activated using CsChrimson. (A-C) All data sets for Figure 7D-F. Frequency of lunges (A) or UWEs (B) and the SBPI (C) during indicated conditions. For C, error bars denote ± S.D. (D, E) Frequency of lunges (D) and UWEs (E) during pre-stimulation period (Pre) and post stimulation intervals (PSI). Data points indicate mean ± S.E.M.

(F) Baseline locomotor activity before P1 activation (Pre-stimulation period) measured by average velocity under indicated conditions.

(G, H) Effect of OA feeding on UWEs induced by P1 photostimulation in solitary flies. OA feeding was combined with activation of P1 neurons using *P1^{a} split-GAL4* and *UAS-CsChrimson*. (G) Raster plots showing bouts of UWEs (red) triggered by P1 stimulation alone (*P1^{a} split-GAL4* and *UAS-CsChrimson*) or P1 stimulation + OA 5mM (*P1^{a} split-GAL4* and *UAS-CsChrimson* with 5 mM OA feeding). Blocks of frequency (1 Hz, 5 Hz) and intensity (5.0 mW/cm^2, 9.5 mW/cm^2) titrated 30 s photostimulation trials (grey bars, 655 nm) with 2.5 min inter-trial intervals were delivered as indicated. (H) Frequency of UWEs evoked by P1 stimulation alone or by P1 stimulation in 5 mM OA fed flies during indicated periods.

For A-C, F and H, Kruskal-Wallis one-way ANOVA and post hoc Mann-Whitney U tests were performed. For D and E, two-way ANOVA and post hoc Mann-Whitney U tests were used for statistical analysis.