Figures and figure supplements

GnRH pulse generator activity in mouse models of polycystic ovary syndrome

Ziyue Zhou *et al*.

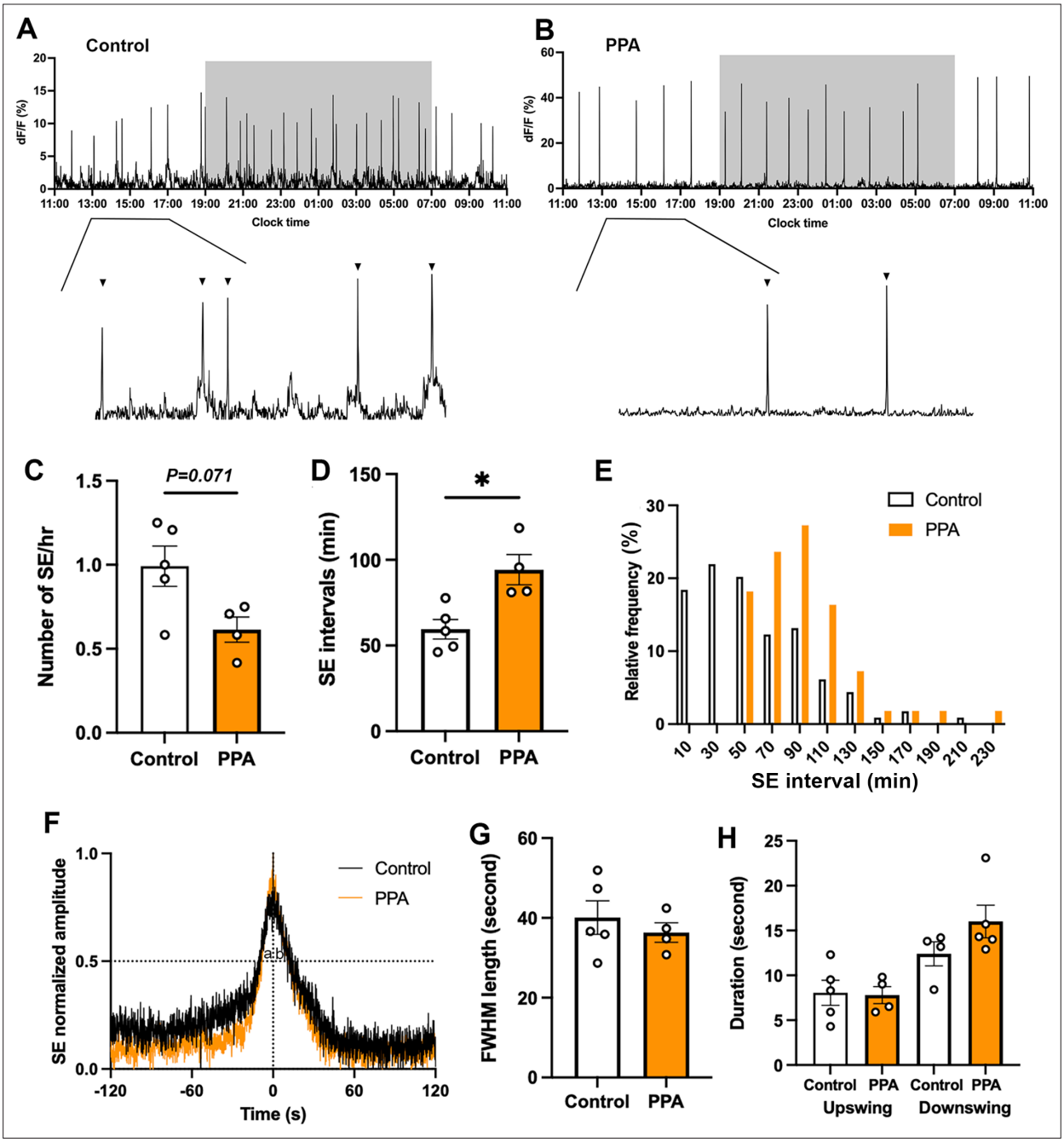

Figure 1. Slowerpulse generator activity in PPA animals. Representative 24 hr photometry recordings showing synchronization events (SEs) observed in diestrous (**A**) control and (**B**) PPA females with the light-off period (19:00-07:00) represented by the shaded area and expanded views of the traces (13:00-17:00) given below. Triangles point to identified SEs. (**C**) SE frequency and (**D**) SE intervals in control (n=5) and PPA (n=4) mice. Mann-Whitney U tests. (**E**) Frequency histograms showing relative SE frequencies occurring in 20 min bins, calculated separately for controls (white, n=114, 5 mice) and PPA (orange, n=55, 4 mice). X-axis represents the bin centers. (**F**) Continuous recordings at 10 Hz sampling rate showing normalized profile of SE overlaid from control (black, 7 SEs from 5 animals) and PPA (orange, 5 SEs from 4 animals). (**G**) FWHM length of control (n=5) and PPA (n=4) animals. Mann-Whitney U test. (**H**) Durations of upswing and downswing for control (n=5) and PPA (n=4) animals, respectively. Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data show mean ± SEM. Each circle is an individual animal. * p<0.05.

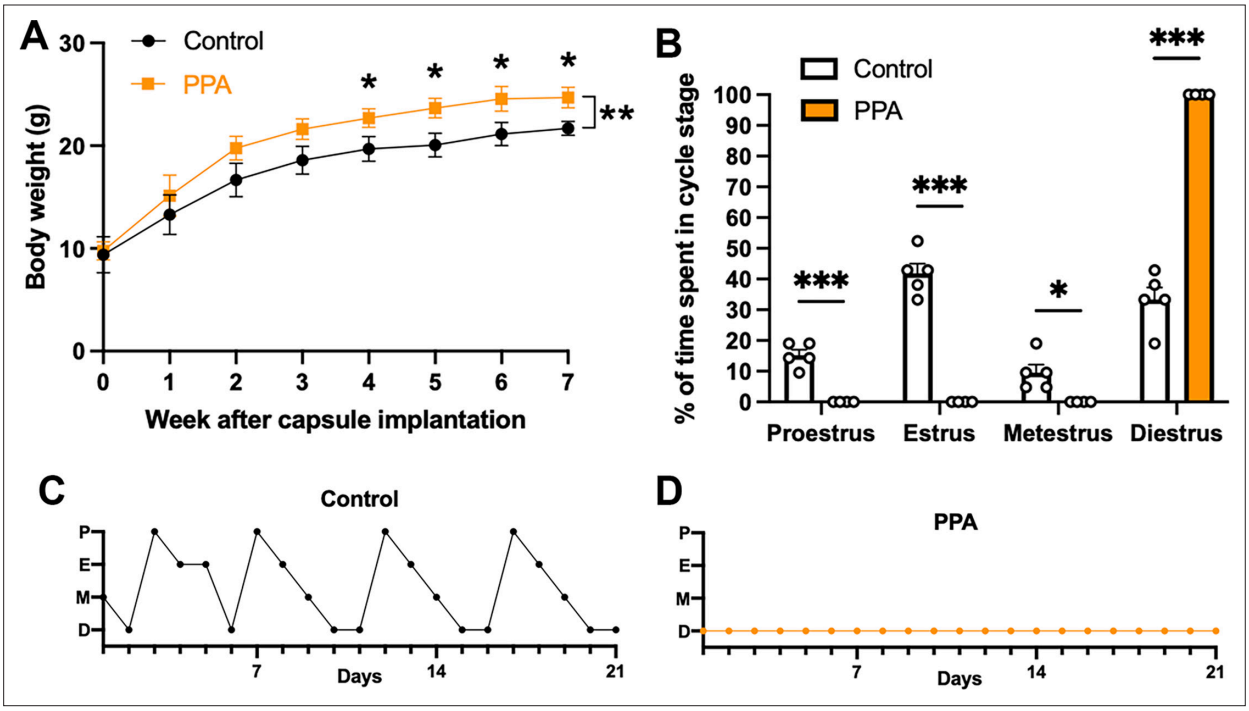

Figure 1—figure supplement 1. Peripubertal androgen (PPA) treatment causes an increase in body weight and a loss ofestrous cyclicity. (**A**) Weekly body weight of animals (mean ± SEM) with blank (control: n=5) or dihydrotestosterone (PPA: n=4) capsules. Asterisks above each weekly data point denote significance using Sidak's multiple comparisons test. Asterisks between lines denote the mean effect of the capsule over 7 weeks. Two-way repeated-measure ANOVA with Sidak's multiple comparisons test. (**B**) The proportion of time spent at each stage of the estrous cycle over 21 days (n=4–5 per group). Two-way ANOVA followed by Sidak's multiple comparisons tests. (**C–D**) Representative graphs of estrous cycle pattern over 21 days in (**C**) control and (**D**) PPA females. P, proestrus; E, estrus; M, metestrus; D, diestrus. Data show mean ± SEM. Each circle is an individual animal. * p<0.05, ** p<0.01, *** p<0.001.

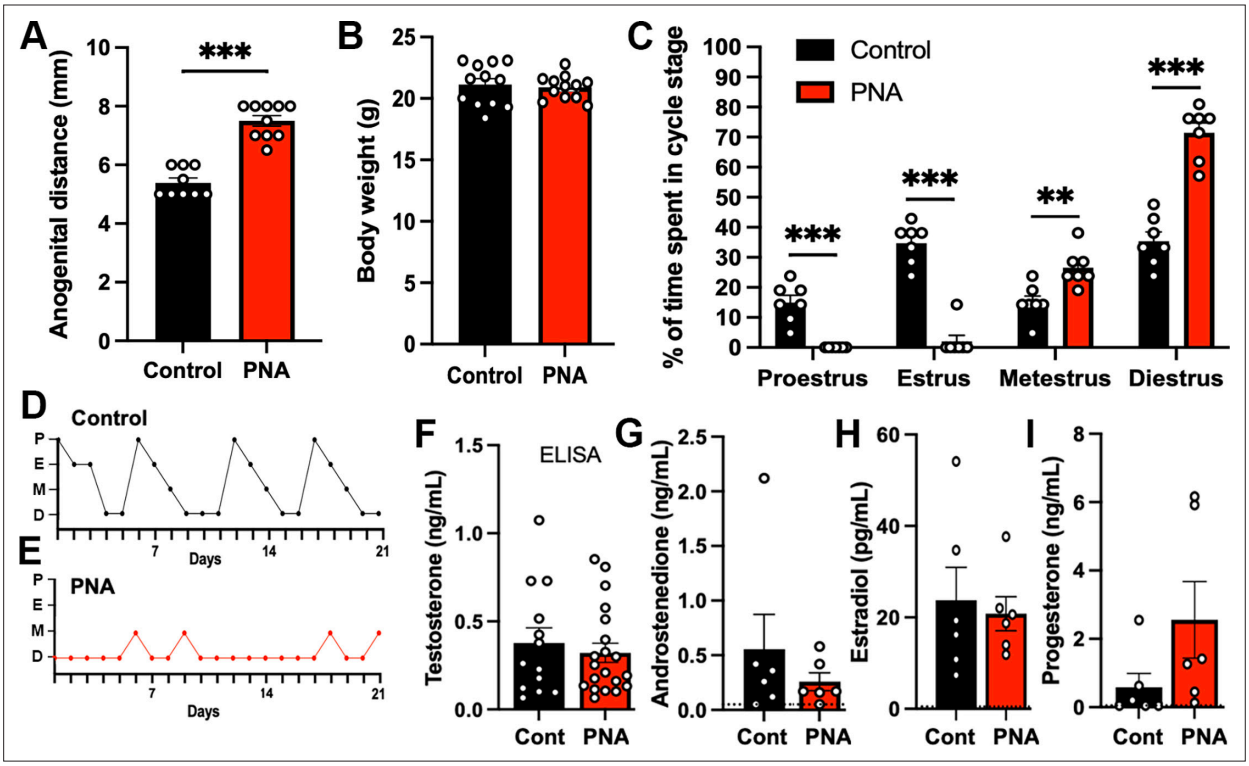

Figure 2. Prenatal androgen exposure leads to increased anogenital distance and disrupted estrous cyclicity. (**A**) Anogenital distance (control: n=9; PNA: n=10) and (**B**) Body weight of control and PNA animals (n=12 per group). Mann-Whitney U tests. (**C**) Proportion of time spent at each estrous cycle stage over 21 days (n=7 per group). Two-way ANOVA followed by Sidak's post-hoc tests. (**D–E**) Representative graphs of estrous cycle pattern in (**D**) control and (**E**) PNA females. P, proestrus; E, estrus; M, metestrus; D, diestrus. (**F**) Serum testosterone level of control (n=13) and PNA (n=20) animals in diestrus measured by enzyme-linked immunosorbent assay (ELISA). The limit of detection for testosterone ELISA is 0.066 ng/mL. (**G–I**) Plasma levels of (**G**) androstenedione, (**H**) estradiol, and (**I**) progesterone in control and PNA animals measured using liquid chromatography-mass spectrometry (LC-MS). Blood samples were collected at 10:00 on diestrus (n=6 per group). Dotted lines represent the limit of detection for each hormone. Androstenedione: 0.05 ng/mL; estradiol: 0.50 pg/mL; progesterone: 0.05 ng/mL. All samples were below limit of detection for testosterone LC-MS measurement (<0.01 ng/mL). Mann-Whitney U tests. Data show mean ± SEM. Each circle is an individual animal. ** p<0.01, *** p<0.001.

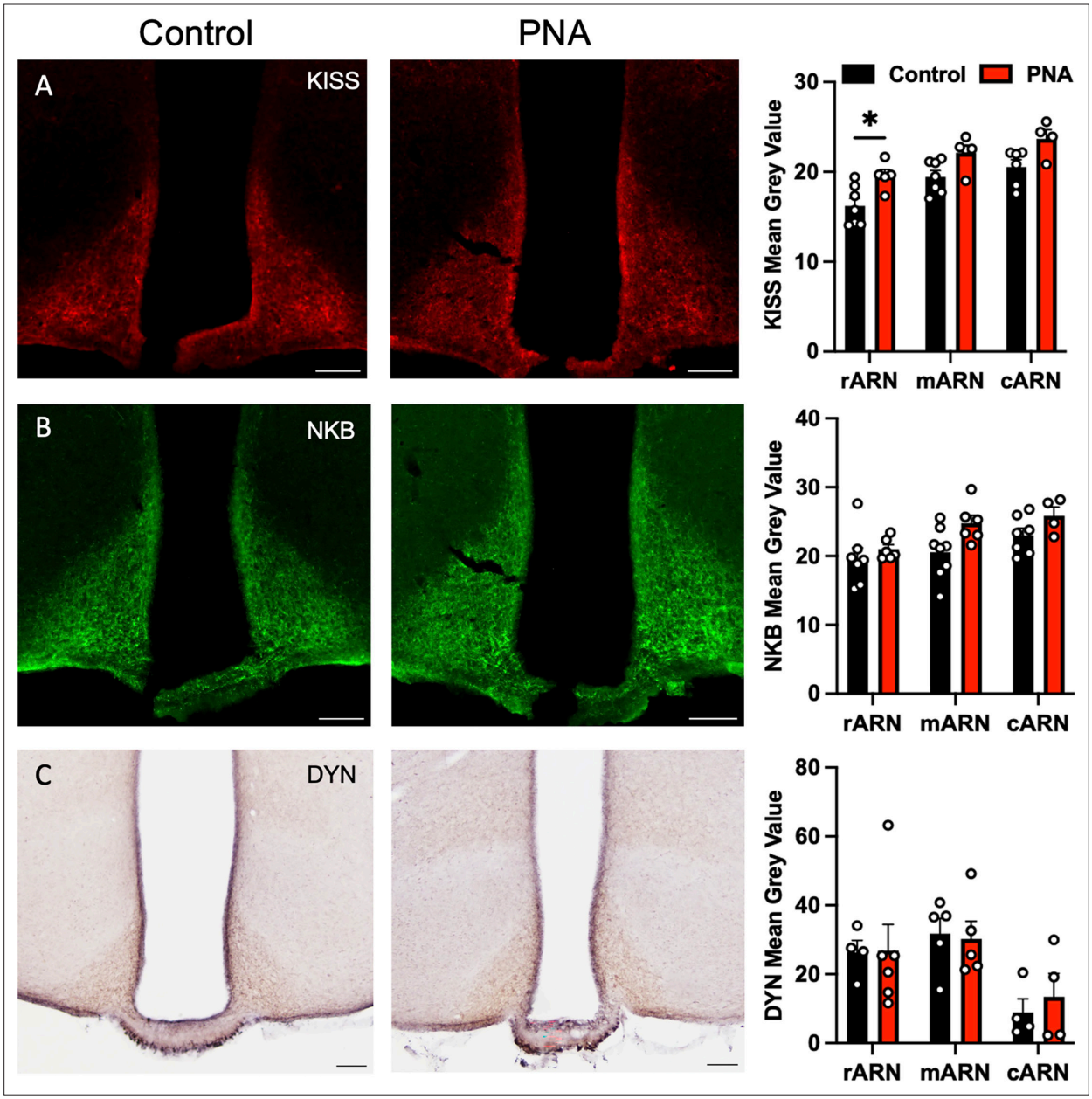

Figure 2—figure supplement 1. Slightly increased kisspeptin immunofluorescence in the rostral arcuate nucleus of PNA mice. Representative arcuate nucleus images of (**A**) Kisspeptin (KISS), (**B**) Neurokinin B (NKB) immunofluorescent staining, and (**C**) Dynorphin (DYN) Nickel-3,3′-Diaminobenzidine (DAB) staining from control and PNA animals, along with the quantified immunoreactive level in rostral, middle, and caudal arcuate nucleus (rARN, mARN, cARN) in control (n=4–8) and PNA (n=4–6) animals. Scale bars represent 100 μm. Two-way ANOVA followed by Sidak's multiple comparisons test. Data show mean ± SEM. Each circle is an individual animal. Asterisk represents significant multiple comparisons results. * $p<0.05$.

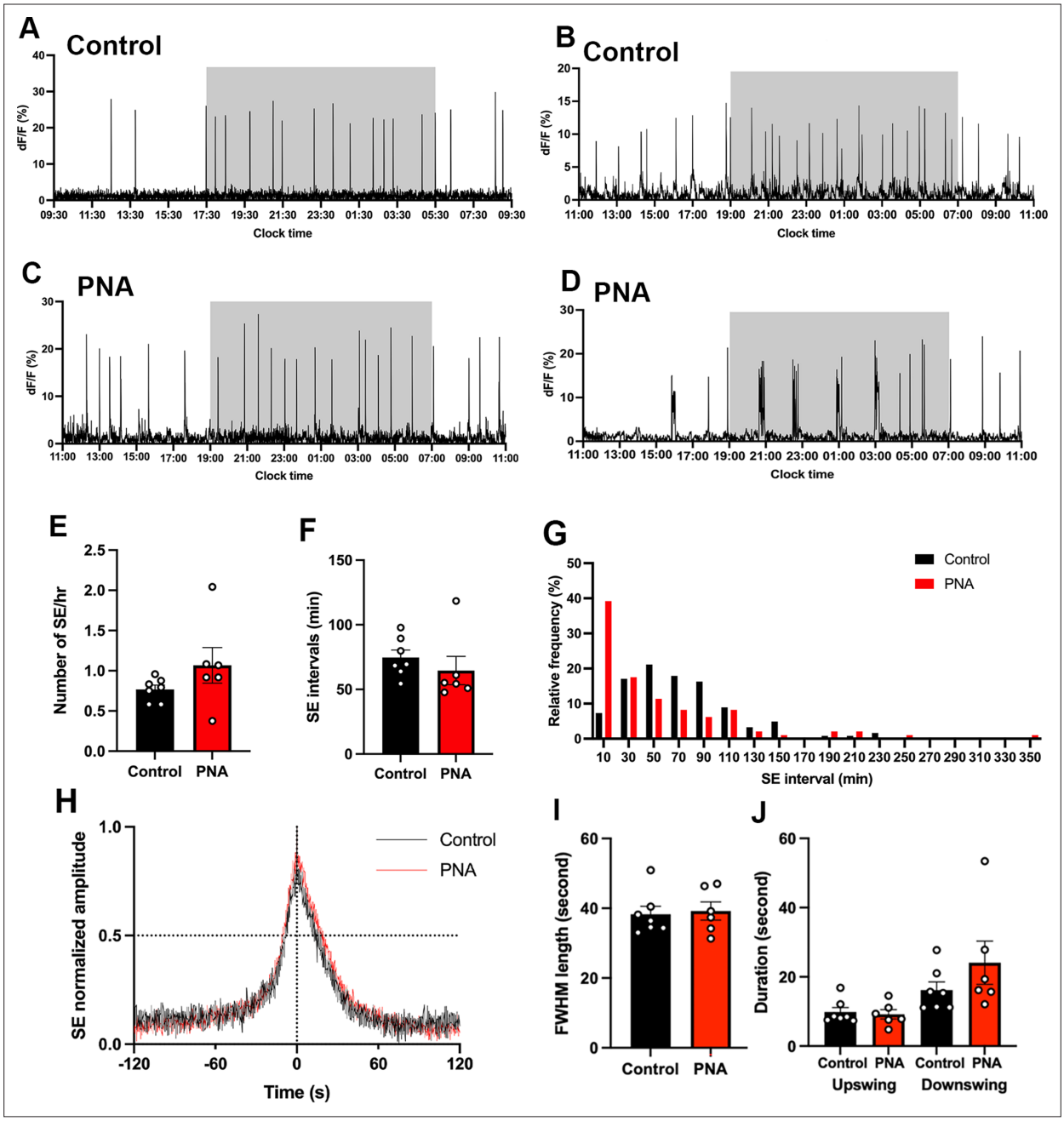

Figure 3. PNA animals exhibit highly variable patterns of ARN^{KISS} neuron SEs. Representative 24 hr photometry recordings showing SEs observed in (**A,B**) two control and (**C,D**) two PNA females with the light-off period (17:30-05:30 or 19:00-07:00) represented by the shaded area. Recordings were performed when vaginal cytology indicated diestrus. (**E**) SE frequency and (**F**) SE intervals in control (n=7) and PNA (n=6) mice. Mann-Whitney U tests. (**G**) Frequency histograms showing relative SE frequencies occurring in 20 min bins, calculated separately for controls (black, n=123, 7 mice) and PNA (red, n=98, 6 mice). X-axis represents the bin centers. Multi-peak SEs (mpSE) with peaks occurring within 160 s were considered as one SE with interval calculated from the first peak. (**H**) Continuous (10 Hz) recording showing normalized mean profile of SE overlaid from control (black, 22 SEs from 7 animals) and PNA (red, 20 SEs from 6 animals). (**I**) FWHM length (upswing +downswing) in control (n=7) and PNA (n=6) animals. Mann-Whitney U test. (**J**) Durations of FWHM upswing and downswing for control (n=7) and PNA (n=6) animals, respectively. Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data show mean ± SEM. Each circle is an individual animal.

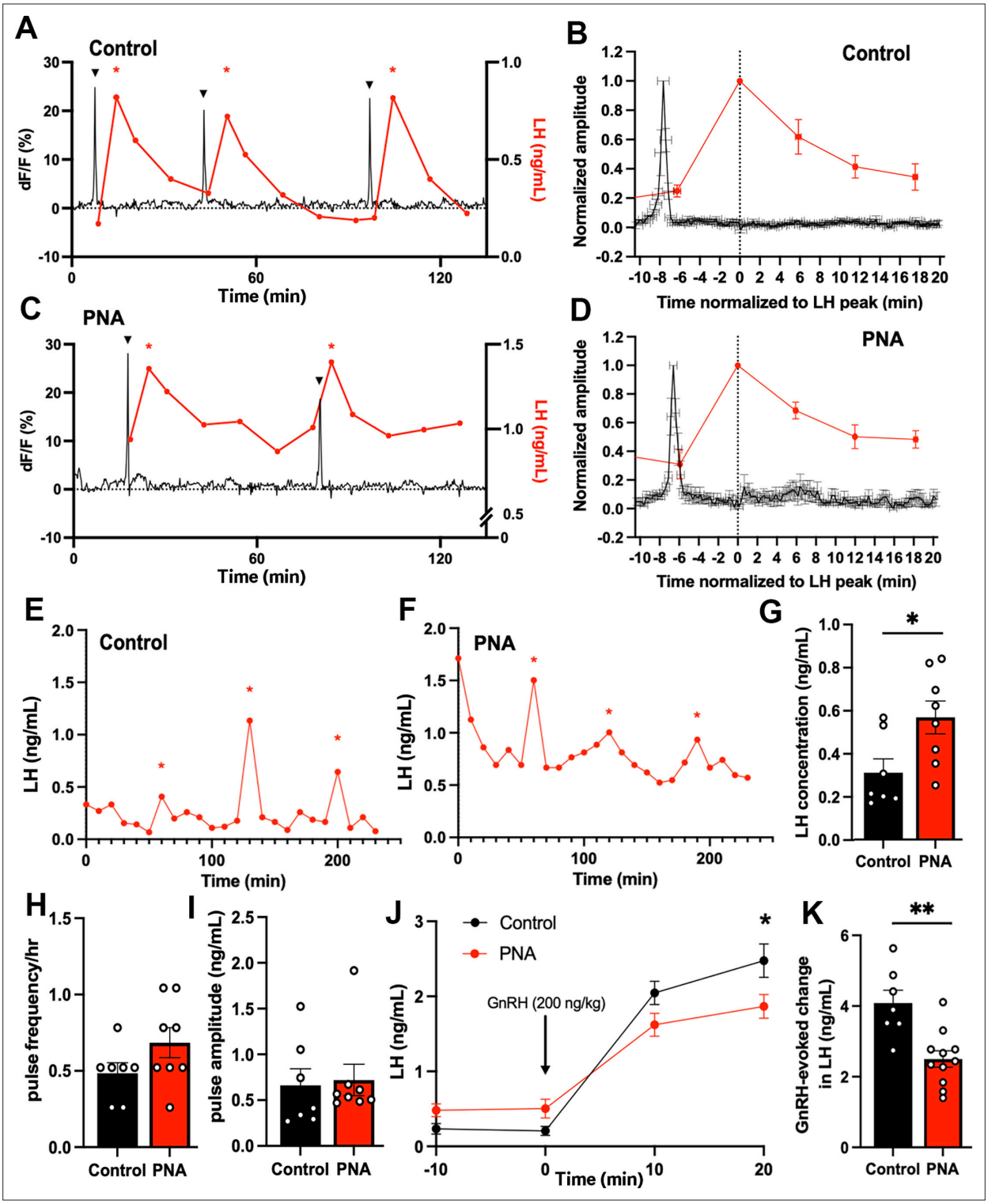

Figure 4. Increased total LH concentration but normal LH pulse frequency in PNA animals. Representative examples from (**A**) control and (**C**) PNA mice showing the relationship of SEs (black) with LH secretions (red). Triangles and red asterisks indicate identified SEs and LH pulses, respectively. Normalized LH secretion plotted against normalized SEs in (**B**) control and (**D**) PNA animals. The amplitudes of SEs and serum LH levels were normalized to their peaks, with time 0 being the peak of LH in control (n=7 from 4 mice) and PNA (n=7 from 5 mice) animals. Representative examples of LH pulse profiles in (**E**) control and (**F**) PNA animals. Red asterisks indicate identified LH pulses. (**G**) Total LH concentration, (**H**) LH pulse frequency per hour, and (**I**) LH pulse amplitude in diestrous control (n=7, with 13 LH pulses) and PNA (n=8, with 21 LH pulses) mice across 4 hr sampling at 10 min intervals. Mann-Whitney U tests. (**J**) Change in LH levels following an intraperitoneal injection of GnRH (200 ng/kg, i.p.) in diestrous control (n=7) and PNA (n=11)

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mice. An asterisk above the 20 min data point denotes significance between LH levels in control and PNA using two-way repeated-measure ANOVA followed by Sidak's post-hoc tests. (**K**) GnRH-evoked changes in LH levels in control (n=7) and PNA (n=11) mice were calculated by the difference in LH levels before (–10 min and 0 min) and after (10 min and 20 min) GnRH injections. Mann-Whitney U test. Data show mean ± SEM. Each circle is an individual animal. * $p<0.05$, ** $p<0.01$.

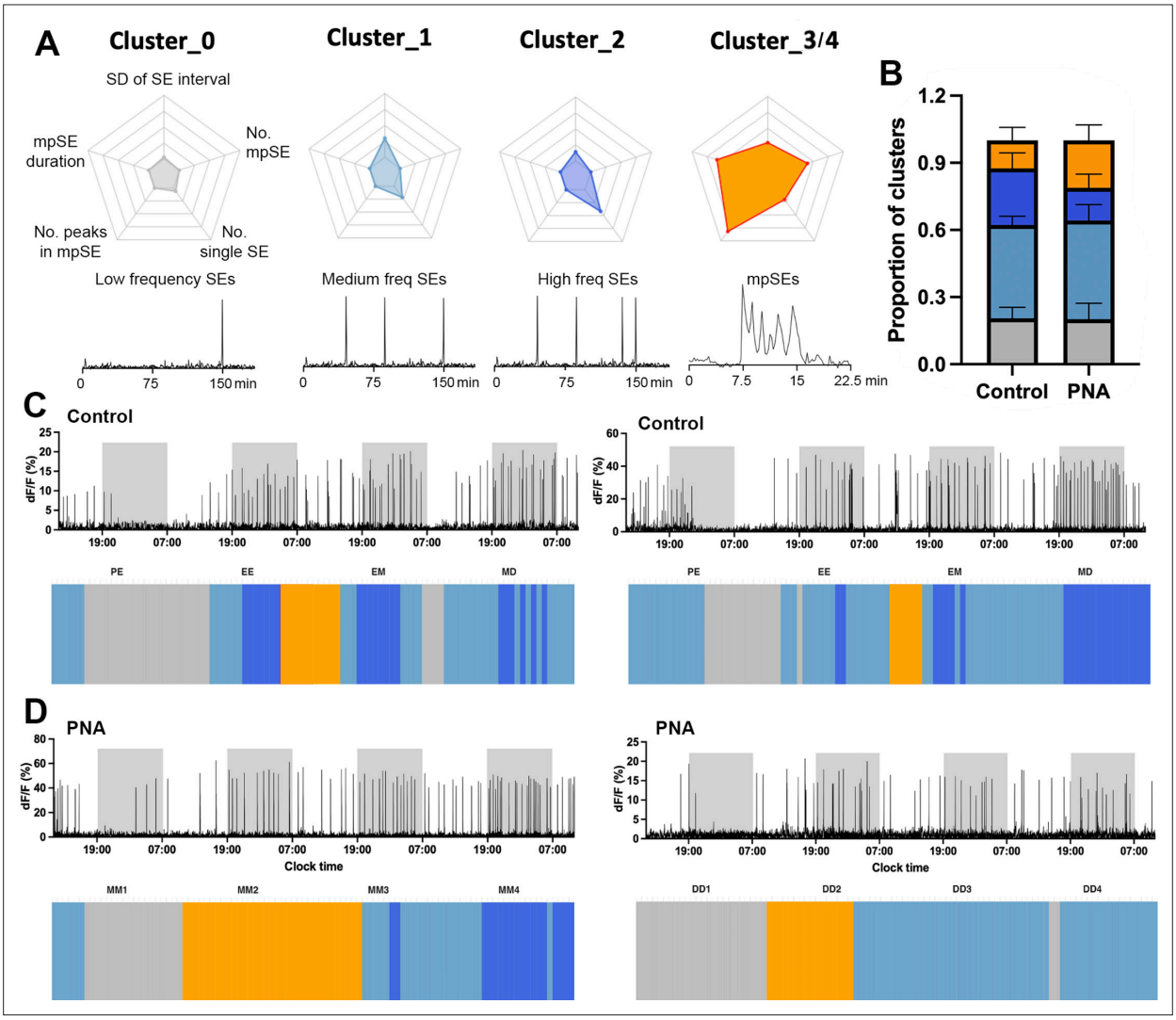

Figure 5. PNA animals exhibit cycling pulse generator activity. (**A**). Cluster centroid values for normalized parameters used in k-means clustering with a schematic plot of corresponding photometry recordings patterns. The parameters used for cluster assignments are labelled in Cluster_0 with the following axes: (top) standard deviation (SD) of SE intervals, (top right) number of multi-peak SEs (mpSEs), (bottom right) number of single SEs, (bottom left) number of peaks in mpSEs, (top left) duration of mpSEs. All axes have a minimum value of 0 and maximum value of 1. (**B**) Bar graph indicating proportion of the cluster-assignments for 4-day recordings in control (n=5) and PNA (n=4) animals. Data show mean ± SEM. (**C–D**) Representative examples of four-day consecutive recording of ARN^{KISS} activity and corresponding hourly k-means cluster assignments in (**C**) two control animals starting in proestrus on day 1 and transiting to diestrus on day 4 according to vaginal smears; (**D**) two PNA animals remaining in either metestrus or diestrus for four days. Light-off periods (19:00-07:00) are represented by the shaded area in the photometry recording. Color fields represent the assigned clusters for the center (4th hour) of the 7 hr moving time windows. Estrous stages of the mice determined by vaginal lavage at the beginning and the end of each 24 hr recording were labelled above the corresponding k-means cluster assignments: proestrus to estrus (PE), estrus to metestrus (EM), metestrus to diestrus (MD), stuck in metestrus (MM), or stuck in diestrus (DD). K-means clustering was performed without vaginal cytology information.

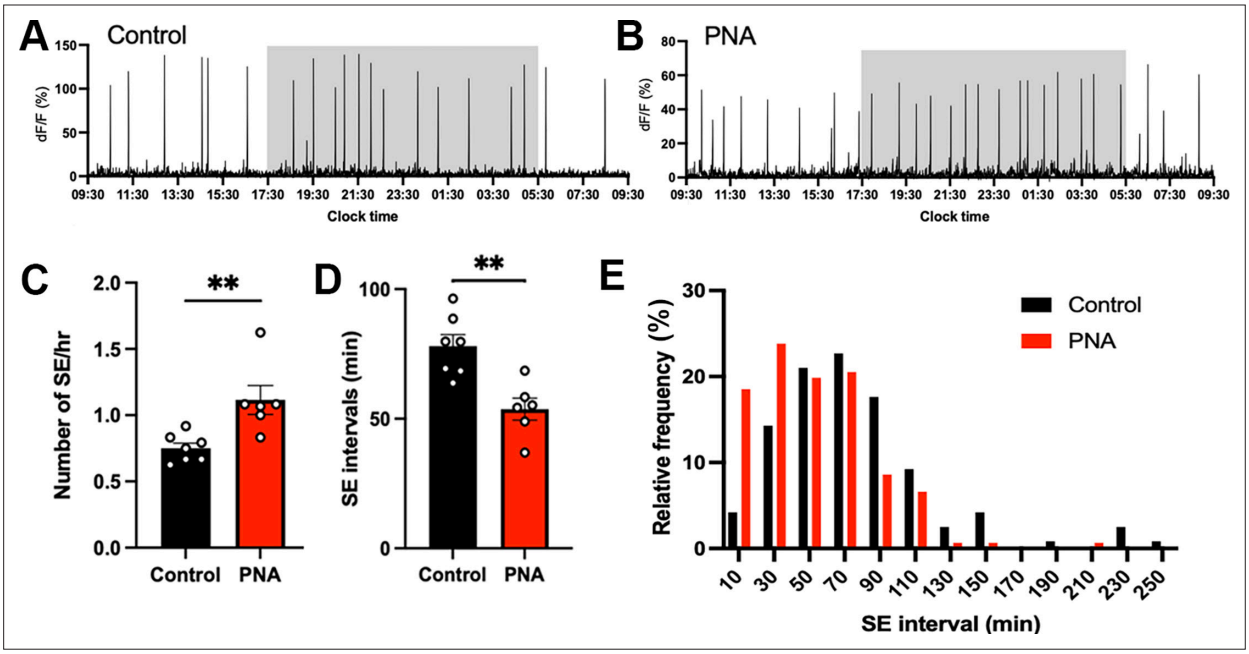

Figure 6. Faster ARN^{KISS} neuron synchronization events in 'diestrous-like' PNA animals. Representative 24 hr photometry recordings showing ARN^{KISS} neuron SEs observed in (**A**) diestrous control and (**B**) algorithm-identified diestrous PNA females. The light-off period (17:30-05:30) is represented by the shaded area. (**C**) SE frequency and (**D**) SE intervals in control (n=7) and PNA (n=6) mice. Mann-Whitney U tests. (**E**) Histogram showing percentage of SE interval frequencies occurring in 20 min bins, calculated separately for controls (black, n=119, 7 mice) and PNA (red, n=151, 6 mice) animals. X-axis represents the bin centers.

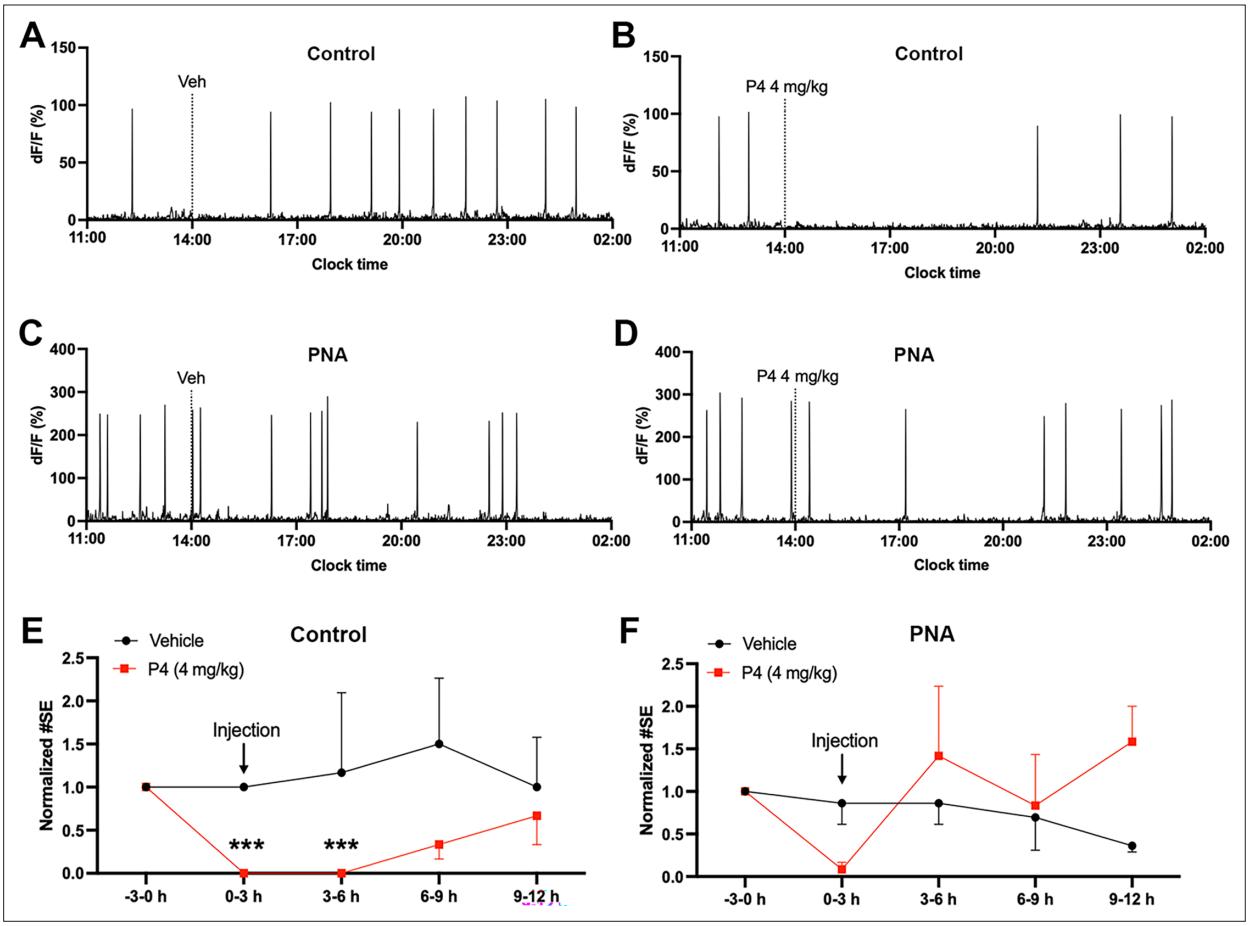

Figure 7. Defective progesterone negative feedback in PNA animals. (**A–D**) Examples of 15 hr photometry recordings of pulse generator activity in (**A–B**) control and (**C–D**) PNA animals receiving (**A,C**) vehicle (Veh) or (**B,D**) progesterone (P4, 4 mg/kg) i.p. injections. Dashed line represents when i.p. injection took place. Normalized number of SEs in (**E**) control (n=3) and (**F**) PNA (n=3) animals before and after vehicle (black) and P4 (red) injections. Data were analyzed in 3 hr bins and normalized to the number of SEs before injections (−3–0 hr) for each mouse. The asterisks above the data denote significant effects at indicated time periods compared to the pre-treatment period (−3–0 hr) by Sidak's multiple comparisons test. Data represent mean ± SEM. Two-way repeated-measure ANOVA with Sidak's multiple comparisons tests. *** p<0.001.

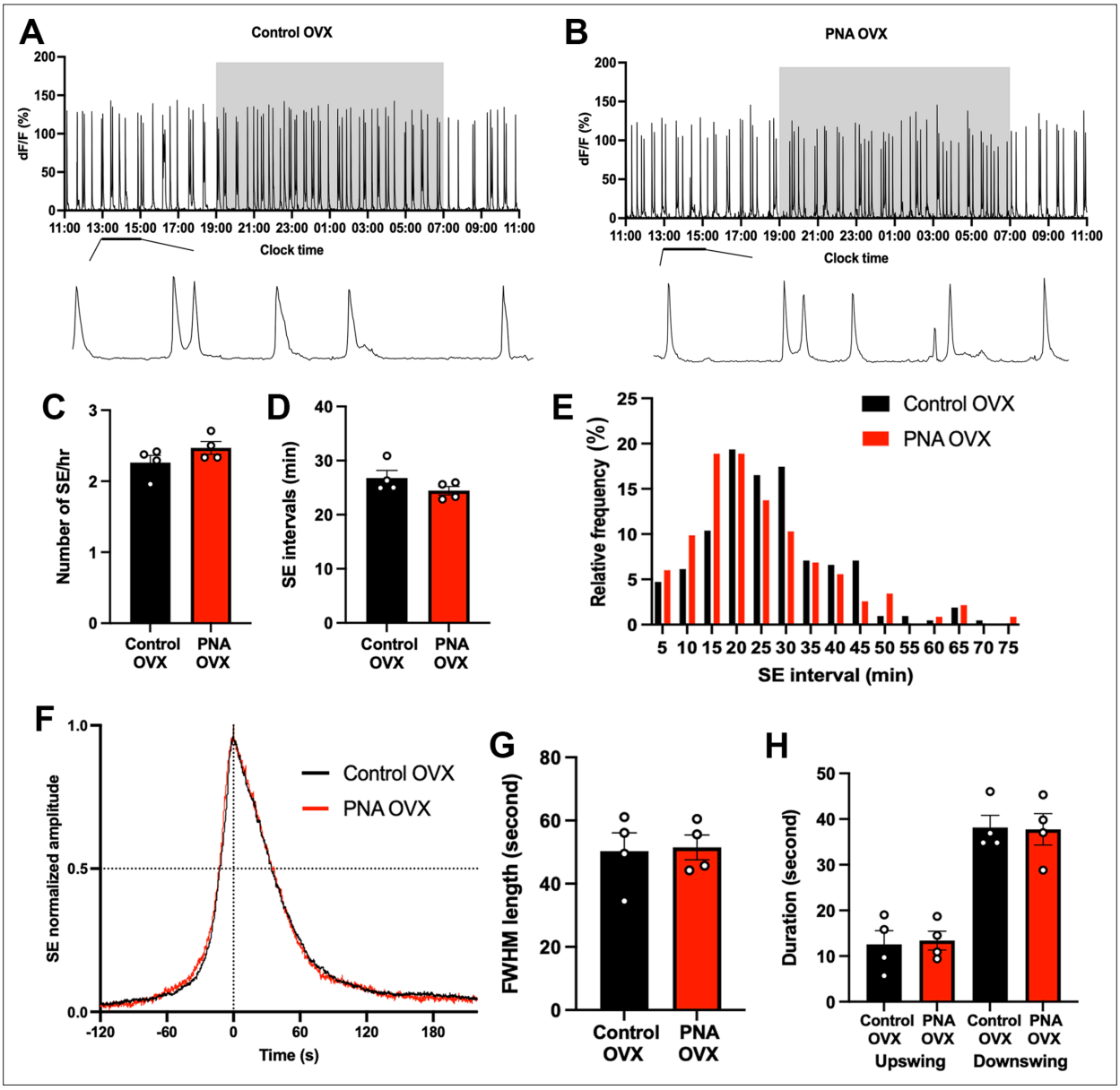

Figure 8. Similar ARN^{KISS} activity and SE profiles in control and PNA animals following ovariectomy. Representative 24 hr photometry recordings showing ARN^{KISS} neuron SEs observed in (**A**) control and (**B**) PNA females 3 weeks after ovariectomy (OVX) with the light-off period (19:00-07:00) represented by the shaded area and expanded views of the traces (13:00-15:00) given below (**A–B**). (**C**) SE frequency and (**D**) SE intervals in control and PNA mice (n=4), Mann-Whitney U tests. (**E**) Histogram showing relative SE frequencies occurring in 5 min bins, calculated separately for controls (n=212, 4 mice) and PNA (n=233, 4 mice) animals. X-axis represents the bin centers. Each SE cluster was analyzed as a single SE with the interval calculated from the first peak. (**F**) Continuous recordings at 10 Hz sampling rate showing normalized profile of SEs following OVX overlaid from control (black, 14 SEs from 4 animals) and PNA (red, 10 SEs from 4 animals). (**G**) FWHM length of OVX control and PNA animals (n=4). Mann-Whitney U test. (**H**) Durations of upswing and downswing for control and PNA animals (n=4), respectively. p>0.05, Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data show mean ± SEM. Each circle is an individual animal.