PNAS

Supporting Information for

Sexual differentiation of neural mechanisms of stress sensitivity during puberty

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Supporting Information Text

Supplementary Methods

Steroid hormone analysis

Steroid hormone analysis was conducted as previously described **(1, 2)**. Briefly, internal standard was added to serum samples (45-400 µl) followed by two liquid-liquid extractions using methyl tert butyl ether and then dichloromethane. Extracts were derivatized using dansyl chloride and then analyzed by LC-MS/MS (Sciex QTRAP 5500). Individual calibration curves were constructed for each analyte with at least 8 points. The linearity was $r > 0.9990$ and the curve fit was linear with $1/x$ weighting. None of the compounds of interest were detected in blank or double blank samples. Inter-assay coefficient of variation was determined by a pool of human serum and ranged from 8.62-11.88%.

Oxytocin and c-fos immunohistochemistry

Sections were blocked in 10% normal goat serum for 20 minutes and then incubated in rabbit anti-cfos (Synaptic Systems, 1:K) in PBS 0.5% TritonX overnight at room temperature. For oxytocin double staining, sections were washed in PBS and then incubated in goat anti-rabbit AlexaFluor 555 (Molecular Probes, 1:500 in PBX-TX) for 2 hrs. Sections were then incubated in mouse anti-oxytocin (Sigma-Aldrich 1:2000 in PBS-TX) overnight at 4 C. Sections were then washed in PBS and incubated goat anti-mouse AlexaFluor 488 (Molecular Probes, 1:500 in PBS-TX) for 2 hrs. For BNSTam sections, sections were washed in PBS and incubated in biotinylated goat anti-rabbit (Vector labs, 1:500 in PBS-TX for 2 hrs). Sections were washed in PBS and then incubated in avidin-conjugated peroxidase (Vector labs) for 30 min. After washing in PBS sections were developed in nickel enhanced diaminobenzidine (Vector labs) for 2 min.

Photometry data analysis

Photometry data was analyzed using a custom Python script, down sampled to 30 samples per second to match the 30fps video framerate. 405nm was used as the isosbestic wavelength and 470nm as the excitatory wavelength. To correct for motion artifact and bleaching of the fluorophore, the 405nm signal was fit to a biexponential model and subtracted from the excitatory output. Such that $\Delta F/F$ = [100*((470nm signal – fitted signal)/fitted signal)]. Z-scores were

calculated using the formula: z-score = ((ΔF/F - mean ΔF/F of baseline period)/standard deviation of ΔF/F of baseline period). For behavior non-specific analysis, the baseline period was 5 to 25 seconds after recording started. For behavior-specific analysis, the baseline period was -10 to -5 seconds before the behavior onset. Area under the curve (AUC) was calculated using the trapezoidal method for specified time points across behaviors. Linear mixed effects models were used to determine the effects of surgery, social condition, and time point using the statsmodels package **(3)** in Python using one of the following formulas:

- Z-Score=surgery condition + social condition + distance + surgery condition*social condition + surgery condition*distance + social condition*distance + surgery condition*social condition*distance + (1|mouse)
- AUC=surgery condition + time point + surgery condition*time point + (1|mouse)
- AUC=social condition + time point + social condition*time point + (1|mouse)
- AUC=time point + (1|mouse)

Supplementary Table

Supplementary Table 1. Antibodies and reagents used for immunohistochemistry

Reagent	Dilution	Company (catalog #)
rabbit anti-c-fos	1:1000	Synaptic Systems (226008)
mouse anti-oxytocin	1:2000	Sigma-Aldrich (MAB5296)
goat anti-rabbit biotinylated	1:500	VectorLabs (BA-1000)
goat anti-rabbit AlexaFluor555	1:500	Invitrogen (A-21428)
goat anti-mouse AlexaFluor488	1:500	Invitrogen (A-11011)

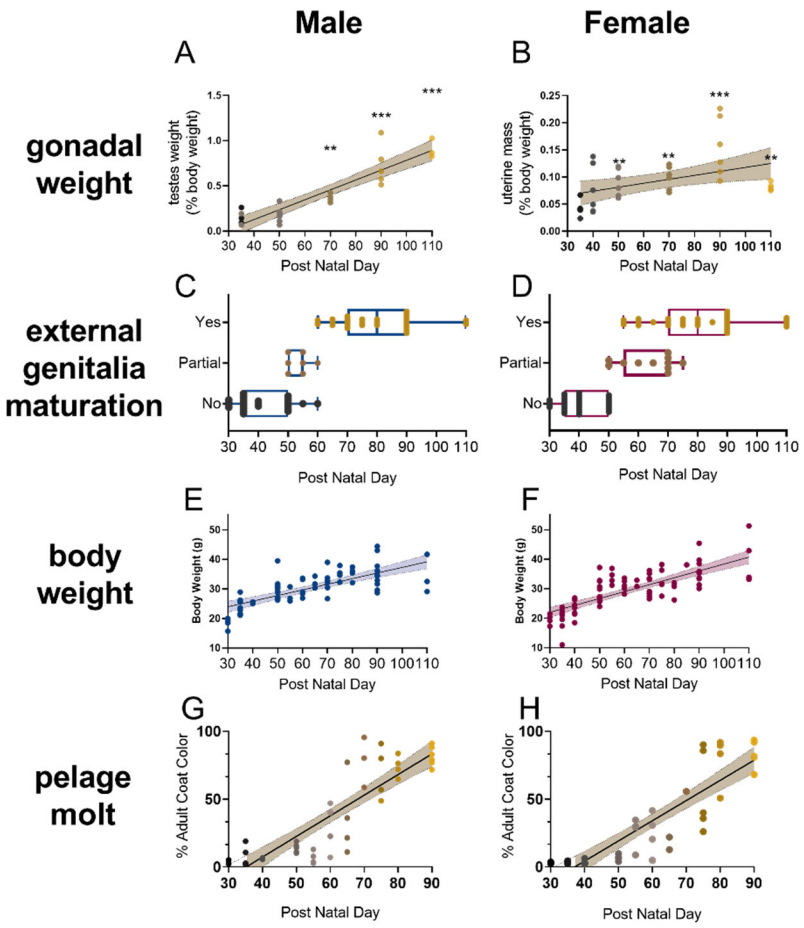

Fig. S1: Metrics of pubertal development in male and female California mice. Male testes weight (A) and female uterine (B) weight increases with age. Mice of different ages were assessed for preputial separation (C) or vaginal opening (D) to reveal that external genitalia maturation generally occurs after post-natal day 60. Measures of body weight in male (E) and female (F) mice increases with age. California mice molt from a gray pelage to a brown pelage, with the fastest rate of change occurring after post-natal day 60. ** $p<0.01$, *** $p<0.001$ versus post-natal day 35. Data available at doi:10.6084/m9.figshare.23681493**.**

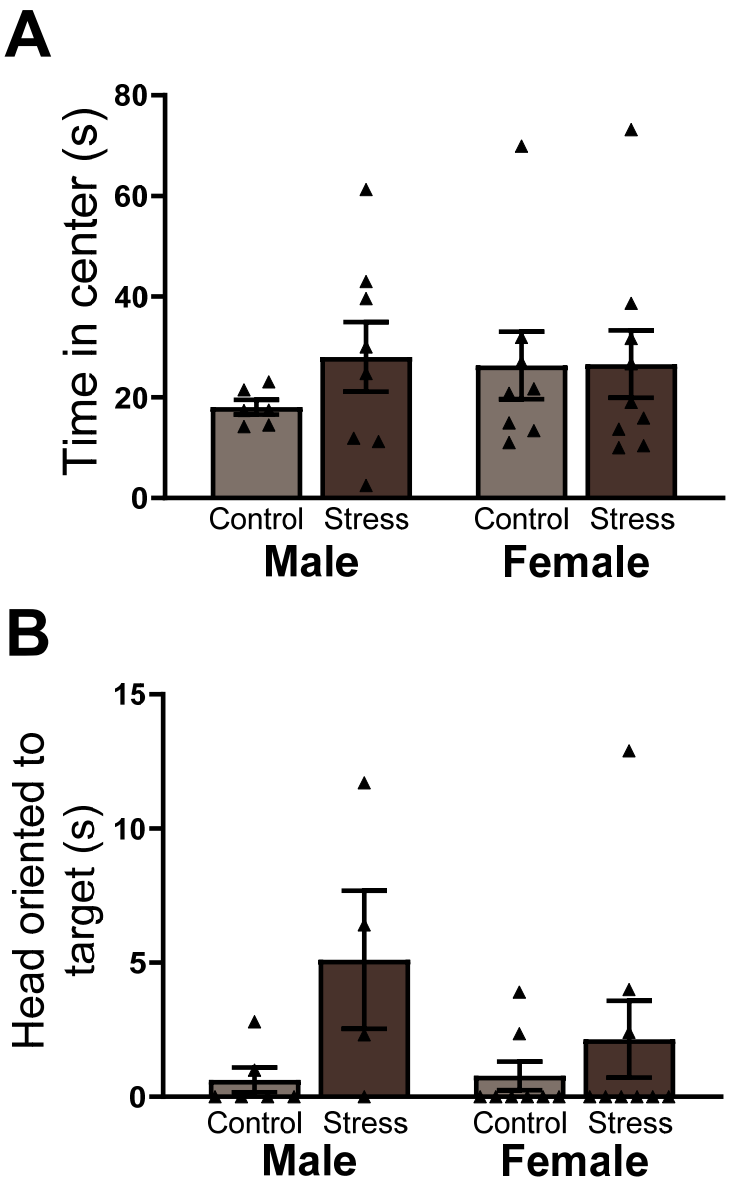

Fig. S2: For juvenile mice there were no differences in time spent in the center of the arena during open field (A) or in vigilance during the acclimation phase (B). Data available at doi:10.6084/m9.figshare.23681493

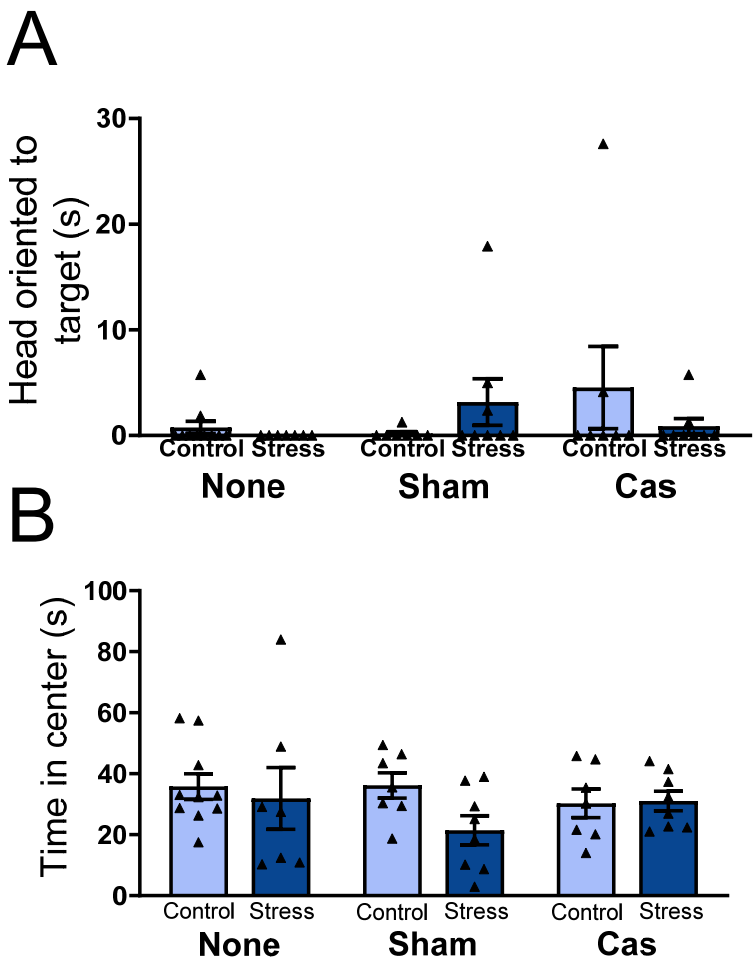

Fig. S3: For adult male mice, there were no differences in vigilance during the acclimation phase (A) or in time spent in the center of the arena during the open field phase (B). Data available at doi: 10.6084/m9.figshare.23681514.

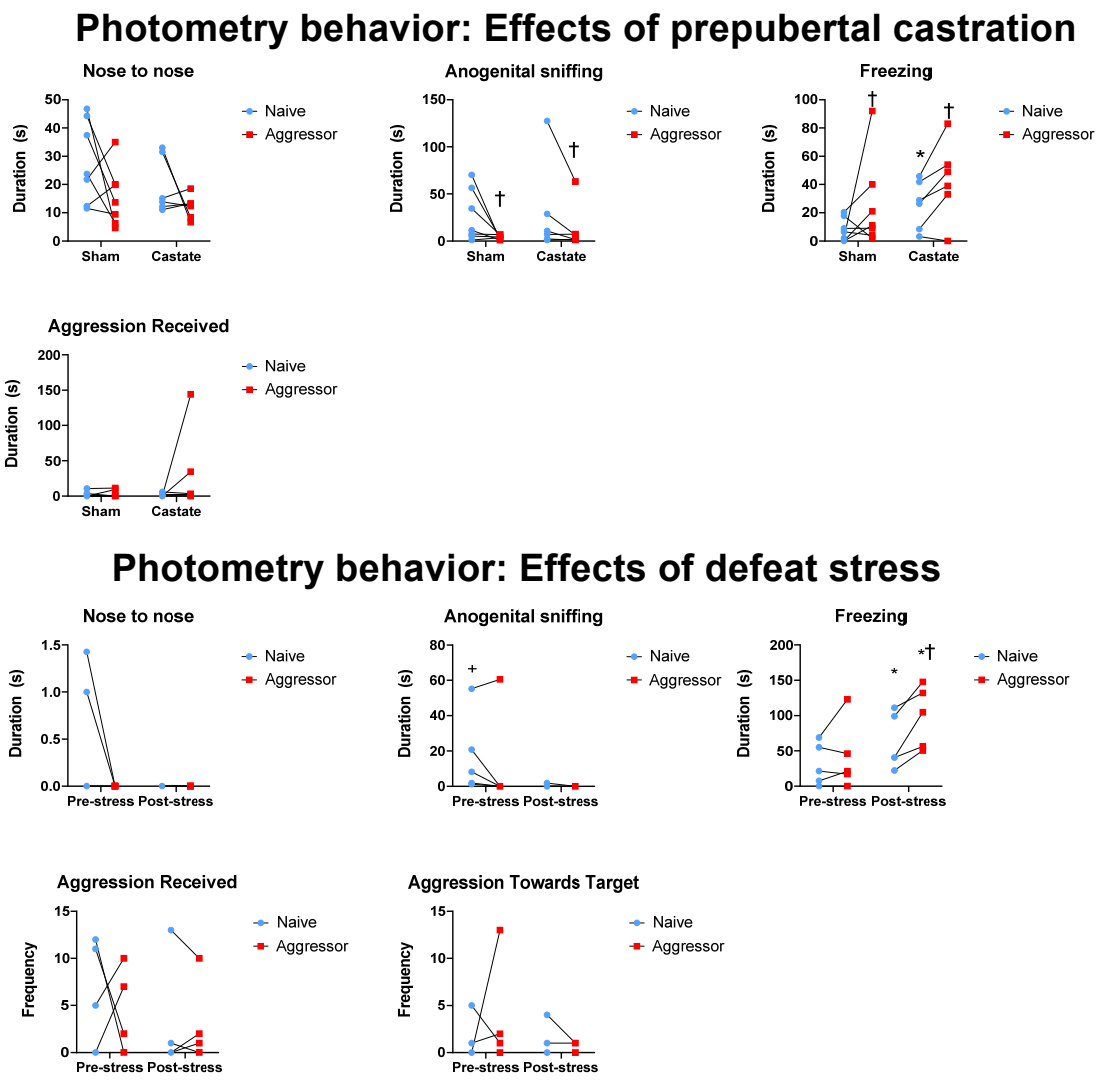

Fig. S4: Top: Behavior of sham and prepubertal castrated male mice during photometry observations with naïve or aggressor target mice. Bottom: Behavior of intact males mice before and after stress during photometry observations with naïve or aggressor target mice. * p <0.05 vs. sham, † p<0.05 vs naïve target mice, + p=0.06 vs. aggressor target mice. Data are available at doi: 10.6084/m9.figshare.23669292

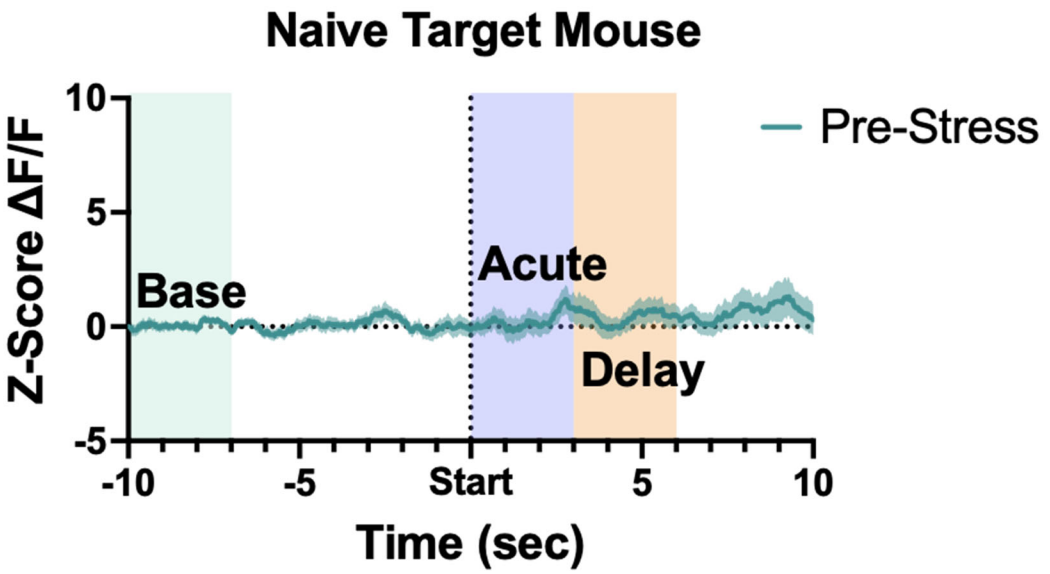

Fig. S5: Unstressed focal mice showed no changes in ΔF/F when engaging in anogenital sniffing with an aggression naïve target mouse. Data available at doi: 10.6084/m9.figshare.23983032

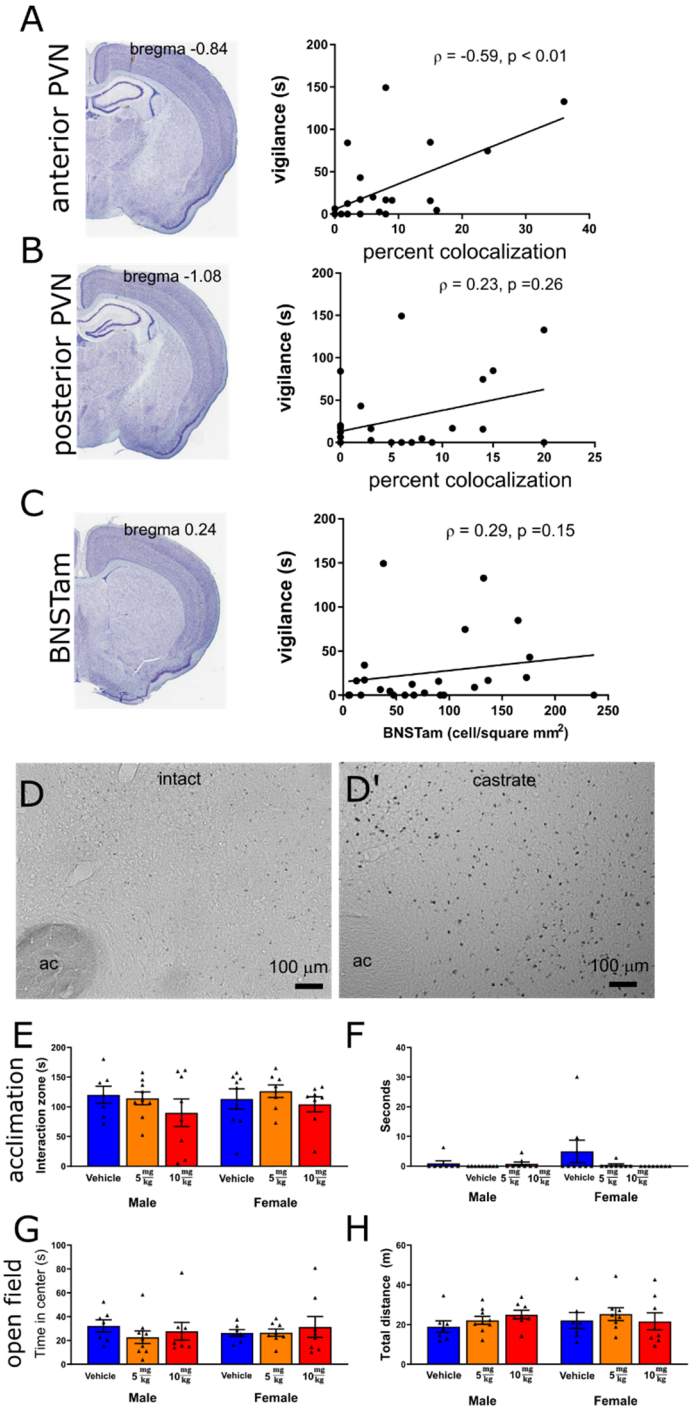

Fig. S6: Social vigilance was positively correlated with oxytocin/c-fos colocalizations in the anterior (A) but not posterior (B) PVN, and did not correlate with c-fos in anteromedial BNST (C). Castration increased c-fos immunoreactivity in the anteromedial BNST (D, D'). Oxytocin receptor antagonists did not affect time in the interaction zone (E) or vigilance (F) during the acclimation stage and did not affect time in the center (G) or locomotor behavior (H) during the open field phase. Data available at doi:10.6084/m9.figshare.23929644.

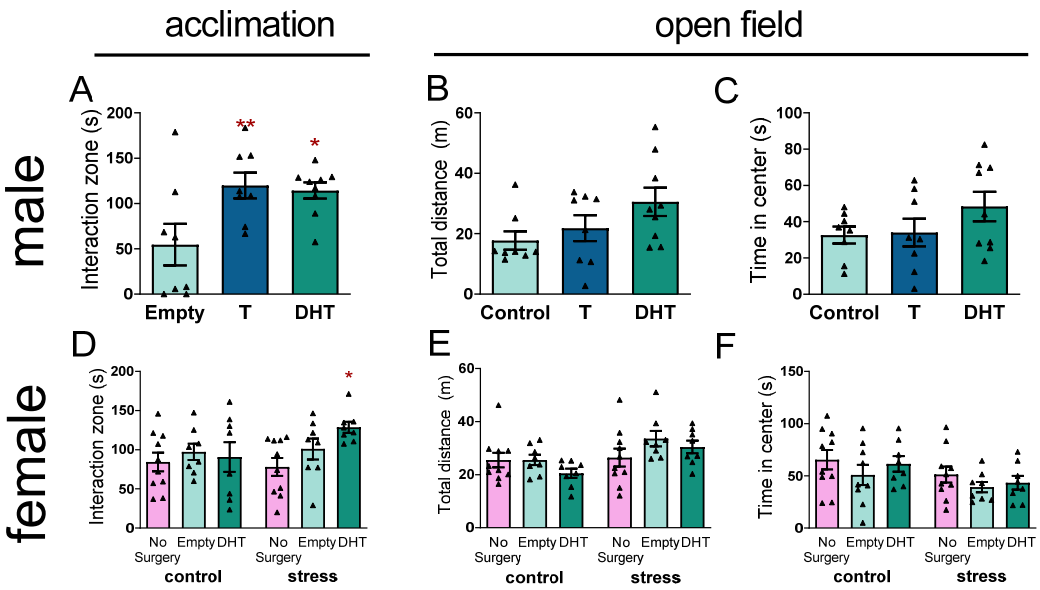

Fig. S7: In stressed males, T and DHT treatment increased approach to an empty cage (A) but had not effects on locomotor behavior (B) or time in the center (C) during the open field phase. For females, DHT treatment had no effect on approach to an empty cage (D) or behavior in the open field phase (E,F). After stress DHT increased approach to an empty cage. * $p<0.05$, ** $p<0.01$ vs empty implant. Data available at doi:10.6084/m9.figshare.23926101

Supplementary References

1. B. P. Kenealy, K. L. Keen, J. P. Garcia, L. K. Kohlenberg, E. Terasawa, Obligatory role of hypothalamic neuroestradiol during the estrogen-induced LH surge in female ovariectomized rhesus monkeys. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 13804–13809 (2017).

2. B. P. Kenealy, *et al.*, Neuroestradiol in the Hypothalamus Contributes to the Regulation of Gonadotropin Releasing Hormone Release. *J. Neurosci.* **33**, 19051–19059 (2013).

3. S. Seabold, J. Perktold, Statsmodels: Econometric and Statistical Modeling with Python in (2010), pp. 92–96.