Supplementary Material

Activation of arcuate nucleus GABA neurons promotes luteinizing hormone secretion and reproductive dysfunction: implications for polycystic ovary syndrome

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*equal contribution

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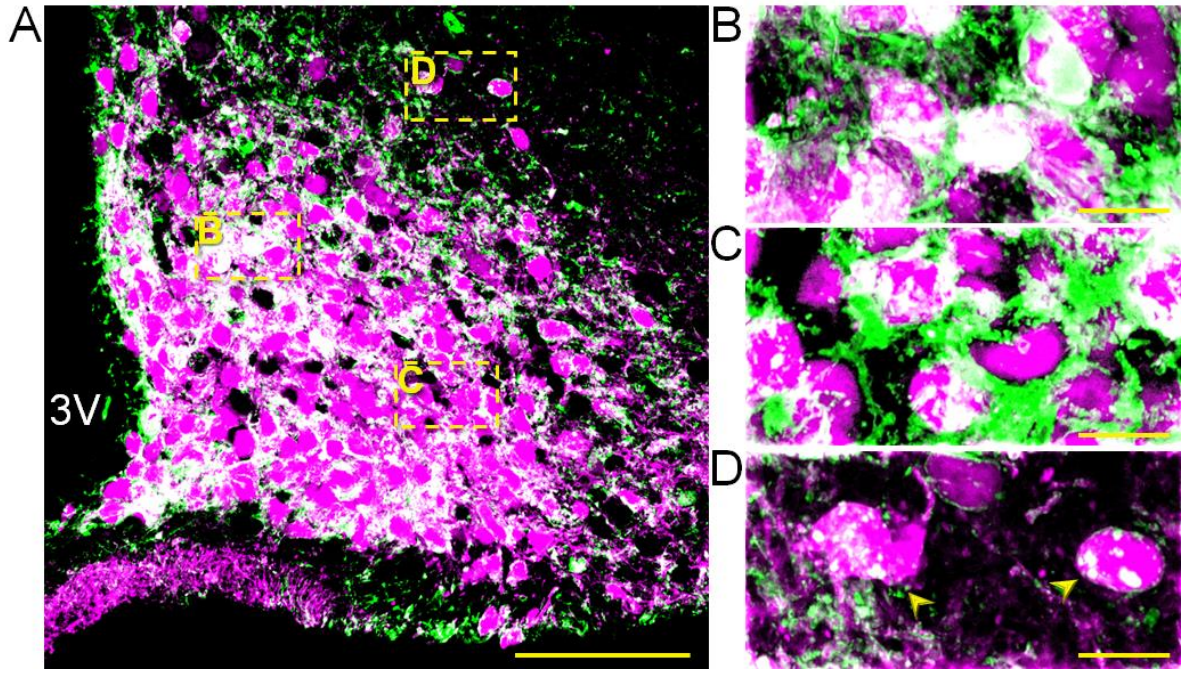
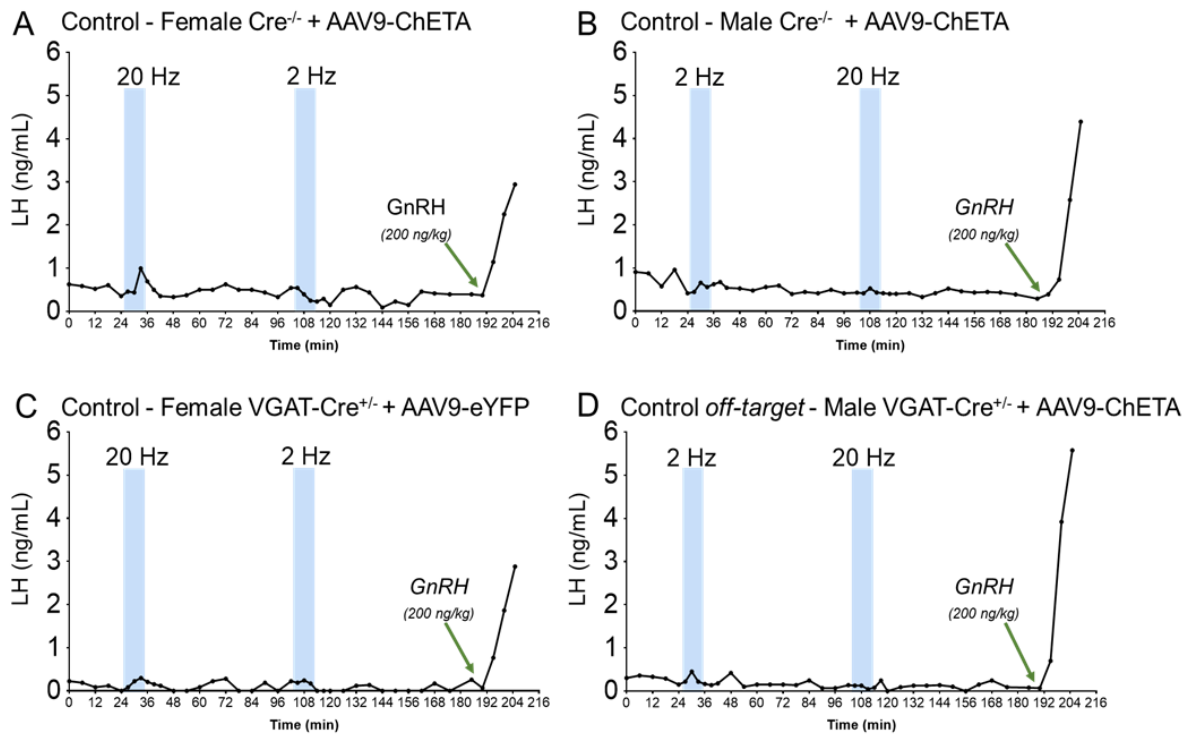
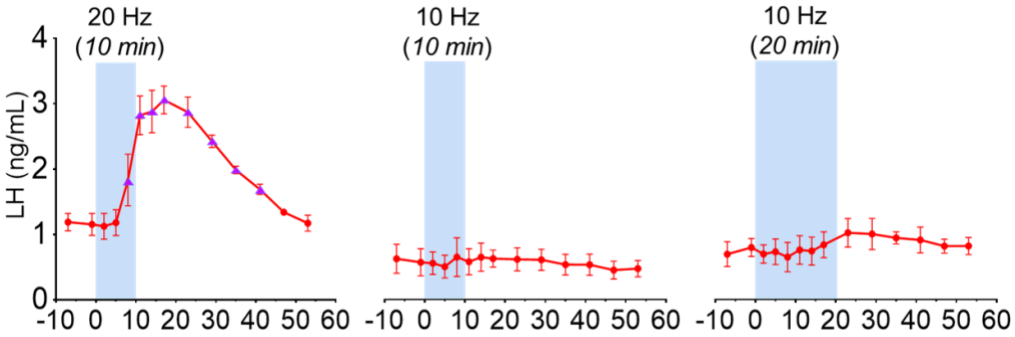


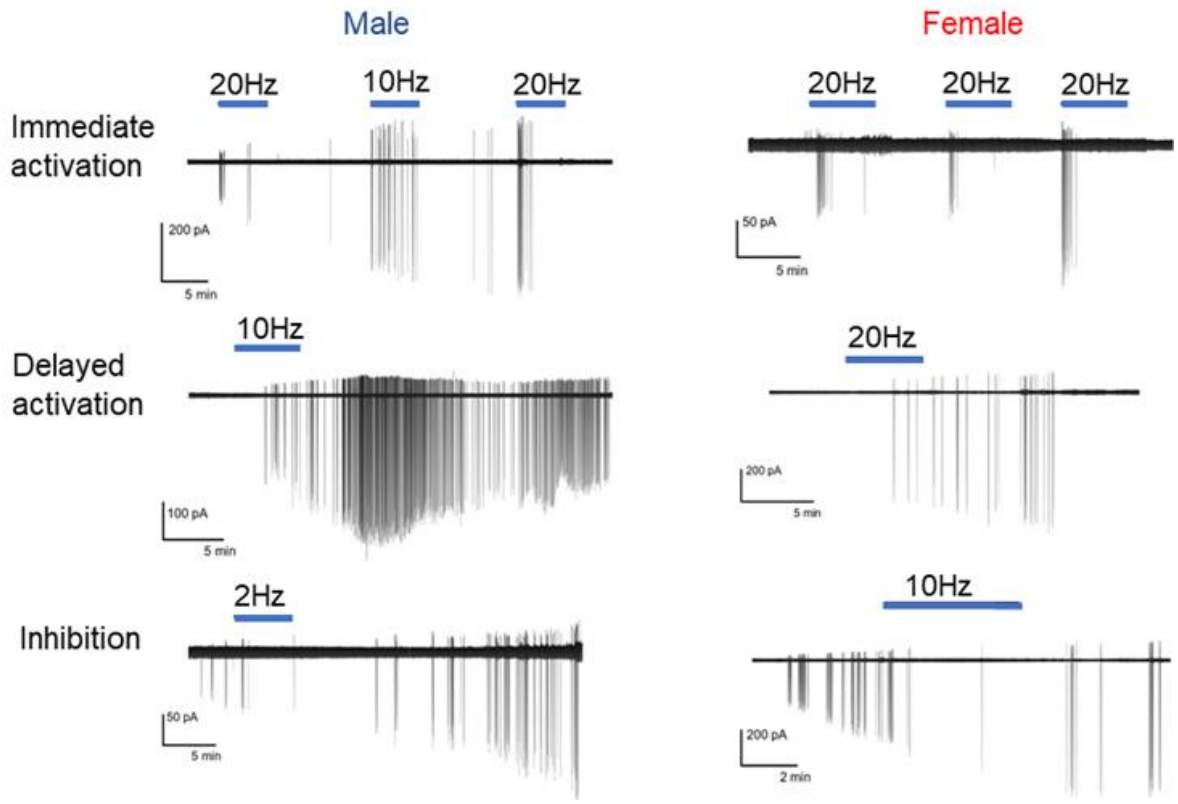
Supplementary Figure 1: Expression of AAV9-ChETA-eYFP in ARN GABA neurons. (*A*) Representative confocal image (12.5-µm optical thickness) of ARN GABA neurons (*magenta*) co-expressing AAV9-ChETA-eYFP (*green*) in a transfected VGAT-Cre;tdTomato male mouse (scale bar = 100 µm). (*B, C*) Higher magnification confocal projections demonstrating transfection near the injection site (scale bar = 10 µm). (*D*) Non-transfected ARN GABA neurons that appear to receive contact (*yellow* arrows) from ChETA-expressing fibers within the ARN. (Scale bar = 10 µm).



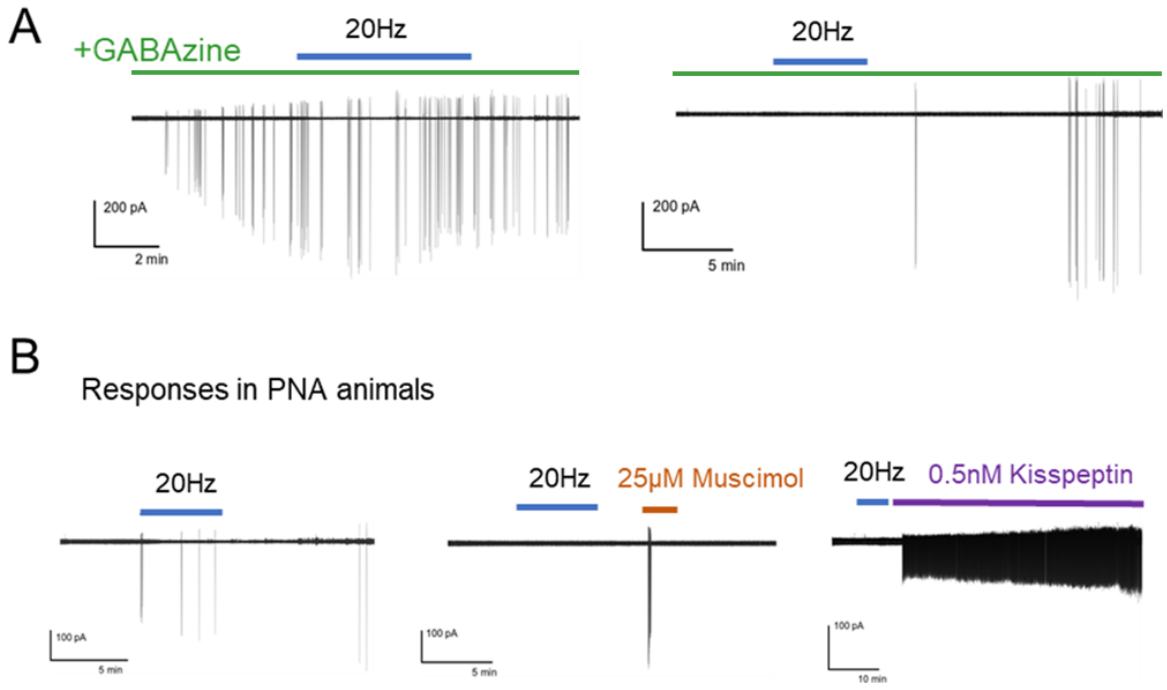
Supplementary Figure 2: Examples of LH release in response to photostimulation in control animals. (*A*, *B*) Representative traces of LH secretion pattern from $Cre^{-/-}$ diestrous female (N = 4) and male (N = 5) mice, previously transfected with AAV9-ChETA-eYFP in the ARN. (*C*) Representative LH secretion in control VGAT-Cre diestrous female mice (N = 3) injected with control virus (AAV9-eYFP). (*D*) Representative LH secretion in off-target VGAT-$Cre^{+/-}$ male (N = 5) and female (N = 4) mice. Mice received s.c. GnRH (200 ng/kg) at the end of the protocol to confirm normal pituitary gland responses. Blue bars indicate 5-ms photostimulation delivered at 2 or 20 Hz over 10 min.



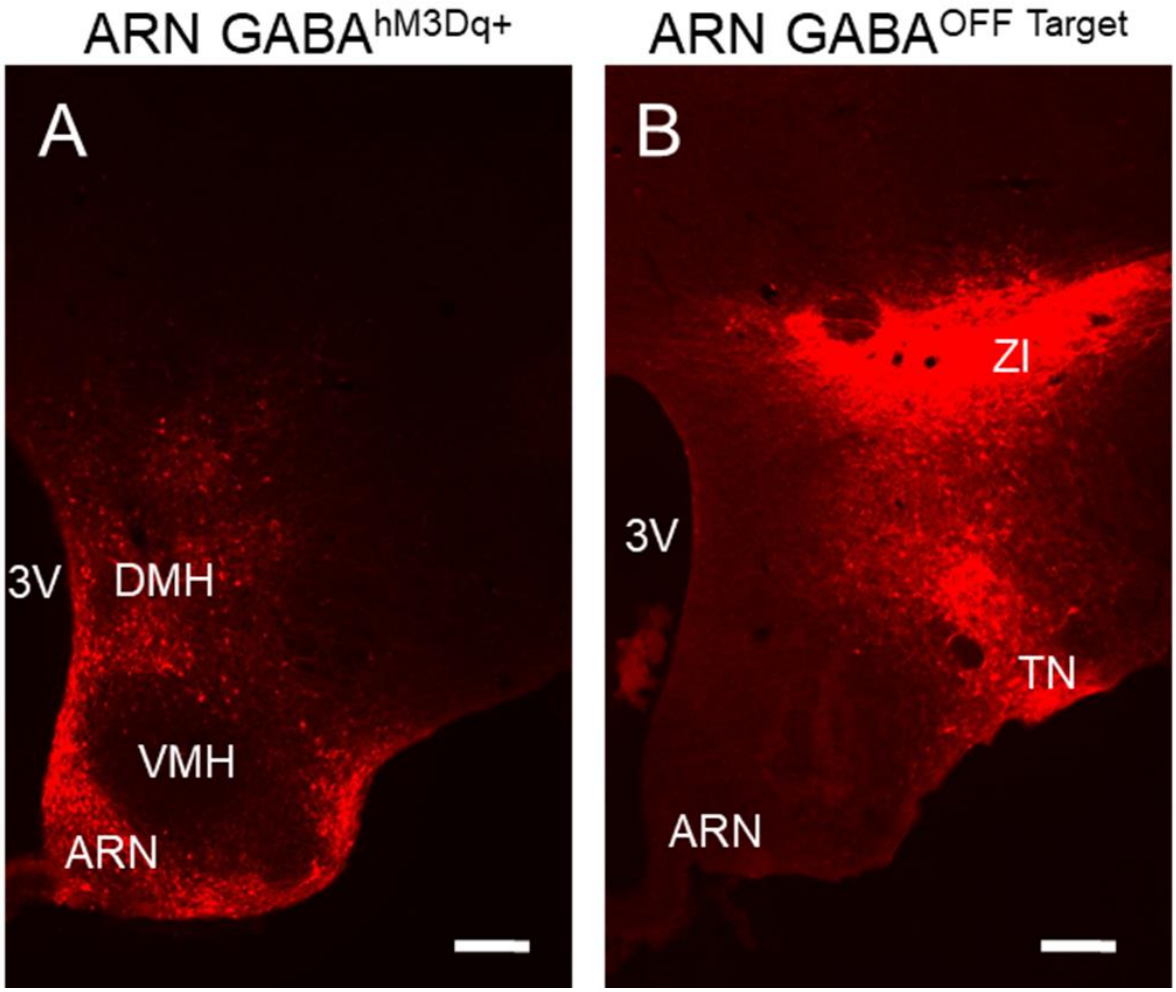
Supplementary Figure 3: Frequency of photostimulation determines LH response to optogenetic activation of ARN GABA neurons. Summary data showing the pattern of mean ± SEM LH levels following photostimulation of AAV9-ChETA-eYFP-transfected ARN GABA neuron fibers in VGAT-Cre$^{+/-}$ female mice (N = 3). Three different paradigms of photostimulation were tested: 20 Hz over 10 min, 10 Hz over 10 min, and 10 Hz over 20 min (i.e. conveying the same number of light pulses as the former). Magenta triangles indicate LH levels significantly higher than baseline levels. One-way ANOVA with Tukey's post hoc test.



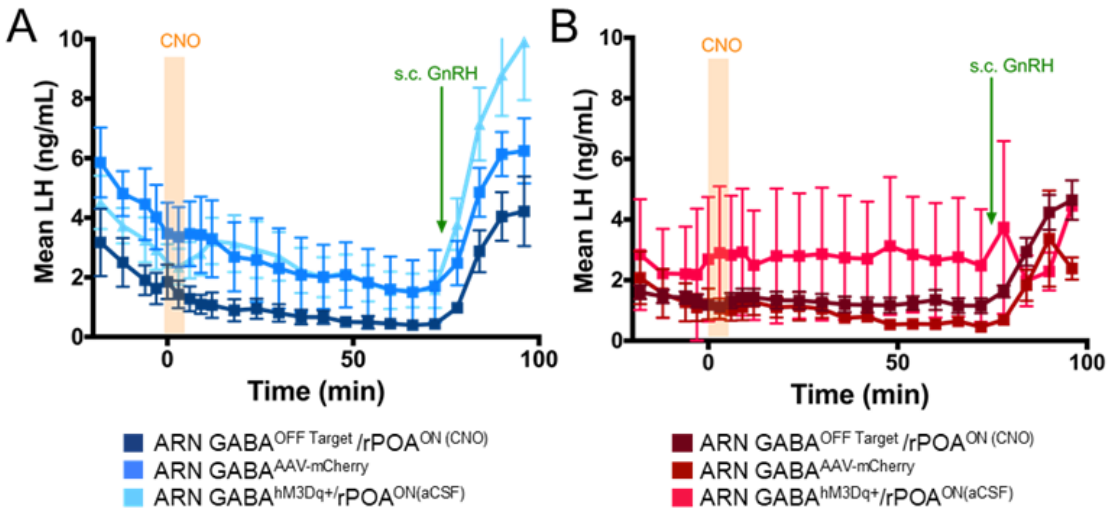
Supplementary Figure 4: GnRH neurons exhibit a range of responses to optogenetic activation of VGAT-fibers in male and female mice. Examples of the different patterns of GnRH neuron firing observed after 2, 10, and 20 Hz blue light activation of ARN GABA fibers located in the rPOA of male and female mice. Bars above the trace indicate the duration of light-stimulation exposure with the frequency indicated.



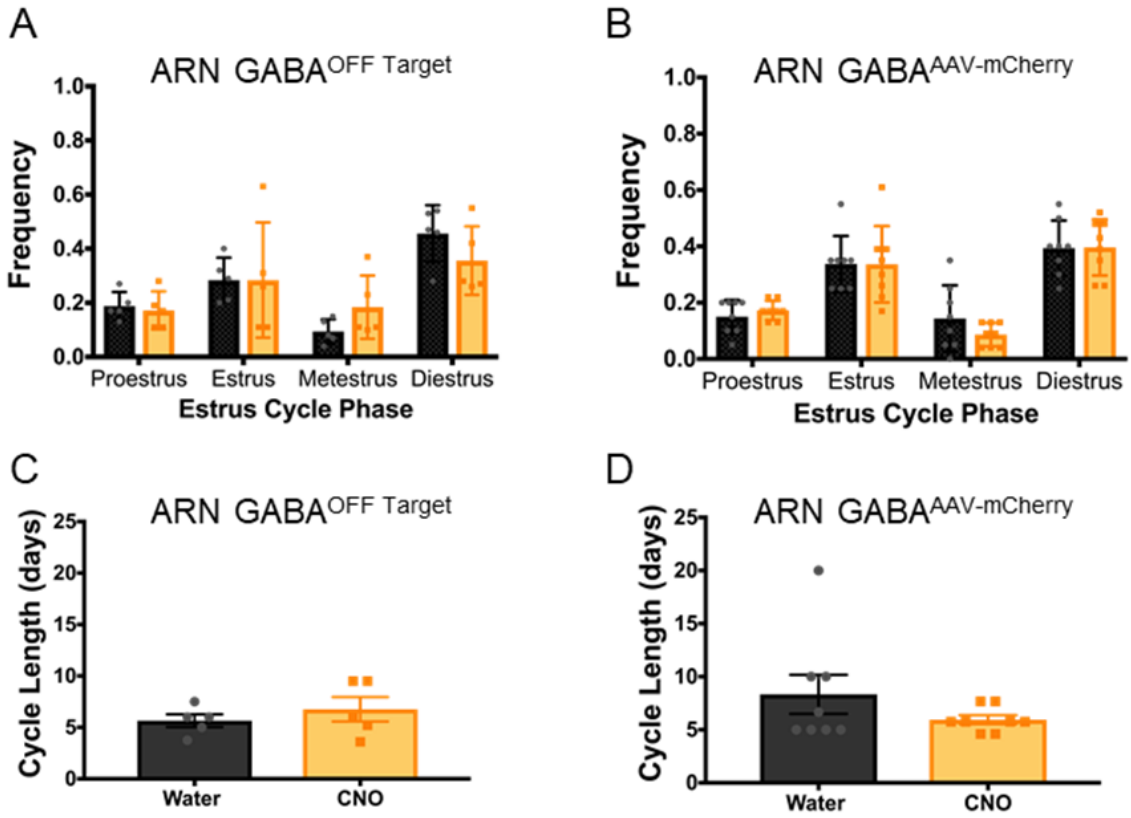
Supplementary Figure 5: Effects of optogenetic activation of VGAT fibers on GnRH neurons are abolished in the presence of GABAzine and reduced in PNA female mice. *(A)* Two example traces showing an absence of change in GnRH neuron firing following 20 Hz blue light illumination (horizontal bars) when 5 μM GABAzine is included in the bathing solution. *(B)* Three traces showing effects of 20 Hz blue light illumination on GnRH neuron firing. The first shows an immediate and transient activation while the remaining cells did not respond to optogenetic activation but responded to muscimol and kisspeptin.



Supplementary Figure 6. Mapping AAV5-hM3Dq-mCherry expression in the mediobasal hypothalamus. *(A, B)* Examples of typical patterns of ARN and extra-ARN transfection observed following bilateral injection of AAV5-hM3Dq-mCherry into the ARN of VGAT-Cre$^{+/-}$;τGFP mice. Mice displaying AAV5-hM3Dq-mCherry expression largely restricted to the ARN were considered targeted and termed ARN GABA^{hM3Dq+} and those with little (less than 10 cells) or no transfection in the ARN and some or no transfection in other hypothalamic areas were termed ARN GABA$^{OFF\ Target}$. Panel B shows an example of strong transfection of GABA neurons in the zona incerta, but a complete absence of labelling in the ARN, classified as ARN GABA$^{OFF\ Target}$. DMH = dorsomedial nucleus of the hypothalamus, VMH = ventromedial nucleus of the hypothalamus, ARN = arcuate nucleus, 3V = third ventricle, scale bar = 100 µm.



Supplementary Figure 7: Examples of LH release in response to intra-rPOA CNO or aCSF administration in control animals. (*A*,*B*) Mean ± SEM values of LH secretion patterns before and after delivery of CNO or aCSF into the rPOA of different male (*A*) and female (*B*) VGAT-Cre$^{\tau GFP}$ control groups. No significant changes in LH secretion were observed following CNO administration to ARN GABA$^{OFF\ Target}$/rPOA$^{ON(CNO)}$ (N = 4 males, N = 12 females) or ARN GABA$^{AAV\text{-}mCherry}$ mice (N = 4 males, N = 3 females), or following aCSF administration to ARN GABA^{hM3Dq+}/rPOA $^{ON(aCSF)}$; N = 4 males, N = 2 females). GnRH was administered s.c. as a positive control for LH secretion. Only animals exhibiting an LH response to GnRH administration were included.



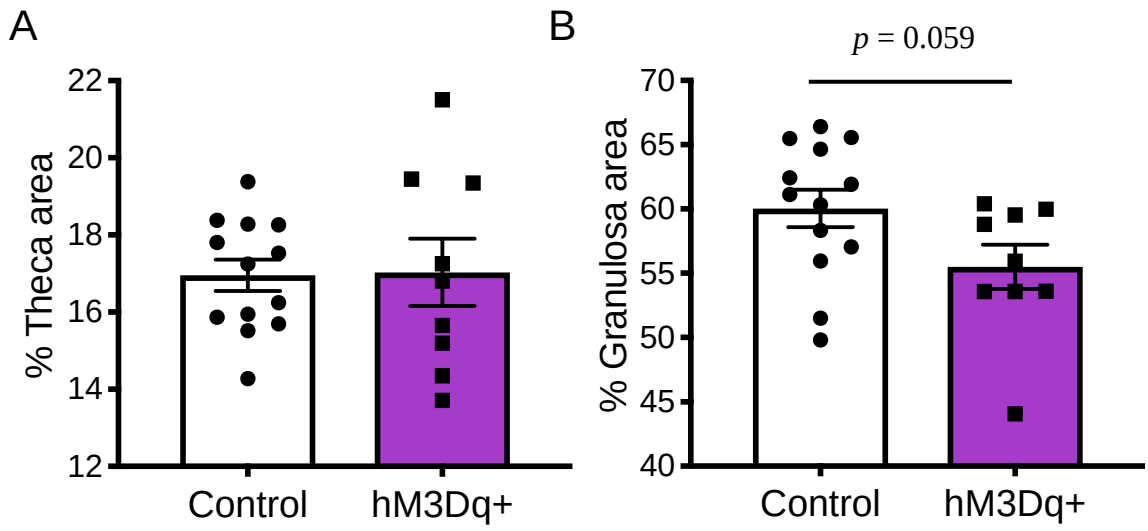
Supplementary Figure 8: Chronic activation of ARN GABA neurons has no impact on estrous cyclicity in control mice. (*A,B*) Mean ± SEM frequency of each estrous cycle stage before (water; grey bars) and during CNO exposure (CNO; yellow bars) in ARN GABA$^{\text{OFF Target}}$ (N = 5) and ARN GABA$^{\text{AAV-mCherry}}$ (N = 8) controls. (*C,D*) Mean ± SEM cycle length in days before (water; grey bars) and during CNO exposure (CNO; yellow bars) of control animals. (*A,B*) Multiple Student's t-tests; (*C,D*) Mann-Whitney test.

FEMALES	No. VGAT neurons	No. ChETA neurons	% VGAT with ChETA	%ChETA with VGAT
tARN	226.3 ± 29.9	213.9 ± 28.1	93.3 ± 1.6	98.6 ± 0.3
cARN	260.4 ± 51.3	208.0 ± 33.6	82.4 ± 4.7	98.7 ± 0.8
MALES	**No. VGAT neurons**	**No. ChETA neurons**	**% VGAT with ChETA**	**%ChETA with VGAT**
tARN	250.0 ± 35.2	237.1 ± 31.9	94.4 ± 1.5	99.5 ± 0.3
cARN	282.6 ± 59.8	239.1 ± 55.4	84.7 ± 6.2	99.2 ± 0.2

Supplementary Table 1: Expression of channelrhodopsin-2 E123T accelerated (ChETA) variant in ARN GABA neurons of transfected VGAT-Cre;tdTomato mice. Mean ± SEM number per section of single-labelled VGAT and ChETA-expressing neurons in the tuberal ARN (tARN) and caudal ARN (cARN) from transfected VGAT-Cre;tdTomato diestrous female (N = 5) and male (N = 4) mice. VGAT neurons were identified by endogenous tdTomato reporter expression (red fluorescence). The table also provides the mean ± SEM percentage of dual-labelled VGAT and ChETA neurons in the tARN and cARN regions in both sexes. Cell counts and co-localization analysis included two representative sections from each of the tARN and cARN regions.

	ARN GABA^{hM3Dq+}	ARN GABA$^{OFF\ Target}$
Acute intra-rPOA CNO delivery- Male	47.54 ± 7.24 (N = 6)	2.83 ± 1.97 (N = 4)
Acute intra-rPOA CNO delivery- Female	26.17 ± 6.03 (N = 5)	1.62 ± 1.45 (N = 5)
Chronic CNO delivery via drinking water	45.17 ± 6.49 (N = 13)	5.27 ± 1.20 (N = 5)

Supplementary Table 2. Number of hM3Dq+mCherry cells per section of ARN. (Mean ± SEM, N = number of animals)



Supplementary Figure 9: The impact of chronic ARN GABA neuron activation on follicle wall composition. Mean ± SEM proportion of the follicle wall made up of theca cells (*A*) or granulosa cells (*B*) from control (ARN GABA^{mCherry} and ARN GABA^{OFFTarget}; N=13) and ARN GABA^{hM3Dq+} (N=9) mice following CNO administration. Student's t-test.