CRISPR-Cas9 knockdown of ESR1 in preoptic GABA-kisspeptin neurons suppresses the preovulatory surge and estrous cycles in female mice

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Abstract

Evidence suggests that estradiol-sensing preoptic area GABA neurons are involved in the pre-ovulatory surge mechanism necessary for ovulation. In vivo CRISPR-Cas9 editing was used to achieve a 60-70% knockdown in estrogen receptor alpha (ESR1) expression by GABA neurons located within the region of the rostral periventricular of the third ventricle (RP3V) and medial preoptic nuclei (MPN) in adult female mice. Mice exhibited variable reproductive phenotypes with the only significant finding being those mice with bilateral ESR1 deletion in RP3V GABA neurons that had reduced cFos expression in GnRH neurons at the time of the surge. One sub-population of RP3V GABA neurons expresses kisspeptin. Re-grouping ESR1-edited mice on the basis of their RP3V kisspeptin expression revealed a highly consistent phenotype; mice with a near complete loss of kisspeptin immunoreactivity displayed constant estrus and failed to exhibit surge activation but retained pulsatile LH secretion. These observations demonstrate ESR1-expressing GABA-kisspeptin neurons in the RP3V are essential for the murine preovulatory LH surge mechanism.

INTRODUCTION

Understanding how circulating estradiol levels feedback on the brain to regulate the pulse and surge profiles of gonadotropin-releasing hormone (GnRH) secretion is of central importance to elucidating the neural control of mammalian fertility. Studies in genetic mouse models have clearly identified that estrogen receptor alpha (ESR1) is the key receptor underlying both estrogen positive and negative feedback (Couse, Yates et al. 2003, Herbison 2015). Despite significant progress (Herbison 2015, Moenter, Silveira et al. 2019, Goodman, Herbison et al. 2022, Kauffman 2022), defining the precise cellular and molecular mechanisms through which ESR1 ultimately modulates GnRH secretion remains a significant challenge. This is particularly intriguing given that the final output neuron of the network, the GnRH neuron, expresses ESR2 but not ESR1 (Herbison and Pape 2001).

Recent experiments using live cell imaging and *in vivo* CRISPR gene editing have demonstrated that estradiol acts directly at ESR1-expressing kisspeptin neurons in the arcuate nucleus to suppress the activity of the GnRH pulse generator and bring about estrogen negative feedback at the level of the brain (McQuillan, Clarkson et al. 2022). This same level of definition has not yet been possible for the positive feedback mechanism where only the general location of estradiol action is known with certainty. Viral retrograde labelling demonstrated that ESR1-expressing afferents to the GnRH neuron cell bodies are concentrated in the anteroventral periventricular (AVPV) and preoptic periventricular (PVpo) nuclei of the hypothalamus (Wintermantel, Campbell et al. 2006); collectively termed the rostral periventricular area of the third ventricle (RP3V) (Herbison 2008). As the selective knockdown of ESR1 in the RP3V results in acyclic mice with an absent luteinizing hormone (LH) surge (Porteous and Herbison 2019), it seems very likely that the RP3V is the area within which estradiol acts to enable the surge mechanism in mice.

The identity of the key ESR1-expressing populations within the RP3V mediating the estrogen positive feedback mechanism has not been defined conclusively. Most evidence supports a role for the RP3V kisspeptin neurons. These cells express ESR1, are activated at the time of the surge, and have their kisspeptin synthesis strongly upregulated by estradiol (Smith, Popa et al. 2006, Adachi, Yamada et al. 2007, Clarkson, d'Anglemont de Tassigny et al. 2008). Further, RP3V kisspeptin neurons project directly to GnRH neuron cell bodies where they provide a strong and lasting excitatory influence while the selective activation of RP3V kisspeptin neurons *in vivo* evokes a surge-like increment in LH secretion (Piet, Kalil et al. 2018). Given these findings, it was surprising to find that selective *in vivo* CRISPR knockdown of ESR1 in AVPV kisspeptin neurons blunted the amplitude of the LH surge but had no impact on estrous cyclicity (Wang, Vanacker et al. 2019). This suggested that kisspeptin neurons may not be the key or sole phenotype in the RP3V responsible for positive feedback in mice.

There is a long-standing evidence implicating GABAergic inputs to the GnRH neuron cell bodies in the rodent positive feedback mechanism. This ranges from studies showing alterations in $GABA_A$ receptor transmission at GnRH neurons around the time of the daily LH surge (Christian and Moenter 2007) to *in vivo* studies demonstrating a functionally significant fall in GABA release within the preoptic area just prior to the LH surge (Herbison and Dyer 1991, Jarry, Hirsch et al. 1992). More recently, the genetic deletion of ESR1 in all

GABA neurons was found to result in failure of the positive feedback mechanism (Cheong, Czieselsky et al. 2015).

The present study was designed to test directly whether ESR1 GABA neurons located in the medial preoptic area are critical for the positive feedback mechanism. We employed *in vivo* CRISPR gene editing to knockdown ESR1 in the RP3V and adjacent medial preoptic nucleus (MPN) and evaluated estrous cyclicity alongside pulsatile and surge patterns of LH secretion.

RESULTS

CRISPR knockdown of ESR1 in preoptic area GABA neurons

Design and testing of gRNA

The design and characterization of guide RNAs for *Esr1* (NM_007956) have been reported previously (McQuillan, Clarkson et al. 2022). In brief, gRNA directed at both the sense and antisense strands around exon 3 were tested for efficacy *in vitro* using an ESR1-expressing hypothalamic cell line mHypo-CLU189-A genetically modified to stably express Cas9 (CLU189-Cas922C). Transduction with AAV-U6-gRNA-EGFP identified gRNA-1, -2, -3 and -6 to be the most effective in reducing *Esr1* mRNA levels by 20-30% *in vitro*. The efficacy of gRNA-2, -3 and -6 were assessed *in vivo* by giving unilateral injections AAV1-U6-gRNA(2, 3, or 6)-Ef1α-mCherry into the medial preoptic area of *Vgat-Cre,LSL-Cas9-EGFP* mice (N=4 per gRNA). This was found to result in 79±7%, 72±5% and 78±3% reductions, respectively, in the numbers of ESR1-immunoreactive EGFP-expressing neurons on the injected side of the brain compared with the non-injected side. Our prior studies have shown that this CRISPR-Cas9-AAV platform enables the knockdown of ESR1 in a selective manner in genetically targeted hypothalamic kisspeptin neurons (McQuillan, Clarkson et al. 2022).

ESR1 knockdown in preoptic GABA neurons

In the present study, gRNA-2 and gRNA-3 were used for ESR1 knockdown with their effects in medial preoptic GABA neurons evaluated in *Vgat-Cre,LSL-Cas9-EGFP* mice at the end of the *in vivo* series of studies. In keeping with the predominant GABAergic phenotype of preoptic area neurons (Moffitt, Bambah-Mukku et al. 2018), large numbers of cells expressing EGFP/Cas9 were detected throughout the region (Fig.1A,B), including the AVPV, PVpo, MPN and bed nucleus of the stria terminalis. Analyses of the MPN in mice that did not receive AAV injections found that 52.1±4.2% of VGAT-EGFP neurons expressed ESR1 (N=12).

The percentage of VGAT-EGFP neurons expressing ESR1 was evaluated within AAV-injected regions of the RP3V and MPN in mice receiving gRNA-2, gRNA-3, or control gRNA-LacZ. In keeping with prior studies showing that up to one half of medial preoptic area GABA neurons express ESR1 (Herbison 1997, Cheong, Czieselsky et al. 2015), we found in gRNA-LacZ mice that 54.9±3.7% and 59.7±3.9% of VGAT-EGFP neurons in the RP3V (N=6) and MPN (N=7), respectively, were immunoreactive for ESR1 (Fig.1A,C). In mice receiving gRNA-2 these values were reduced to 23.1±3.3% (N=8) and 22.2±4.7% (N=9) while gRNA-3 resulted in 17.6±1.8% (N=8) and 19.3±2.9% (N=8) of VGAT-EGFP neurons with ESR1 in the RP3V and

MPN, respectively (Fig.1B,C). Inexplicably, one gRNA-3 mouse with correct AAV targeting maintained normal levels of ESR1 expression (52%) and was excluded from the analysis. Overall, gRNA2&3 generated a significant 62% and 68% reduction ESR1 expression by RP3V VGAT neurons ($p<0.0001$; one-way ANOVA $F_{(2, 19)}= 41.91$, post-hoc Dunnett's test versus LacZ $p<0.0001$ (gRNA2 & gRNA3), Fig.1C) and a 63% and 71% reduction ESR1 expression by MPN VGAT neurons ($p<0.0001$; one-way ANOVA $F_{(2, 21)}= 29.79$, post-hoc Dunnett's test versus LacZ $p<0.0001$ (gRNA2 & gRNA3), $p<0.0001$ (gRNA3); Fig.1C). Together, these data demonstrate that the two gRNAs result in a very similar 60-70% reduction in ESR1 expression in preoptic VGAT neurons.

Locations of VGAT ESR1 knockdown

Bilateral AAV injections occurred in an heterogenous manner within the medial preoptic area of individual mice (Fig.1D-G). Due to the established importance of the RP3V in the estrogen positive feedback mechanism and possible role of the MPN (Wintermantel, Campbell et al. 2006, Porteous and Herbison 2019), mice were categorized according to the unilateral or bilateral spread of AAV in the AVPV, PVpo and MPN. Injection sites including parts of the AVPV and/or PVpo were termed an "RP3V" hit while AAV spread in central aspects of the medial preoptic area involving at least part of the MPN and were termed an "MPN" hit (see Fig.1D-G).

Nine gRNA-2 mice had injection sites involving both the RP3V and MPN; in three mice this included bilateral hits of the "RP3V" and "MPN" (Fig.1D) while four mice had one region with a bilateral hit and a unilateral or miss for the other brain region (Fig.1E). The remaining two mice received solely unilateral injections and were excluded from functional correlations. Eight gRNA-3 mice had injection sites involving RP3V and MPN. This included bilateral hits of the "RP3V" and "MPN" (Fig.1F) in three mice with another three mice having one region with a bilateral hit and the other unilateral. The remaining two gRNA-3 mice received solely unilateral injections and were excluded from functional correlations. Of eight gRNA-LacZ mice, four had bilateral injections involving the RP3V and/or MPN (Fig.1G). Two mice had unilateral injections involving both the AVPV and MPN but as ESR1 expression in VGAT neurons is not changed by gRNA-LacZ injections were included in functional analyses. Two further gRNA-LacZ mice with small unilateral injections in only the MPN were excluded from functional correlations. This resulted in experimental groups of 6 gRNA-LacZ, 7 gRNA-2 and 6 gRNA-3 mice.

Effects of VGAT ESR1 knockdown on estrous cyclicity

The estrous cycles of *Vgat-Cre,LSL-Cas9-EGFP* mice were determined over a three-week period before stereotaxic injection of AAV-gRNA and then again for 3 weeks after a three-week post-surgical interval. Mean cycle length for all animals prior to AAV injection was 5.9±1.5 days. No significant differences ($p>0.05$, Wilcoxon paired tests) were detected in cycle length for mice given gRNA-LacZ (n=6), gRNA-2 (N=7) or gRNA-3 (n=6) (Fig.2A-C). Whereas individual gRNA-LacZ mice exhibited relatively stable cycles before (5.9±0.7 days) and 3-weeks after (5.6±0.7 days) AAV injection (Fig.2F), gRNA-2 and -3 mice displayed considerable variability in estrous cycle length after AAV injection with some mice entering constant estrous or diestrus (Fig.2G); scored as zero for cycle length. We considered that this heterogeneity may have resulted from variations in ESR1 knockdown across the RP3V

and MPN in individual mice. To assess this, all animals that had bilateral AAV injections involving the RP3V (n=9) and those with bilateral MPN injections (n=10) were considered as separate groups. These groupings also exhibited great variability with no significant differences (p>0.05, Wilcoxon paired tests) in cycle length (Fig.2D,E). The four mice displaying constant estrous did not exhibit any unique gRNA, bilateral/unilateral injection location, or level of ESR1 in RP3V or MPN VGAT neurons.

Effects of VGAT ESR1 knockdown on pulsatile LH secretion

Following the post-AAV estrous cycle monitoring, pulsatile LH secretion was evaluated in diestrus, or in estrus for the four mice that had stopped cycling, using 6-min interval tail tip bleeding for 180 min (Steyn, Wan et al. 2013, Czieselsky, Prescott et al. 2016) and analyzed with PULSAR-Otago using the "Intact Female" parameters (Porteous, Haden et al. 2021).

All mice exhibited typical profiles of pulsatile LH secretion (Fig.3 A-D) with the exception of three mice (one in each gRNA group) that had no LH pulse during the 180 min sampling period. The gRNA-lacZ mice exhibited mean LH levels of 0.39±0.10 ng/mL (n=6) and had LH pulses with an interval of 32.6±3.1 min and amplitude of 0.92±0.17 ng/mL (n=5), as found in wild-type mice (Czieselsky, Prescott et al. 2016). No differences were detected in mean LH (p=0.35, H=2.17 Kruskal-Wallis test), pulse amplitude (p=0.034, H=6.26 with no post-hoc differences between gRNA-LacZ and gRNA2 (p=0.883, Dunn's test) or gRNA3 (p=0.207)), or pulse interval (p=0.34, H=2.24) between gRNA-LacZ and gRNA-2 (n=6-7) or gRNA-3 mice (n=5-6) (Fig.3E-G). To test whether bilateral ESR1 knockdown in VGAT neurons in the RP3V or MPN might be more selective in identifying a pulse phenotype, we re-grouped mice as above depending on their bilateral involvement of the RP3V and MPN and correlated pulse parameters with ESR1 expression in VGAT neurons. No significant correlations were detected between any parameter in either region (Pearson correlation r values of 0.06-0.41; p>0.05)(Fig.3H-M).

Effects of VGAT ESR1 knockdown on the LH surge

After pulse bleeding studies, mice were ovariectomized (OVX) and given an estradiol replacement regimen that evokes the LH surge (Czieselsky, Prescott et al. 2016) with a terminal blood sample analyzed for LH around the time of lights off and the brain processed for dual GnRH–cFos immunohistochemistry.

All gRNA-lacZ mice had increased cFos expression in rostral preoptic area GnRH neurons (43±8%, range 24-84%; 21±0.7 GnRH neurons/section), although two of the mice had LH levels below 1.5 ng/mL indicating that they had likely surged before lights off (Fig.4A,B). Unexpectedly, huge variation was found between gRNA-2 and gRNA-3 treated mice in the generation of the surge (Fig.4A,B). The number of GnRH neurons detected in the rPOA was the same in all groups (gRNA2, 20±1.9 GnRH neurons/section; gRNA3, 20±1.7 GnRH neurons/section). However, a significant reduction in the number GnRH neurons with cFos was found (p=0.0005, H=11.81 Kruskal Wallis) with gRNA-2 (p=0.0023 Dunn's multiple comparisons) but not gRNA-3 (p=0.91) compared with gRNA-LacZ mice (Fig.4A). The wide variation in single point LH values in all three groups did not reveal any significant

differences between groups (Fig.4B). This was a curious result given that gRNA-2 and -3 generated an identical 60-70% ESR1 knockdown in VGAT neurons (Fig.1C). To explore this effect of gRNA-2 further and parcellate between the RP3V and MPN, we again re-grouped the mice depending on whether they had bilateral involvement of the RP3V and MPN. A significant correlation was found between ESR1 expression in RP3V VGAT neurons and cFos in GnRH neurons (p=0.008, Pearson r = 0.66) (Fig.4C) but not LH secretion (p=0.26, Pearson r=0.31) across all mice (Fig.4D). No significant correlation was found for ESR1 expression in MPN VGAT neurons and cFos in GnRH (p=0.18, Pearson r = 0.36) or LH secretion (p=0.60, Pearson r=0.14) (Fig.4E,F).

CRISPR knockdown of ESR1 in RP3V GABA-kisspeptin neurons

The above observations suggested that ESR1 in RP3V VGAT neurons was required for the activation of GnRH neurons at the time of the surge. However, it remained unclear why so much heterogeneity remained in this group; some mice with significant ESR1 knockdown >80%) continued to show surge levels of cFos in GnRH neurons (>40%). Further the mice entering constant estrus had some of the highest levels of remaining ESR1 in RP3V VGAT neurons. Given the importance of RP3V kisspeptin neurons for the GnRH surge (Clarkson, d'Anglemont de Tassigny et al. 2008, Wang, Vanacker et al. 2019) and evidence that these cells are a GABAergic phenotype (Moffitt, Bambah-Mukku et al. 2018, Stephens and Kauffman 2021), we considered that one possible explanation for the heterogeneity was that the CRISPR gene editing had knocked down ESR1 variably in the sub-population of RP3V GABA neurons co-expressing kisspeptin.

ESR1 knockdown in preoptic kisspeptin neurons

The last set of brain sections from all gRNA mice underwent dual label immunohistochemistry to assess ESR1 expression in RP3V kisspeptin neurons. The normal periventricular distribution of kisspeptin neurons was detected within the AVPV and PVpo of gRNA-lacZ mice (N=6) with 20.1±2.0 and 24.0±1.9 kisspeptin cells/section, respectively and 74.5±3.3% and 63.3±5.1% of kisspeptin neurons positive for ESR1 immunoreactivity ("lacZ" group, Table 1)(Fig.5A). The 17 mice receiving gRNA-2 or gRNA-3 clearly fell into one of three groups;

- "Normal" group (2 x gRNA-2, 5 x gRNA-3) exhibited a normal number of kisspeptin neurons in the AVPV and PVpo with normal ESR1 expression (Table 1).
- "Unilateral" group (4 x gRNA-2, 2 x gRNA-3) had a substantial >75% reduction in kisspeptin-immunoreactive neuron number on only one side of the brain in either the AVPV (p=0.007, Kruskal-Wallis ANOVA) and/or the PVpo (p=0.002, Kruskal-Wallis with Dunn's tests) (Table 1). The expression of ESR1 in remaining kisspeptin neurons was highly variable (Table 1).
- "Bilateral" group (3 x gRNA-2, 1 x gRNA-3), had almost no kisspeptin neurons apparent in either the AVPV (0.4±0.1 kisspeptin neurons/section; p=0.0006) or PVpo (0.8±0.3 kisspeptin neurons/section; p=0.003) (Table 1.) (Fig.5B).

We also determined ESR1 knockdown in RP3V and MPN VGAT neurons in these new groups.

As expected, the "lacZ" mice were normal with ESR1 expressed in 50-61% of VGAT neurons while both the "normal" and "unilateral" groups had 18% co-expression (p=0.0002 to 0.0242, Kruskal-Wallis ANOVA), and the "bilateral" group exhibited an average of 25% co-expression that was not different to either "normal" or "unilateral" groups (Table 2).

Effects of ESR1 knockdown on estrous cycles, pulsatile LH secretion and the LH surge

The groups segregated by kisspeptin expression exhibited highly consistent reproductive phenotypes. The two gRNA groups with normal RP3V kisspeptin expression ("lacZ" and "normal"), exhibited normal estrous cycles 3 weeks after AAV injection (Fig.5C), the usual variable LH surge levels (4.8±1.7 and 6.3±1.3 ng/mL; range 0.1-14.3 ng/mL)(Fig.5D) and ~40-50% of GnRH neurons with cFos at the time of the expected LH surge (Fig.5E). This was accompanied by normal pulsatile LH (Fig.5F-H). In contrast, the "bilateral" mice with essentially no RP3V kisspeptin expression were all found to enter constant estrus (Figs.2D & 5C) and have both low LH levels (1.4±0.3 ng/mL; range 0.8-2.1 ng/mL) and low cFos expression in GnRH neurons (4.3±2.6%; p=0.0004, H=18.24, Kruskal-Wallis with p=0.0185 post-hoc Dunn's test)(Fig.5E) at the time of the expected surge. The LH surge levels across the groups were significantly different (p=0.0320, H=8.802, Kruskal-Wallis ANOVA; Fig.5D) but the high variability in lacZ and normal mice prevented significant differences between individual groups (p=0.698, post-hoc Dunn's test). Pulsatile LH release in "bilateral" mice was variable with one mouse having no pulses in the 180 min recording period while the others displayed pulses not significantly different to gRNA-lacZ mice despite an apparent trend for reduced LH pulse amplitude (p=0.1409) (Fig.5H). Finally, the "unilateral" mice displayed a phenotype intermediate between that of "normal" and "bilateral" groups. Estrous cyclicity was normal (Fig.5C) except for one mouse that went into constant diestrus and LH pulsatility was unaffected (Fig.5F-H), while LH levels (1.6±0.5 ng/mL) and the % GnRH neurons with cFos (10.3±3.7%, p=0.0297, post-hoc Dunn's tests) at the time of the expected LH surge were similar to the "bilateral" group.

DISCUSSION

We find here that CRISPR knockdown of ESR1 in preoptic area GABAergic neurons results in a variable reproductive phenotype with most mice exhibiting normal estrous cycles and LH secretion. However, re-sorting individual mice on the basis of their RP3V kisspeptin expression provided highly consistent reproductive traits; mice with absent RP3V kisspeptin expression were acyclic and failed to exhibit an LH surge but retained pulsatile LH secretion. These data demonstrate an essential role for the RP3V GABA-kisspeptin neuronal phenotype in the murine estrogen positive feedback mechanism.

We show here that gRNA-2 and gRNA-3 are effective at knocking down ESR1 expression in preoptic GABA neurons in adult female mice; both gRNA achieved a 60-70% reduction in ESR1 within the RP3V and MPN compared to gRNA-lacZ mice. We had previously reported that gRNA-3 generated an 80% knockdown in ESR1 within arcuate kisspeptin neurons

(McQuillan, Clarkson et al. 2022). This indicates the general utility of the CRISPR approach for *in vivo* gene editing of ESR1 in the mouse brain.

When examined on the basis of the original GABA neuron groupings, the only significant observation was that expression of ESR1 in RP3V GABA neurons correlated positively with the percentage of GnRH neurons expressing cFos at the time of the surge. The significant correlation between the ESR1 expression in RP3V GABA neurons and cFos in GnRH neurons when all gRNA-2 and gRNA-3 mice were combined revealed that this was not likely to be due to any unique actions of gRNA-2. While providing evidence that ESR1 in RP3V GABA neurons was required for normal activation of the GnRH neurons at the time of the surge, this was not the case for the LH surge. We note that single-point LH measurements are unreliable even for the OVX+E2 paradigm with individual mice exhibiting quite marked variability in surge onset and peak LH levels (Czieselsky, Prescott et al. 2016). As such, we favor cFos expression in GnRH neurons as a more reliable index of whether the surge has commenced at the time of investigation.

The highly variable reproductive phenotypes of the ESR1 knockdown mice became consistent when sorted on the basis of their RP3V kisspeptin expression. Four mice exhibited a near complete lack of kisspeptin immunoreactivity within the entire RP3V. As the RP3V kisspeptin neurons are a GABAergic phenotype (Moffitt, Bambah-Mukku et al. 2018), they will all be targeted for Cas9 expression in the *Vgat-Cre,LSL-Cas9-EGFP* mice. Prior estimates have indicated that 20-75% of RP3V kisspeptin neurons express *Vgat* in adult mice (Cravo, Margatho et al. 2011, Cheong, Czieselsky et al. 2015) although it is very likely that all RP3V kisspeptin neurons express VGAT during development and, as such, will all express Cas9 as adults. Hence, when the bilateral stereotaxic AAV-gRNA injections happen to include the entire extent of the RP3V, all GABA-kisspeptin neurons will be targeted. The almost complete lack of kisspeptin immunoreactivity is due to the critical dependence of kisspeptin expression on ESR1 in RP3V kisspeptin neurons (Smith, Cunningham et al. 2005, Dubois, Acosta-Martinez et al. 2015, Greenwald-Yarnell, Marsh et al. 2016). The CRISPR-mediated knockdown in ESR1 within these RP3V cells would gradually result in the suppression of kisspeptin biosynthesis. However, one curious observation is that essentially all cytoplasmic kisspeptin immunoreactivity is absent despite the expectation that some kisspeptin neurons would retain functional ESR1 due to the inherent mosaicism of this CRISPR strategy (Platt, Chen et al. 2014). Hence, the near complete absence of kisspeptin might also result from disordered circulating estradiol levels in acyclic mice.

We also noted an interesting reproductive phenotype in mice with unilateral knockdown of ESR1 in RP3V kisspeptin neurons resulting in unilateral reductions in kisspeptin-immunoreactive neuron numbers. Although showing no change in estrous cycles, these mice had somewhat similar deficits in GnRH neuron activation levels at the time of the surge compared to mice with no RP3V kisspeptin. Prior studies have indicated that 30-50% reductions in RP3V kisspeptin neuron number are compatible with a normal surge mechanism (Szymanski and Bakker 2012, Hu, Li et al. 2015). It is possible that a substantial reduction in RP3V kisspeptin input to GnRH neurons from one side of the brain results in weaker surge activation. It may also alter the timing of surge onset outside the window

examined in this study as the mice maintain normal estrous cycles implying that ovulation is occurring.

It is widely anticipated that that RP3V kisspeptin neurons are the critical estradiol-sensing component underlying the preovulatory surge mechanism (Uenoyama, Inoue et al. 2021, Goodman, Herbison et al. 2022, Kauffman 2022). We now provide data demonstrating that ESR1-expressing RP3V kisspeptin neurons are essential for the surge mechanism and estrous cyclicity in mice. It is unclear why the previous CRISPR-Cas9 study by Wang and colleagues did not observe the same result (Wang, Vanacker et al. 2019). However, that study did not examine kisspeptin expression so it is difficult to estimate the degree or level of functional knockout within the population, and only the AVPV was targeted whereas the majority of RP3V kisspeptin neurons are found in the PVpo.

We find that pulsatile LH secretion is maintained in the absence of RP3V kisspeptin expression. A trend towards a reduced LH pulse amplitude was observed but, alongside pulse frequency, no significant differences were detected. This observation agrees with the "two-compartment model" of the GnRH neuron network in which the kisspeptin pulse and kisspeptin surge generators are thought to operate in relative independence at different compartments of the female GnRH neuron to bring about LH pulses and the LH surge, respectively (Herbison 2020).

A caveat to an exclusive role of RP3V kisspeptin neurons in surge generation is the possibility that non-kisspeptin ESR1-expressing GABA neurons immediately adjacent to RP3V kisspeptin cells are also required for the surge. However, we note that acyclic mice with no kisspeptin ("bilateral loss") had similar levels of ESR1 knockdown in RP3V GABA neurons found in normal mice that exhibited normal surge activation. Nevertheless, we cannot be sure that the significant correlation between the degree of ESR1 knockdown in RP3V GABA neurons and GnRH neuron activation at the surge is attributable entirely to GABA-kisspeptin neurons. If RP3V GABAergic non-kisspeptin neurons are involved, it seems that they may play an intrinsic role within the RP3V as GABA release from the RP3V to GnRH neurons is inconsequential for surge induction (Piet, Kalil et al. 2018).

In summary, we show here that CRISPR gene editing can be used to efficiently knockdown ESR1 in GABAergic neurons. Characterizing reproductive phenotypes on an individual animal basis in relation to GABA neuron sub-populations revealed that a loss of kisspeptin in RP3V GABA neurons was perfectly correlated with a absent surge mechanism and estrous acyclicity. These observations provide definitive evidence for ESR1-expressing RP3V GABA-kisspeptin neurons being essential for the preovulatory surge mechanism while having no role in pulse generation.

METHODS

Animals

Vgat-Cre;LSL-Cas9-EGFP mice were generated by crossing 129S6Sv/Ev C57BL6 *Vgat-Cre* mice (JAX stock #026175)(Vong, Ye et al. 2011) with B6J.129(B6N) *Rosa26-LSL-Cas9-EGFP* mice

(JAX stock #026175)(Platt, Chen et al. 2014). All mice were provided with environmental enrichment under conditions of controlled temperature (22±2^{0}C) and lighting (12-hour light/12-hour dark cycle; lights on at 6:00h and off at 18:00h) with *ad libitum* access to food (Teklad Global 18% Protein Rodent Diet 2918, Envigo, Huntingdon, UK) and water. Daily vaginal cytology was used to monitor the estrous cycle stage. All animal experimental protocols were approved by the Animal Welfare Committee of the University of Otago, New Zealand (96/2017).

Experimental protocol

The estrous cycles of adult female *Vgat-Cre;LSL-Cas9-EGFP* mice were assessed for three weeks and mice exhibiting regular 4- to 6-day cycles given bilateral stereotaxic injections of AAV1-U6-gRNA-LacZ/ESR1-2/ESR1-3-Ef1α-mCherry into the medial preoptic area. Three weeks later, estrous cycles were again monitored for 3 weeks. Pulsatile LH secretion was then assessed using 6-minute tail-tip bleeding for 180 min. Mice were then ovariectomized and given an estrogen replacement regimen to induce the LH surge at which time they were killed, a terminal blood sample taken, and perfusion-fixed for immunohistochemical analysis.

Stereotaxic surgery and injections

Adult mice (3-4 months old) were anaesthetized with 2% Isoflurane, given local Lidocaine (4mg/kg, s.c.) and Carprofen (5mg/kg, s.c.) and placed in a stereotaxic apparatus. Bilateral injections of 1.5 µL AAV1-U6-gRNA-LacZ/ESR1-2/ESR1-3-Ef1α-mCherry-WPRE-SV40 (1.3-2.5 x 10^{13} GC/mL) were given into the medial preotic area and mice allowed to recover for 3 weeks before commencing the experimental protocol. A custom-made bilateral Hamilton syringe apparatus holding two 29- gauge needles 0.9 mm apart was used to perform bilateral injections into the preoptic area. The needles were lowered into place over 2 min and left *in situ* for 3 min before the injection was made. The AAV was injected at a rate of ~100 nl/min with the needles left *in situ* for 10 min before being withdrawn. Carprofen (5 mg/kg body weight, s.c.) was administered for post-operative pain relief for two days.

Pulsatile hormone measurement, ovariectomy, estrogen replacement, and LH assay

Profiles of pulsatile LH secretion were determined by tail-tip bleeding at 6-min intervals for 180 min in diestrus, or where cycles had stopped, estrous-stage mice as reported previously (Czieselsky, Prescott et al. 2016). Bilateral ovariectomy was performed under Isoflurane anesthesia with pre- and post-operative Carprofen (5mg/kg body weight, s.c.). Estradiol replacement was provided by s.c. implantation of an ~1 cm length of Silastic capsule (Dow Corning, USA) filled with 0.4µg/mL 17-β-estradiol to provide 4 µg 17-β-estradiol/20g body weight. This protocol returns the plasma profile of pulsatile LH secretion and 17-β-estradiol concentrations to that found in diestrous females (Porteous, Haden et al. 2021). Six days later, mice were given an s.c. injection of estradiol benzoate (1 µg in 100 µL) at 09:00 and killed at 19:00 the following evening at the time of lights off (Czieselsky, Prescott et al. 2016) when a terminal blood sample was taken. Plasma LH concentrations were determined using the ultrasensitive LH ELISA of Steyn and colleagues (Steyn, Wan et al. 2013, Czieselsky, Prescott et al. 2016) and had an assay sensitivity of 0.04 ng/mL and intra-and inter-assay coefficients of variation of 4.6% and 9.3%. Pulse analysis was undertaken with PULSAR Otago (Porteous, Haden et al. 2021) using the following validated parameters for intact

female mice: smoothing 0.7, peak split 2.5, level of detection 0.04, amplitude distance 3 or 4, assay variability 0, 2.5, and 3.3, G values of 3.5,2.6,1.9,1.5,and 1.2.

Immunohistochemistry

Mice were given a lethal overdose of pentobarbital (3mg/100µL, i.p.) and perfused through the heart with 20mL of 4% paraformaldehyde in phosphate-buffered saline. Three sets of 30 µm-thick coronal sections were cut through the full extent of the preoptic area and incubated overnight in a cocktail of chicken anti-EGFP (1:5,000; AB13970, Abcam; RRID:AB_300798) and rabbit anti-ESR1 (1:1,000; #06-935, Merck-Millipore; RRID:AB_310305) followed by 2h incubation in goat anti-chicken 488 (1:400; Molecular Probes) and biotinylated goat anti-rabbit immunoglobulins (1:400, Vector Laboratories) and then 2h in Streptavidin 647 (1:400, Molecular Probes). This was followed by placing in 5% normal goat serum for 1h and labelling for mCherry with rabbit anti-mCherry (1:10,000; Abcam; RRID:AB_2571870) and goat anti-rabbit 568 (1;400; Molecular Probes) using the same incubation periods. The use of a second anti-rabbit antisera resulted in cytoplasmic 568/mCherry labelling that was used for defining the extent of AAV transduction and potentially some 568 fluorescence in ESR1-positive nuclei that was avoided by analyzing only in the far red 647nm spectrum.

To assess ESR1 expression in kisspeptin neurons, dual-label chromogen immunohistochemistry was undertaken with rabbit anti ESR1 (1:5,000), and peroxidase-labelled goat anti-rabbit (1:400, Vector Labs) revealed with nickel-DAB followed by rabbit anti-kisspeptin antisera (AC566, 1:10,000; RRID:AB_2314709), and biotinylated goat anti-rabbit immunoglobulins (1:400, Vector Labs) and Vector Elite avidin-peroxidase (1:100) with DAB as the chromogen.

To assess activation of GnRH neurons dual-label chromogen immunohistochemistry was undertaken with rabbit anti cFos (sc-52, 1:5000, Santa Cruz; RRID:AB_2106783) and biotinylated goat anti-rabbit immunoglobulins (1:400, Vector Labs) and Vector Elite avidin-peroxidase (1:100) with NiDAB, followed by rabbit anti-GnRH (GA01, 1:1,000, RRID:AB_2721114) and peroxidase-labelled goat anti-rabbit (1:400, Vector Labs) revealed with DAB.

Quantitative analyses of ESR1 expression in the RP3V and MPN neurons were undertaken on confocal images captured on a Nikon A1R multi-photon laser scanning microscope using 40x Plan Fluor, N.A. 0.75 objective using, software Nikon Elements C. The numbers of EGFP-labelled cells with and without immunoreactive ESR1 nuclei were counted by an investigator blind to the experimental groupings. Cell counts were undertaken by analyzing all EGFP-positive cells through 10 z-slices of 2µm thickness in sections at each of the levels of the RP3V-MPN for each mouse. The number of kisspeptin neurons with ESR1 was assessed under brightfield microscopy by counting the number of kisspeptin-immunoreactive cells (brown DAB) with and without black (nickel-DAB) ESR1-positive nuclei bilaterally in 3-4 sections throughout the AVPV and PVpo in each mouse. The number of GnRH neurons with cFos was assessed under brightfield microscopy by counting the number of GnRH-immunoreactive cells (brown DAB) with and without black (NiDAB) cFos-positive nuclei bilaterally in 2 sections of the rostral preoptic area in each mouse.

Statistical analysis

Statistical analysis was undertaken on Prism 10 using parametric or non-parametric tests as appropriate. This included one-way ANOVA with post-hoc Dunnett's test, Kruskal-Wallis with post-hoc Dunns tests, Wilcoxon paired tests, and Pearson correlations. Data are presented as mean±SEM.

Data Availability

All data generated or analysed during this study are included in this published article and source data in supplementary information files.

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Acknowledgements
This work was supported by the New Zealand Marsden Fund (16-UOO-201), New Zealand Health Research Council (17-285) and the Wellcome Trust (212242/Z/18/Z).

Author contributions
JC, SHY and RP contributed *in vivo* CRISPR studies and analysis, AK and AKH contributed CRISPR design, *in vitro* studies and analysis, AEH contributed design, funding, and wrote the manuscript.

Competing interests
The authors have no competing interests.

Figure Legends

Figure 1. *CRISPR knockdown of ESR1 in preoptic GABA neurons.* **A,B.** Photomicrographs showing distribution of mCherry (gRNA from AAV) and expression of GFP (Cas9) in VGAT neurons and nuclear-located ESR1 (blue) in PVpo of two mice receiving either gRNA-LacZ (A) or gRNA-2 (B). The mCherry signal is removed in the adjoining plates **a.** and **b.** so that the VGAT neurons (green) co-expressing ESR1 (blue or teal nuclei) are more easily identified. **C.** Individual data points and mean±SEM percentage of GFP/VGAT neurons expressing ESR1 within injected regions of the RP3V and MPN for the three gRNA groups. *** $p<0.001$ versus gRNA-LacZ (ANOVA, post-hoc Dunnett's tests). **D-G,** representative examples of AAV injection sites (pink) in four mice; 16337 (bilateral gRNA-2), 14945 (unilateral RP3V, bilateral MPN gRNA-2), 13631 (bilateral gRNA-3), 13786 (bilateral gRNA-LacZ).

Figure 2. *Deletion of ESR1 from preoptic GABA neurons and estrous cyclicity.* **A-C.** Individual paired data points and mean±SEM estrous cycle length before and after AAV gRNA injection of lacZ, gRNA-2, and gRNA-3 into the RP3V and MPN. **D-E**, Individual paired data points and mean±SEM estrous cycle length before and after AAV gRNA injection in mice with bilateral AAV injections in the RP3V and MPN analyzed separately. Four mice (3 x gRNA-2, 1 x gRNA-3) enter constant estrus and one gRNA2 mouse was in constant diestrus, scored as a cycle length of 0. No significant effects of gRNA injection were detected ($p>0.05$ Wilcoxon paired tests). **F-H,** Examples of estrous cycle patterns from three mice including one (**G**) that entered constant estrous following gRNA-2 injection. The individual animal number is given in each frame.

Figure 3. *Deletion of ESR1 from preoptic GABA neurons does not alter pulsatile LH secretion.* **A-D.** Representative LH pulse profiles from female mice given AAV gRNA-lacZ, gRNA-2 and gRNA-3. The mouse identification number is given in brackets. **E-G.** Histograms show the individual data points and mean±SEM for parameters of pulsatile LH secretion in mice given gRNA-lacZ, gRNA-2 and gRNA-3 into the RP3V and MPN. No significant effects are detected ($p>0.05$, Kruskal-Wallis test). **H-J.** Correlations between the % VGAT neurons with ESR1 in the RP3V and parameters of pulsatile LH secretion. Individual mice are color-coded according to their gRNA treatment. No significant correlations were detected (Pearson $r < 0.34$ in all cases). **K-M.** Correlations between the % VGAT neurons with ESR1 in the MPN and parameters of pulsatile LH secretion. Individual mice are color-coded according to their gRNA treatment. No significant correlations were detected (Pearson $r < 0.41$ in all cases).

Figure 4. *Effects of ESR1 deletion in preoptic GABA neurons on surge parameters.* **A,B**. Individual data points and mean±SEM values showing the percentage of GnRH neurons with cFos and single-point LH levels for mice killed at the time of the expected surge given gRNA-LacZ (black), gRNA-2 (blue) and gRNA-3 (green) injections centered on the RP3V and MPN. ** $p=0.0023$ (Krusakl-Wallis) compared with LacZ. **C,D**. Correlations between the % of RP3V VGAT neurons with ESR1 and cFos expression by GnRH neurons or LH secretion. Individual mice are color-coded according to their gRNA treatment. A significant correlation for cFos in GnRH neurons exists ($p=0.008$, Pearson $r = 0.66$) but not for LH ($p=0.26$, Pearson $r = 0.31$). **E,F**. Correlations between the % of MPN VGAT neurons with ESR1 and cFos expression by

GnRH neurons or LH secretion. Individual mice are color-coded according to their gRNA treatment. No significant correlations were found.

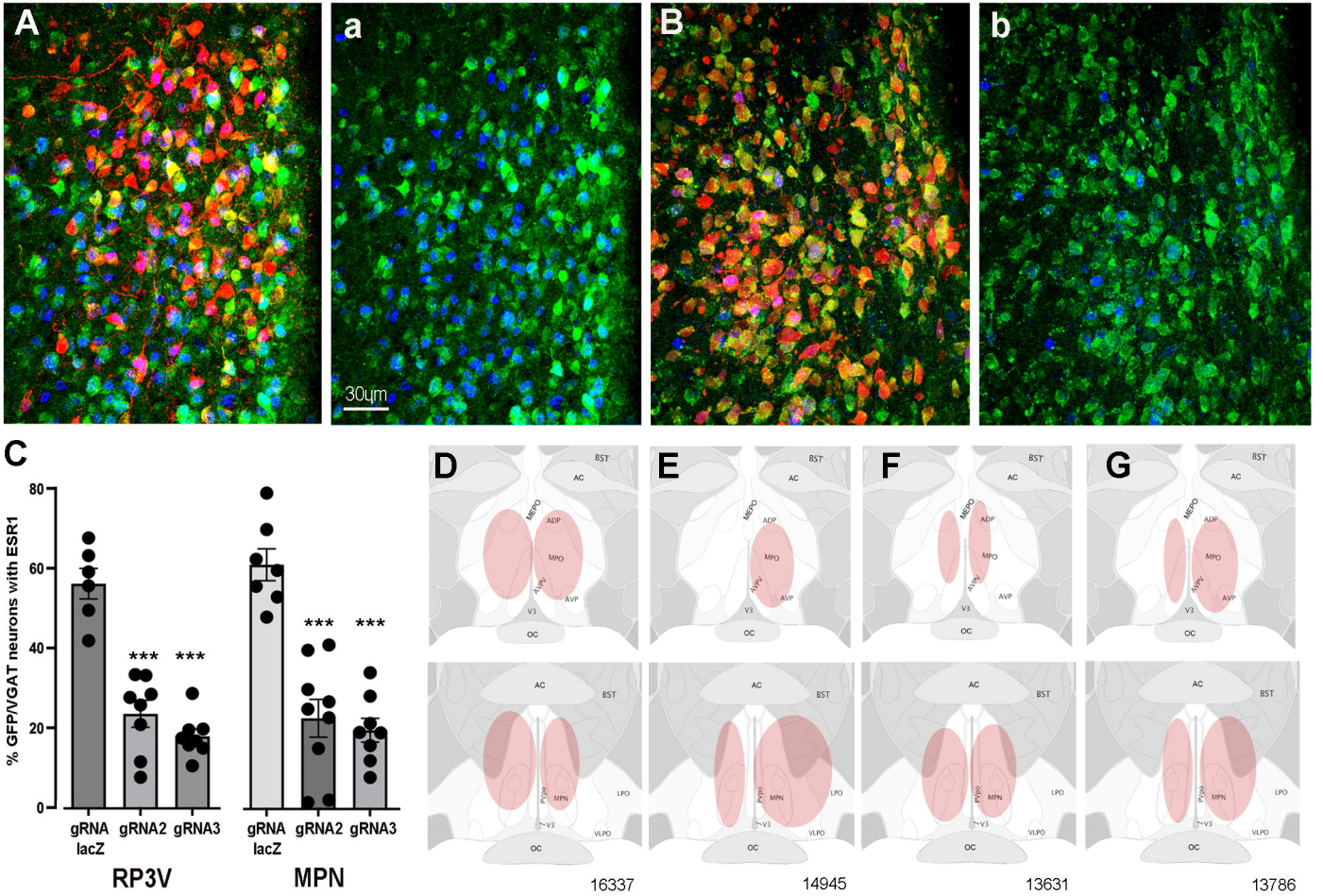
Figure 5. *Suppression of RP3V kisspeptin expression is associated with the loss of estrous cycles and the surge mechanism.* **A,B.** Dual-label immunohistochemistry for kisspeptin (brown) and ESR1 (black) shows the normal high level of co-expression (white arrowheads) in a representative gRNA-lacZ mouse (**A**) but near absence of kisspeptin immunoreactivity in a representative "bilateral loss" mouse (#16337) (**B**). 3V, third ventricle. **C.** Estrous cycle length before and after gRNA injection in gRNA "lacZ" mice, gRNA2/3 mice with "normal" kisspeptin expression, gRNA2/3 mice with a "unilateral" reduction in kisspeptin, and gRNA2/3 mice with a near complete "bilateral" loss of kisspeptin. * $p<0.05$ compared to pre- values. **D,E.** Single point LH levels and % of GnRH neurons with cFos at the time of the expected surge in gRNA "lacZ" mice, gRNA2/3 mice with "normal" kisspeptin expression, gRNA2/3 mice with a "unilateral" reduction in kisspeptin, and gRNA2/3 mice with a near complete "bilateral" loss of kisspeptin. * $p<0.05$ compared to lacZ. **F-H.** Parameters of pulsatile LH secretion in gRNA "lacZ" mice, gRNA2/3 mice with "normal" kisspeptin expression, gRNA2/3 mice with a "unilateral" reduction in kisspeptin, and gRNA2/3 mice with a near complete "bilateral" loss of kisspeptin. No significant differences were detected.

Table 1. Kisspeptin-ESR1 co-expression in re-grouped gRNA mice. Table showing the numbers of kisspeptin neurons/section in the AVPV and PVpo and percentage expression with ESR1. "LacZ" refers to all mice given gRNA-LacZ, "Normal" refers to all gRNA-2/3 mice with normal kisspeptin expression (unilateral cell counts shown), "Unilateral" refers to gRNA-2/3 mice in which only one side of the brain had reduced kisspeptin cell numbers with the cell count on the affected side given, "Bilateral" refers to gRNA-2/3 mice with essentially no cytoplasmic kisspeptin expression bilaterally in the RP3V. Too few kisspeptin neurons existed to reliably determine co-expression with ESR1. ** $p<0.01$, *** $p< 0.001$ compared with lacZ group (Kruskal-Wallis with Dunn's tests, exact p values given below).

		LacZ (n=8)	Normal (n=7)	Unilateral (n=6)	Bilateral (n=4)
AVPV	No. Kisspeptin neurons/section	20.1±2.0	15.4±0.9	5.6±2.5** (p=0.0066)	0.4±0.1*** (p=0.0006)
	% Kiss with ESR1	74.5±3.3	78.5±4.4	57.3±19.7	-
PVpo	No. kisspeptin neurons/section	24.0±1.9	19.6±1.3	4.8±2.8** (p=0.0016)	0.8±0.3** (p=0.0031)
	% Kiss with ESR1	63.3±5.1	70.5±5.2	27.5±17.1	-

Table 2. VGAT-ESR1 in mice grouped on the basis of kisspeptin expression. Table showing the percentage of VGAT neurons in the RP3V and MPN expressing ESR1 in gRNA mice re-grouped on the basis of kisspeptin expression (see Table 1 for explanation of groups). * $p<0.05$, *** $p< 0.001$ compared with lacZ group (Kruskal-Wallis with Dunn's tests, exact p values given below).

		LacZ (n=8)	Normal (n=7)	Unilateral (n=6)	Bilateral (n=4)
RP3V	% VGAT with ESR1	50.4±6.1	17.9±2.3* (p=0.0242)	17.7±3.0* (p=0.0358)	24.5±4.8
MPN	% VGAT with ESR1	61.2±4.2	14.0±3.1*** (p=0.0002)	22.0±5.1* (p=0.0162)	30.1±4.8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a
30μm
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C
% GFP/VGAT neurons with ESR1
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60
40
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0
***
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gRNA lacZ
gRNA2
gRNA3
gRNA lacZ
gRNA2
gRNA3
RP3V
MPN
D
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BST
AC
MEPO
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13631
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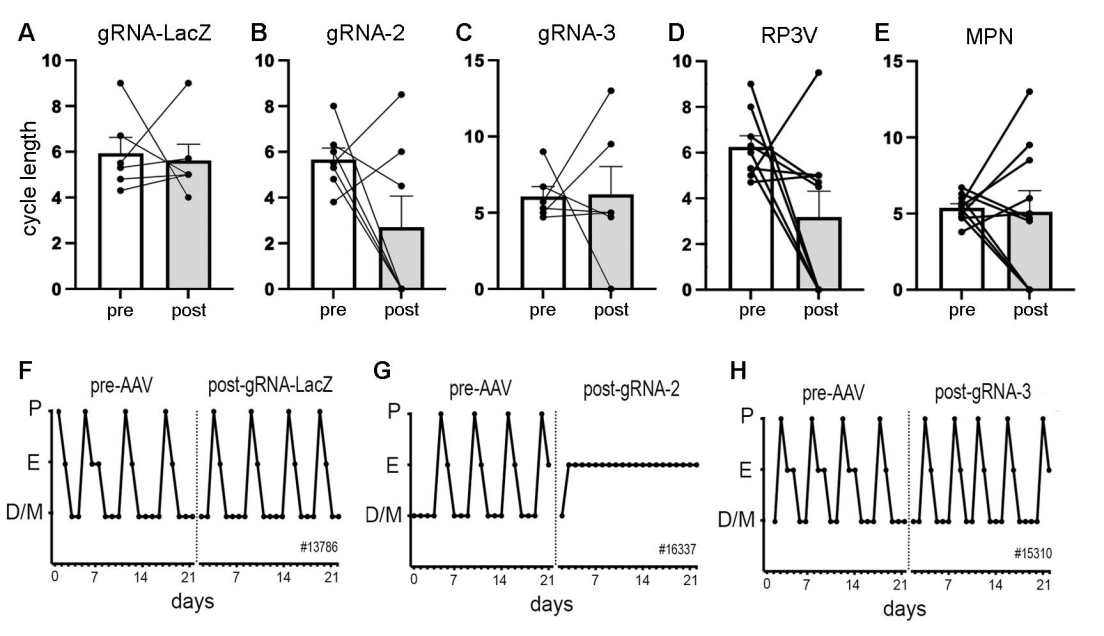
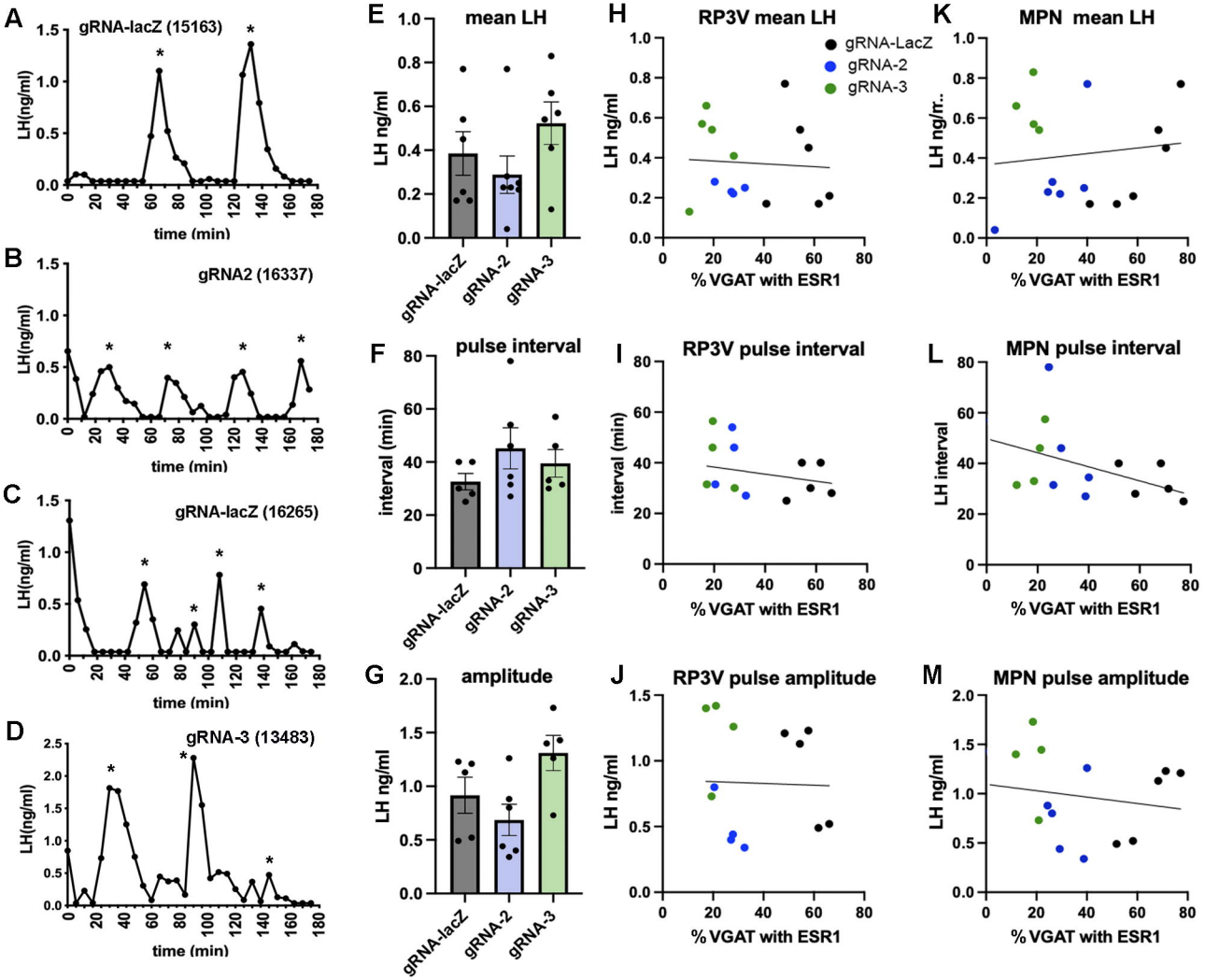

A
gRNA-LacZ
B
gRNA-2
C
gRNA-3
D
RP3V
E
MPN
cycle length
pre
post
F
pre-AAV
post-gRNA-LacZ
G
pre-AAV
post-gRNA-2
H
pre-AAV
post-gRNA-3
P
E
D/M
#13786
#16337
#15310
days


A
gRNA-lacZ (15163)
LH(ng/ml)
time (min)
B
gRNA2 (16337)
LH(ng/ml)
time (min)
C
gRNA-lacZ (16265)
LH(ng/ml)
time (min)
D
gRNA-3 (13483)
LH(ng/ml)
time (min)
E
mean LH
LH ng/ml
gRNA-lacZ
gRNA-2
gRNA-3
F
pulse interval
interval (min)
gRNA-lacZ
gRNA-2
gRNA-3
G
amplitude
LH ng/ml
gRNA-lacZ
gRNA-2
gRNA-3
H
RP3V mean LH
gRNA-LacZ
gRNA-2
gRNA-3
LH ng/ml
% VGAT with ESR1
I
RP3V pulse interval
interval (min)
% VGAT with ESR1
J
RP3V pulse amplitude
LH ng/ml
% VGAT with ESR1
K
MPN mean LH
LH ng/ml
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L
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LH interval
% VGAT with ESR1
M
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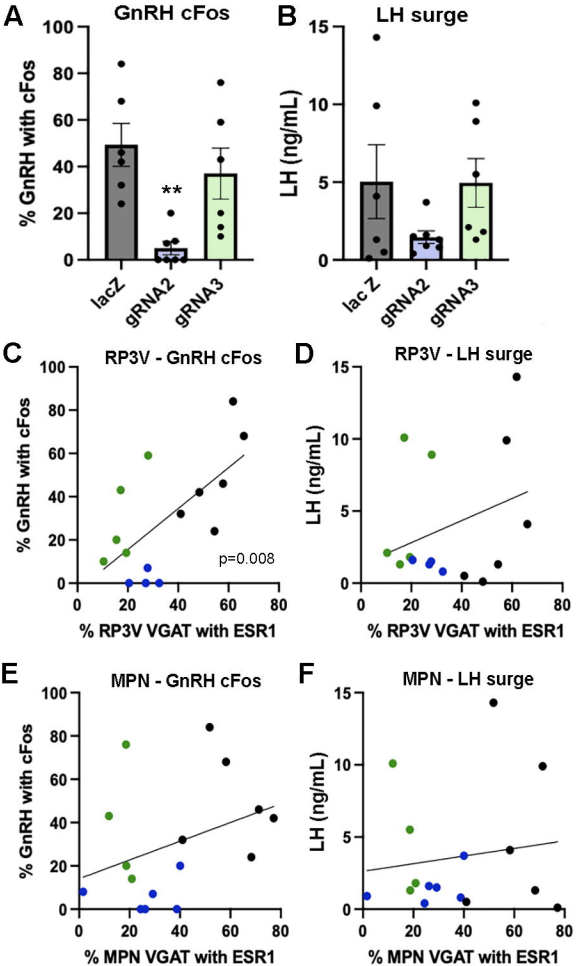
LH ng/ml
% VGAT with ESR1


A
GnRH cFos
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100
80
60
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20
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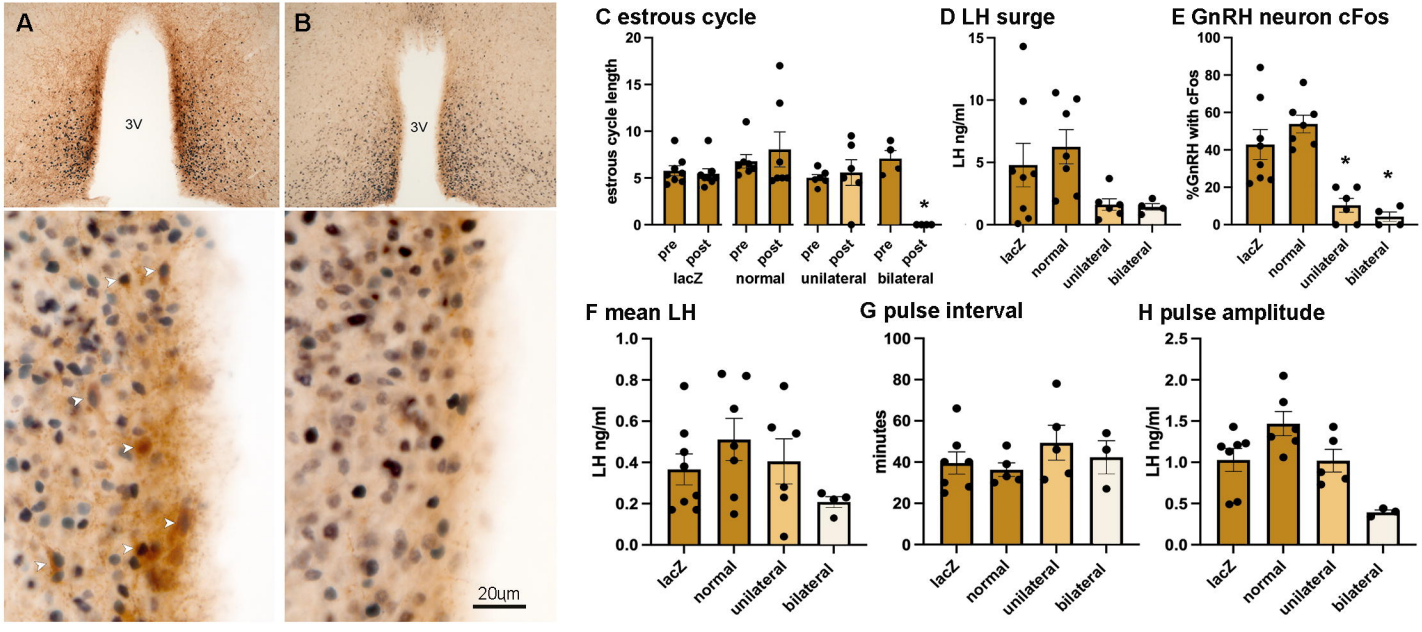
**
lacZ
gRNA2
gRNA3
B
LH surge
LH (ng/mL)
15
10
5
0
lac Z
gRNA2
gRNA3
C
RP3V - GnRH cFos
% GnRH with cFos
p=0.008
% RP3V VGAT with ESR1
D
RP3V - LH surge
LH (ng/mL)
% RP3V VGAT with ESR1
E
MPN - GnRH cFos
% GnRH with cFos
% MPN VGAT with ESR1
F
MPN - LH surge
LH (ng/mL)
% MPN VGAT with ESR1


A
B
3V
3V
20um
C estrous cycle
estrous cycle length
pre
post
lacZ
normal
unilateral
bilateral
D LH surge
LH ng/ml
E GnRH neuron cFos
%GnRH with cFos
F mean LH
LH ng/ml
G pulse interval
minutes
H pulse amplitude
LH ng/ml