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Puberty enables oestradiol-induced progesterone synthesis in female mouse hypothalamic astrocytes

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Progesterone receptors in AVPV kisspeptin neurons are sufficient for positive feedback induction of the LH surge

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Abstract

Kisspeptin, encoded by *Kiss1*, stimulates GnRH neurons to govern reproduction. In female rodents, estrogen-sensitive kisspeptin neurons in the rostral anteroventral periventricular (AVPV) hypothalamus are thought to mediate estradiol (E_2)-induced positive feedback induction of the preovulatory luteinizing hormone (LH) surge. AVPV kisspeptin neurons co-express estrogen and progesterone receptors (PGR) and are activated during the LH surge. While E_2 effects on kisspeptin neurons have been well-studied, progesterone's regulation of kisspeptin neurons is less understood. Using transgenic mice lacking PGR exclusively in kisspeptin cells (termed KissPRKOs), we previously demonstrated that progesterone action specifically in kisspeptin cells is essential for ovulation and normal fertility. Unlike control females, KissPRKO females did not generate proper LH surges, indicating that PGR signaling in kisspeptin cells is required for proper positive feedback. However, since PGR was knocked out from all kisspeptin neurons in the brain, that study was unable to determine the specific kisspeptin population mediating PGR action on the LH surge. Here, we used targeted Cre-mediated AAV technology to re-introduce PGR selectively into AVPV kisspeptin neurons of adult KissPRKO females, and tested whether this rescues occurrence of the LH surge. We found that targeted upregulation of PGR in kisspeptin neurons exclusively in the AVPV is sufficient to restore proper E_2-induced LH surges in KissPRKO females, suggesting that this specific kisspeptin population is a key target of the necessary progesterone action for the surge. These findings further highlight the critical importance of progesterone signaling, along with E_2 signaling, in the positive feedback induction of LH surges and ovulation.

Introduction

In female mammals, ovulation is triggered by an estradiol (E_2)-induced preovulatory luteinizing hormone (LH) surge, which is driven by a surge in gonadotropin-releasing hormone (GnRH) secretion from the brain (i.e., E_2 positive feedback) (1-3). Kisspeptin, encoded by *Kiss1*, potently stimulates GnRH secretion and is essential for the GnRH/LH surge, as female mice lacking *Kiss1* or its receptor, Kiss1r, fail to exhibit LH surges or concurrent GnRH neuron activation (3-5). The hypothalamus contains two populations of kisspeptin-synthesizing neurons, one in the arcuate nucleus (ARC) and another rostrally in the continuum spanning the anteroventral periventricular nucleus and neighboring periventricular nucleus (collectively termed here the AVPV; also called the RP3V) (6-8). E_2's positive feedback effects on the LH surge are thought to be mediated specifically by AVPV *Kiss1* neurons, because 1) E_2 elevates *Kiss1* levels in the AVPV (8-11), 2) AVPV *Kiss1* neurons express ERα (11), 3) AVPV *Kiss1* mRNA and neuronal activation increase in a circadian manner coincident with the LH surge (10, 12-14), and 4) *Kiss1* and kisspeptin levels in the AVPV are sexually dimorphic (greater in females), correlating with the LH surge occurring only in female rodents (8, 15).

While most positive feedback studies focus on E_2, P4 and its receptor (PGR) are also critical contributors to the LH surge. Like ERα knockout (KO) mice, global PR KO female mice are infertile and unable to produce LH surges (16, 17). Converging pieces of evidence indicate that E_2-induced local synthesis of P4 in the hypothalamus is critical for the rodent LH surge (18-20). Specifically, E_2 appears to act in ERα-expressing astrocytes in the hypothalamic AVPV to induce the synthesis and secretion of P4 ("neuroP"), which then acts paracrinely to promote the LH surge (18-23). However, the specific neural population(s) that neuroP4 acts in for this process remains unknown. In immortalized cells representing adult female AVPV kisspeptin neurons (mHypoA51), neuroP augmented E_2 action on the expression and release of kisspeptin (24, 25). Because AVPV kisspeptin neurons *in vivo* express PGR (4, 26-28), we previously tested whether PGR signaling in kisspeptin cells is critical for the LH surge. Using transgenic mice with selective KO of PGR specifically in kisspeptin cells ("KissPRKO"), we demonstrated that P4 signaling directly in kisspeptin cells is necessary for the E_2-induction of the LH surge and ovulation ((27); also see (29)). Moreover, AVPV *Kiss1* cells of E_2-treated KissPRKOs had normal *Kiss1* mRNA levels but reduced neuronal activation (27), indicating that P4 signaling in *Kiss1* cells is critical for activation of AVPV kisspeptin neurons during the LH surge, perhaps due to P4-induced calcium release, as observed in immortalized kisspeptin cells (25).

While our previous studies implicated that PGR signaling in kisspeptin cells as essential for the LH surge, a limitation was that kisspeptin cells are present in several brain regions, including the AVPV, ARC, and extra-hypothalamic areas like the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) (30-33). KissPRKOs lack PGR in all these kisspeptin populations, precluding a definitive conclusion of the specific population that is the direct target for P4 in the LH surge process. Therefore, we tested here whether selective reintroduction of PGR in just one kisspeptin population, the AVPV, of KissPRKOs could rescue the occurrence of the LH surge even with PGR still absent in all other kisspeptin cells.

Materials and Methods

Animals

The same mouse line was used as in our prior study (27), including both KissCre^{+} PR$^{fl/fl}$ females (termed "KissPRKO"; PGR knocked out in *Kiss1* cells) and KissCre^{-} PR$^{fl/fl}$ controls (termed "WT"; PR still present in all cells, including *Kiss1* cells) (**Figure 1**). Our prior study confirmed proper Cre mediated excision of *Pgr* DNA in the AVPV and ARC of KissPRKOs but not in Cre- controls or in tissues known to lack *Kiss1* expression (27). Genotypes and occasional occurrence of germline recombination were determined via PCR analysis of tail DNA; germline-recombined (global PRKO) mice were excluded. Female mice were weaned at 3 weeks and housed 2-3/cage under a 12-12 light-dark cycle (lights off at 1800h). All experiments were approved by the UCSD IACUC.

AAV treatment to induce PR in AVPV kisspeptin cells and mCherry IHC validation

We previously demonstrated that KissPRKO females have impaired ovulatory function and LH surges. Here, we determined if we could rescue the LH surge in KissPRKOs by selectively re-introducing PGR into just AVPV kisspeptin neurons, while all other kisspeptin cells still lack PGR. AAVs were used to deliver a Cre-dependent *Pgr* construct or a control construct to AVPV kisspeptin neurons (which express Cre in Kiss1Cre+ mice) (**Figure 1**). 2-3 month old KissPRKO females (Cre+) and WT controls (Cre-) were

anesthetized with isoflurane and site-specific AVPV-targeted (from bregma: A/P: +0.5 mm; D/V: -5.5 mm, M/L +/-0.3 mm) bilateral injections were made. 500 nL of either AAV1-CMV-DIO-mPGR-2A-mCherry or AAV2/1-CAG-DIO-mCherry (Vector Biolabs) were infused at a flow rate of 100 nL/min; the former AAV, termed "Pgr", allows for *Pgr* and mCherry expression in Cre-expressing cells while the latter AAV, termed "mCh", was a control AAV with the mCherry reporter but no *Pgr* construct. After AAV infusion, the needle was left in place for 10 min to allow diffusion and prevent backflow. To validate the AAV technique, several non-experimental mice were perfused and examined for proper targeting via immunohistochemistry (IHC) analysis of mCherry induction in the AVPV region of Cre+ but not WT (Cre-) mice. Because fixed tissue was needed for IHC, these validation mice were separate from the experimental mice evaluated for LH surges and *Kiss1+Pgr* mRNA co-expression (which required fresh frozen tissue for *in situ* hybridization). IHC was performed on fixed AVPV-containing brain slices using an mCherry primary antibody [1:5000; Abcam ab167453 (34)] and AlexaFluor 594 donkey anti-rabbit secondary [1:2000; Jackson 711-585-152 (35)], following previous methods (36). For assessment of LH surges and *Pgr* mRNA induction after AAV treatment, there were 4 experimental groups: KissPRKO^{Pgr}, KissPRKO^{mCh}, WT^{Pgr}, and WT^{mCh}. Mice were given 4 weeks to recover prior to LH surge testing and brain collection. Double-label ISH evaluation of *Pgr+Kiss1* co-expression was used to confirm successful anatomical targeting of AAV to AVPV *Kiss1* neurons; 1 animal with missed targeting was excluded from the study.

Estradiol-induced LH surge

Four weeks after AAV infusion, mice were anesthetized with isoflurane, ovariectomized (OVX), and given subcutaneous Silastic implants (inner diameter: 0.078 in, outer diameter: 0.125 in) containing 0.75 µg of 17-β E_2 dissolved in sesame oil. This E_2 implant is commonly used to induce LH surges and produces elevated serum E_2 levels ~16-24 pg/ml, resembling mouse proestrus levels (5, 37). This positive feedback paradigm produces an LH surge two days later before lights off (5, 13, 14, 37-39). OVX+E_2 mice from all 4 treatment groups (KissPRKO^{Pgr}, KissPRKO^{mCh}, WT^{Pgr}, and WT^{mCh}) were killed 2 days after E_2 implantation, either in the AM (0900-1000h, control time when the LH surge does not occur; n=4-6/group) or in the PM just before lights off (17:30-18:00h, time of the expected LH surge; n=7-10/group) (**Figure 1)**. Blood was collected at sacrifice and the serum isolated and stored at -20°C. Serum LH was measured by the University of Virginia Ligand Assay Core using a sensitive LH sandwich radioimmunoassay (limit of detectability 0.04 ng/ml) (40, 41). LH surges were defined as >0.70 ng/ml.

Brains were collected fresh frozen onto dry ice and stored at -80°C. Brains were sectioned on a cryostat into alternating sets of 20-µm sections onto Superfrost-plus slides and stored at -80°C.

Double-label In Situ Hybridization (ISH) for Pgr expression in Kiss1 cells

Double-label ISH was used to measure *Kiss1* and *Pgr* co-expression levels in the AVPV. One set of AVPV brain sections was assayed using well-established radio-labeled (^{33}P) *Pgr* (0.04 pmol/ml) and digoxygenin-labeled (DIG; 1:500) *Kiss1* antisense riboprobes, following our standard double-label ISH protocol (14, 27, 42-44). ISH slides were analyzed "blindly" with respect to treatment using microscopy linked to an automated imaging processing system (Dr. Don Clifton, Univ. Washington) that counts the number of DIG-containing (*Kiss1* mRNA) cells under fluorescence microscopy and then quantifies the number of silver grains (*Pgr* mRNA) overlying each DIG cell under dark-field microscopy. A minimum of 3 AVPV sections (mean: 5.5 sections/animal) and 145 *Kiss1* cells (mean: 290 cells/animal) was counted for each animal. Signal-to-background ratios of *Pgr* expression levels for individual DIG (*Kiss1*) cells were auto-calculated by the program, and a *Kiss1* cell considered double-labeled with *Pgr* if its ratio was >3 (13, 14, 43-46).

Statistical Analyses

All data are expressed as mean ± SEM. Differences were analyzed by ANOVA, followed by post-hoc comparisons via Fisher's Protected LSD test. Statistical significance was set at $p < 0.05$.

Results

mCherry localization in the AVPV of Cre+ mice and Pgr upregulation in Kiss PRKO females

Pgr or mCh AAV was infused into the AVPV of adult Cre+ (KissPRKO) females and Cre- (WT) controls (**Figure 1**). We first confirmed proper localization and targeting of the AAV injection, using IHC to identify proper mCherry expression in the AVPV of Cre+ mice but not Cre- controls (**Figure 2A, B**). We also confirmed no spread of mCherry expression in the ARC (**Figure 2C)**.

Next, to verify that our Pgr AAV treatment upregulated *Pgr* levels in *Kiss1* cells of Cre+ mice, *Pgr* + *Kiss1* co-expression levels in the AVPV were determined with double ISH. *Pgr* was co-expressed with *Kiss1* at similar levels between WT^{Pgr} and WT^{mCh} (**Figure 3**), as expected for Cre- controls. As we previously reported, KissPRKOs normally have much less *Prg* co-expression than WTs, evidenced by significantly lower % *Pgr+Kiss1* co-expression in $KissPRKO^{mCh}$ versus either WT^{Pgr} and WT^{mCh} controls ($p < 0.05$ for each; **Figure 3**). Confirming the AAV-mediated upregulation of *Pgr*, $KissPRKO^{Pgr}$ had significantly increased % *Prg+Kiss1* co-expression versus $KissPRKO^{mCh}$ ($p < 0.05$; **Figure 3**), with similar % co-expression as the WT control groups (**Figure 3**). Mean levels of *Pgr* mRNA in *Kiss1* neurons were also similar between $KissPRKO^{Pgr}$ and WT^{Pgr} controls (13.5±1.5 vs 12.0±1.8 grains/cell, p=0.53). Thus, the experimental paradigm effectively re-introduced *Pgr* expression in the AVPV of KissPRKOs that normally lack PR in kisspeptin neurons.

Positive feedback induction of the LH surge is rescued in $KissPRKO^{Pgr}$ females

We hypothesized that the previously-observed loss of LH surges in KissPRKOs (27) reflects an impairment in the AVPV kisspeptin system caused by absent PGR signaling in this specific neural population. We tested whether selective re-introduction of PGR into AVPV kisspeptin neurons, while leaving PGR absent in all other kisspeptin cells, could rescue the LH surge in OVX+E_2 females. All 4 groups exhibited low LH in the AM, as expected for this non-surge time, with no group differences (**Figure 4C**). In the PM, the overall occurrence of an LH surge was greatly increased in $KissPRKO^{Pgr}$ compared with $KissPRKO^{mCh}$ females and was not different between $KissPRKO^{Pgr}$ and WT^{Pgr} and WT^{mCh} control groups (**Figure 4A**). Indeed, virtually all PM $KissPRKO^{Pgr}$ females demonstrated a surge, whereas no PM $KissPRKO^{mCh}$ females surged (**Figure 4B**).

In the PM, mean LH was significantly elevated in both WT control groups versus AM values, whereas mean LH of PM $KissPRKO^{mCh}$ females was lower ($p < 0.05$ versus each PM WT group; **Figure 4C**) and did not differ significantly from AM groups, similar to our previous report (27). By contrast, PM $KissPRKO^{Pgr}$ females exhibited elevated mean LH versus all AM groups and versus PM $KissPRKO^{mCh}$ females ($p < 0.05$ for each; **Figure 4C**). The mean LH of PM $KissPRKO^{Pgr}$ females was not significantly different than PM WT^{mCh} controls but was lower than WT^{Pgr} (**Figure 4C**), due primarily to one very high WT^{Pgr} value (**Figure 4B**; not significantly different if that value excluded). Collectively, these results demonstrate that re-introduction of *Pgr* selectively in AVPV kisspeptin cells rescues the circadian-timed LH surge.

Discussion

Ovulation is dependent on sex steroid-induced positive feedback induction of GnRH/LH surges. Ample evidence implicates the AVPV kisspeptin system in mediating the positive feedback effects of E_2 on the LH surge (reviewed in (3, 9). Along with E_2, P4 is also critical for the LH surge, and we and another group recently reported that P4 action specifically in kisspeptin cells is necessary for females to generate proper LH surges (27, 29). However, neither *in vivo* study determined which exact kisspeptin population mediates this necessary action of PGR. AVPV kisspeptin neurons co-express PGR (27, 28), suggesting they are key targets for P4 action. Supporting this, mHypoA51 cells (representing AVPV kisspeptin cells) respond to P4 by increasing *Kiss1* expression and *in vitro* kisspeptin release (24, 25). To formally test whether AVPV kisspeptin cells are critical for positive feedback induction of the LH surge, we re-introduced *Pgr* expression selectively into AVPV kisspeptin neurons of KissPRKO females using targeted AAV technology. We demonstrate that such upregulation of PGR in kisspeptin neurons specifically within the AVPV is sufficient to rescue the E_2-induced LH surge in KissPRKO females, suggesting these neurons are targets of the endogenous P4 that promotes the surge.

Female KissPRKO mice were previously shown to display impacted fertility, including fewer litters and a notable reduction in litter size (27). The reduced fecundity was traced to diminished ovulatory events in KissPRKO females, reflected by fewer corpora lutea and diminished or absent LH surges in response to elevated E_2 (positive feedback) (27, 29). These sequelae mirrored rats in which hypothalamic P4 synthesis was blocked (19). Moreover, we previously demonstrated that P4 stimulated the release of intracellular calcium in mHypoA51 cells, mirroring our prior *in vivo* results that KissPRKO AVPV kisspeptin neurons were not properly activated by E_2, correlating with impaired LH surges. Here, we both verified and extended that finding to show that the deficit is likely in kisspeptin neurons specifically in the AVPV, as selective re-introduction of PGR in just AVPV kisspeptin neurons was sufficient to rescue both the occurrence and magnitude of LH surges. These findings support prior evidence linking the AVPV kisspeptin population to the circadian-timed E_2-induced LH surge and also supports recent findings that P4 can act *in vitro* in AVPV mHypoA51 kisspeptin cells to activate MAPK and Src kinases and induce kisspeptin release (24). Importantly, our present data also indicate that PGR is not required in other kisspeptin populations, such as the ARC, MeA, or BNST, for proper LH surges, though we cannot rule out if PGR action in those populations is sufficient for surge induction.

Two KissPRKO^{Pgr} females did not show an LH surge, but this was not due to insufficient *Pgr* induction, as *Pgr+Kiss1* co-expression was not different between those 2 non-surging mice and the other 8 surging KissPRKO^{Pgr} mice or surging WT controls (data not shown). Rather, in typical mouse LH surge studies, a small percentage (10-20%) of normal animals sometimes do not surge (for reasons unknown) and the 2 non-surging KissPRKO^{Pgr} animals likely reflect that normal outcome, similar to the 2 non-surging WT^{Pgr} (Figure 4B).

Our findings demonstrate that signaling of endogenous P4 directly in AVPV kisspeptin cells is essential for the positive feedback induction of LH surges. These findings further highlight the critical importance of P4 signaling, along with E_2 signaling, in positive feedback induction of LH surge. We have proposed that the source of P4 in this LH surge process is neural rather than ovarian, derived in astrocytes of the AVPV region, under the influence of elevated E_2 (18-22). Indeed, because all our mice were OVX, any requisite P4 for the surge was non-ovarian in origin, further underlining the role of neuroP in E_2 triggered LH surges and ovulation.

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Figure Legends

Figure 1: Experimental design and timeline for virally re-expressing PGR selectively in AVPV kisspeptin neurons of KissPRKO females and then assessing their ability to generate E_2-induced LH surges.

Figure 2: Double-label IHC verification of proper mCherry expression in AVPV kisspeptin cells of **A)** Cre+ mice (KissPRKO) but not **B)** Cre- mice (WT controls) after targeted AAV injection into the AVPV. Kisspeptin staining is yellow; mCherry is pseudo-colored magenta. White arrowheads in **A** denote kisspeptin neurons. Importantly, there was no spread of AVPV-infused AAV to the ARC region, indicated by **C)** complete absence of mCherry in kisspeptin cells in the ARC of Cre+ animals. Scale bars are shown in white for each image.

Figure 3: Very low co-expression of *Pgr* in AVPV *Kiss1* neurons of KissPRKO^{mCh} females and up-regulation of *Pgr* co-expression in KissPRKOs after "Pgr AAV" treatment, as assessed with double-label ISH. **A)** Representative microscope images of *Pgr* (white silver grains) and *Kiss1* (red fluorescence) co-expression in AVPV neurons of OVX+E_2 females 4 weeks after treatment with either Pgr AAV or control mCh AAV. Yellow arrowheads denote example *Kiss1* cells with *Pgr* co-expression; blue arrowheads denote example non-double labeled *Kiss1* cells. **B)** Mean % of *Kiss1*+*Pgr* co-expression in the AVPV of all 4 PM groups (n=7-10/group). Different letters above the bars signify significantly different ($p < 0.05$) from each other.

Figure 4: Selective re-introduction of PR into AVPV kisspeptin neurons rescues the LH surge in OVX+E_2 females. **A)** The occurrence of LH surges in each group, represented by the % of females demonstrating a surge after treatment with a validated E_2 positive feedback regimen known to elicit an LH surge in the PM but not AM time-point (n=7-10/PM group and n=4-6/AM group). The LH surge occurrence was very low in KissPRKO^{mCh} females and increased to WT levels in KissPRKO^{Pgr} females. **B)** Individual dot plot of PM LH values showing the high proportion of animals achieving an LH surge in both WTs and KissPRKO^{Pgr} (Cre^+ Pgr) females but not KissPRKO^{mCh} (Cre^+ mCh) females. Dashed horizontal line designates LH surge threshold (>0.70 ng/ml). **C)** Mean serum LH levels in OVX+E_2 females at the AM and PM time-periods demonstrating elevated surge-levels of LH in PM KissPRKO^{Pgr} but not PM KissPRKO^{mCh} females (n=7-

10/PM group and n=4-6/AM group). Different letters above the bars signify significantly different ($p < 0.05$) from each other.

Figure 1

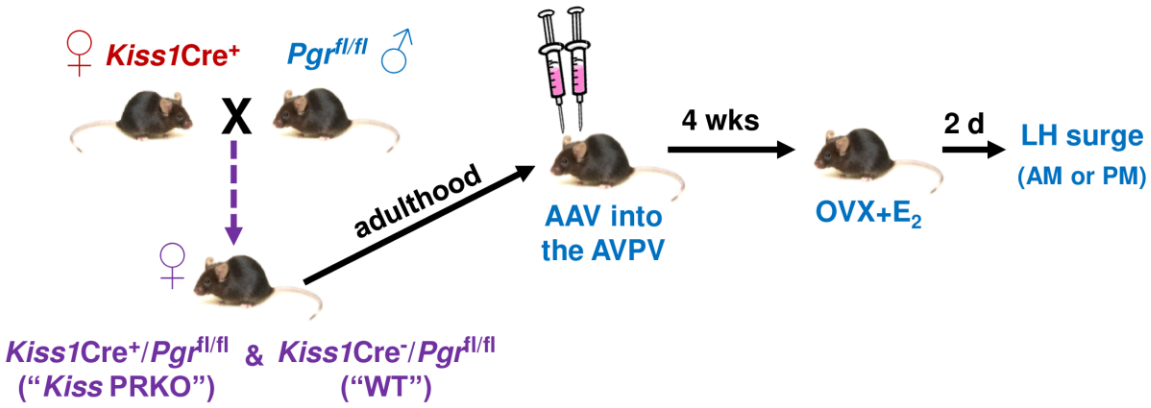

Figure 2

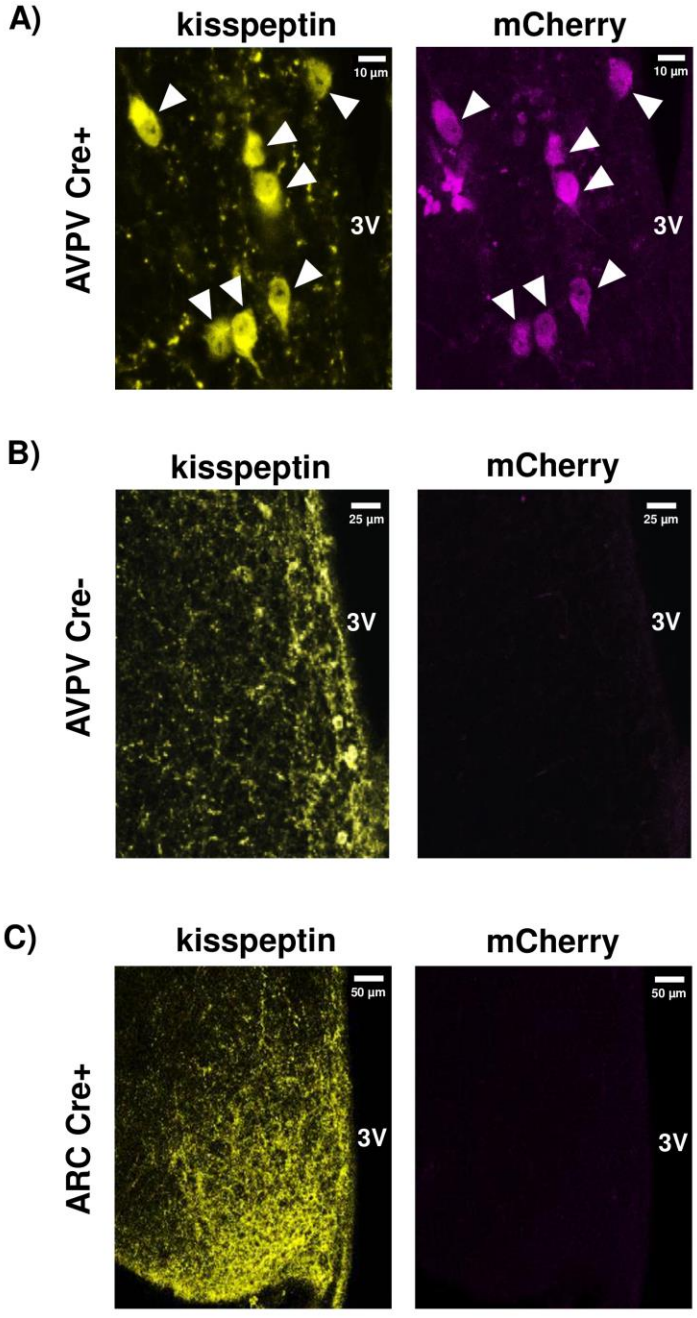

Figure 3

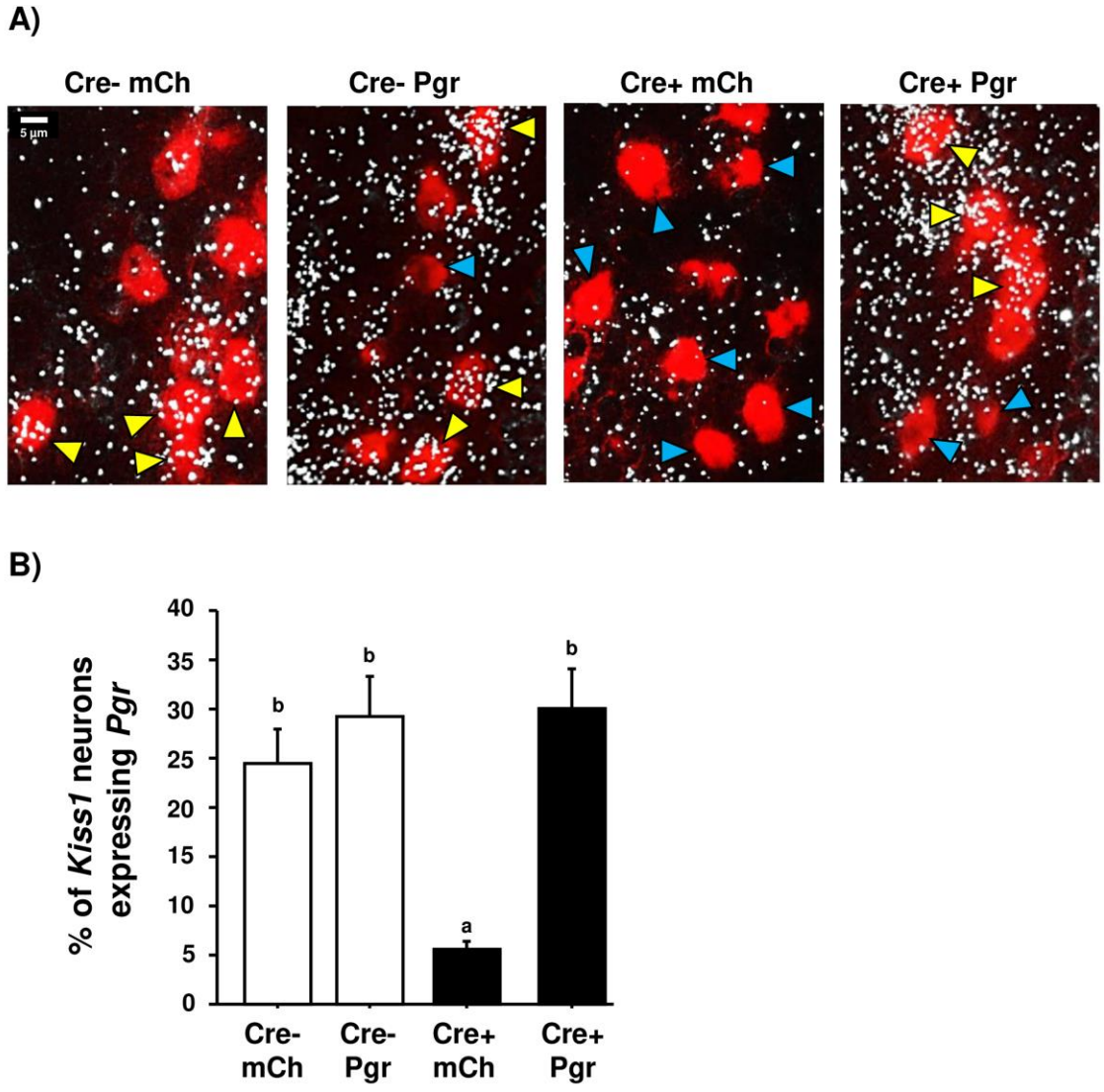

Figure 4