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Glucocorticoids stimulate hypothalamic dynorphin expression accounting for stress-induced impairment of GnRH secretion during preovulatory period

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ABSTRACT

Stress-induced reproductive dysfunction is frequently associated with increased glucocorticoid (GC) levels responsible for suppressed GnRH/LH secretion and impaired ovulation. Besides the major role of the hypothalamic kisspeptin system, other key regulators may be involved in such regulatory mechanisms. Herein, we identify dynorphin as a novel transcriptional target of GC. We demonstrate that only priming with high estrogen (E2) concentrations prevailing during the late prooestrus phase enables stress-like GC concentrations to specifically stimulate *Pdyn* (prodynorphin) expression both *in vitro* (GT1-7 mouse hypothalamic cell line) and *ex vivo* (ovariectomized E2-supplemented mouse brains). Our results indicate that stress-induced GC levels up-regulate dynorphin expression within a specific kisspeptin neuron-containing hypothalamic region (antero-ventral periventricular nucleus), thus lowering kisspeptin secretion and preventing preovulatory GnRH/LH surge at the end of the prooestrus phase. To further characterize the molecular mechanisms of E2 and GC crosstalk, chromatin immunoprecipitation experiments and luciferase reporter gene assays driven by the proximal promoter of *Pdyn* show that glucocorticoid receptors bind specific response elements located within the *Pdyn* promoter, exclusively in presence of E2. Altogether, our work provides novel understanding on how stress affects hypothalamic-pituitary-gonadal axis and underscores the role of dynorphin in mediating GC inhibitory actions on the preovulatory GnRH/LH surge to block ovulation.

1. Introduction

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway for the central regulation of fertility. Pulsatile hypothalamic GnRH release regulates pituitary secretion of gonadotropins (LH, Luteinizing Hormone and FSH, Follicle-Stimulating Hormone) that, in turn control ovarian function during the oestral cycle. The gonadal steroids are critical feedback regulators of the hypothalamus-pituitary activity. Much of the ovulatory cycle is dominated by negative feedback effects of the gonadal estradiol (E2) and progesterone. As the cycle progresses, rising E2 produced by maturing ovarian follicles (at the end of the prooestrus phase) evokes a positive feedback action that triggers generation of the pituitary signal of ovulation (Clarkson and Herbison, 2009). Because GnRH neurons do not possess the required sex steroid receptors (Lehman et al., 1993; Skinner et al., 2001), feedback signals to these neurons rely on transmission through other steroid-sensitive cells within the brain. Kisspeptin neuropeptide secreting neurons within the antero-ventral periventricular (AVPV) nucleus are considered as critical hypothalamic neurons for processing the positive feedback E2 signal during the generation of the GnRH/LH surge in rodents. Another subgroup of kisspeptin neurons, located in the arcuate nucleus (ARC) of the hypothalamus, co-synthesize the endogenous opioid inhibitor dynorphin and the excitatory neuropeptide neurokinin B that autosynaptically coordinate the pulsatile release of kisspeptin to maintain episodic GnRH secretion (Burke et al., 2006; Goodman et al., 2007). These latter kisspeptin neurons are considered key effectors mediating the negative feedback of E2 while dynorphin is reported to mediate the negative feedback of progesterone (Goodman et al., 2004) which slows down GnRH/LH pulsatility frequency (Schulz et al., 1981). Intravenous administration of the opioid receptor antagonist naloxone blocks the suppressive effect of dynorphin on LH release (Kinoshita et al., 1982). Dynorphin specific receptors (κ-opioid opioid receptors or KOR) are expressed in ARC kisspeptin neurons as well as in GnRH neurons in ovine and rats (Weems et al., 2016), indicating that dynorphin could act on both kisspeptin and GnRH secretion levels. Interestingly, prodynorphin gene *Pdyn* (dynorphin precursor

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gene) is a direct target of sex steroids in rodents. E2 exerts a positive or a negative effect on *Pdyn* expression depending on E2 concentrations and/or brain regions (Kanaya et al., 2017; Simerly et al., 1996). For instance, in the ARC, low E2 concentrations increase *Pdyn* immunoreactive cell number whereas higher concentrations decrease *Pdyn* expression (Kanaya et al., 2017). Besides, testosterone also regulates hypothalamic *Pdyn* expression suggesting that dynorphin could play a major role in female as well as in male reproduction (Iwasa et al., 2017).

In mammals, physical or emotional stresses are pivotal triggers of reproductive dysfunctions (Whirledge and Cidlowski, 2013). Chronic stress induces activation of the corticotrope axis which directly controls the hypothalamic-pituitary-gonadal (HPG) activity. Stress-induced increases in plasma glucocorticoids (GC) plays a major role in mediating suppression of GnRH and LH secretion. Reproductive stress responses depend on the reproductive state of female individuals, highlighting the crucial role of ovarian steroids (Kajantie and Phillips, 2006). Contrary to the well described direct effects of GC on GnRH responsiveness and pituitary gonadotropin expression (Breen and Karsch, 2006), their effects at the level of hypothalamus remain unclear. The lack of glucocorticoid receptors (GR) expression in GnRH neurons (Dufourny and Skinner, 2002) suggests that GC act indirectly, possibly *via* an interneuronal system to lower GnRH pulse frequency, involving for instance corticotrophin-releasing factor (Kinsey-Jones et al., 2006), RFamide related peptide-3 (Leon et al., 2014) or kisspeptin (Gottsch et al., 2004; Navarro et al., 2005a, b). Studies on rodents as well as in domestic animals demonstrate that exogenous administration of stress-like GC levels disrupts the preovulatory LH surge and oestrous cyclicity (Breen et al., 2005; Luo et al., 2016; Saketos et al., 1993). Other studies report that GC down-regulate kisspeptin gene (*Kiss1*) expression (Kinsey-Jones et al., 2009) and inhibit AVPV kisspeptin neurons activity at the time of the LH surge (Luo et al., 2016), preventing them to elicit the E2-induced surge release of GnRH and LH (Luo et al., 2016). GC may therefore block ovulation by acting directly on kisspeptin neurons. Hypothalamic deletion of GR (*Nr3c1*) in kisspeptin-expressing neurons prevents GC induced *Kiss1* suppression but does not impair the acute suppression of the HPG axis following stress, suggesting that kisspeptin neurons are not the sole effectors of stress-induced GC actions (Whirledge and Cidlowski, 2013). Interestingly, stress-like immobilization or elevation of GC levels significantly increases dynorphin concentrations in various brain areas, particularly in hypothalamus (Nabeshima et al., 1992; Shirayama et al., 2004), associated with increased number of dynorphin expressing neurons within the ARC (Oakley et al., 2009a, b; Ralph et al., 2016). Hence, dynorphin could be a good candidate to trigger GC inhibitory effects on HPG activity.

In the present study, we investigate the potential role of dynorphin in mediating stress response during a specific period of the ovulatory cycle. We use the GC-sensitive and dynorphin expressing hypothalamic cell line GT1-7 and provide evidence that only high levels of E2 enable GC to up-regulate *Pdyn* expression. We further confirm these results *ex vivo* in E2-supplemented ovariectomized mouse brains that exhibit an up-regulation of *Pdyn* in the AVPV upon GC treatment. We also explore E2 and GC crosstalk at a molecular level and show that E2 allows GR to access to glucocorticoid response elements located within the proximal region of mouse *Pdyn* promoter, thereby demonstrating that dynorphin relays and participates to GC activities in controlling female reproduction.

2. Materials and methods

2.1. Animals and treatment

RjOrl:SWISS female mice at 8 weeks of age (Janvier Breeding Centre, Le Genest-Saint-Isle, France) were housed in nest-enriched cages under a 12:12 h light-dark cycle, maintained at 22 °C, and fed a standard diet with free access to food and water. Experiments were conducted in accordance with the French and European legal requirements (Decree 2010/63/UE) and were approved by the "Charles Darwin" Ethical committee (project number 01490-01).

Mice were ovariectomized under general anaesthesia (xylazine 10 mg/kg – ketamine 100 mg/kg, i.p.) and implanted with Silastic (Dow Corning) implants filled with 50 µg of estradiol-benzoate (Sigma-Aldrich, Saint-Louis, MA, USA) in 30 µL of sesame oil as previously described (Naule et al., 2015; Raskin et al., 2009).

Three weeks after ovariectomy and E2 supplementation, female mice were euthanized (in the morning, at 10.00 a.m.) and the brain and uterine horns were collected. Uterus is an estrogen-responsive tissue usually used to indirectly monitor circulating oestradiol levels. Therefore, uterine horns collected for each female were weighed in order to ensure that all studied females were comparably impregnated with E2 implants. Ovariectomized and E2-supplemented females showed an average percentage of relative uterine weight of 0.73 ± 0.04% of body weight, about 2.4-fold increase when compared with eight weeks old RjOrl:SWISS female mice at dioestrus (Lemini et al., 2015).

2.2. Slice preparation, drug application and RNA preparation

The brain was immersed in cooled artificial cerebrospinal fluid (aCSF) containing: 117 mM NaCl; 4.7 mM KCl; 1.2 mM NaH_2PO_4; 25 mM $NaHCO_3$; 2.5 mM $CaCl_2$, $2H_2O$; 1.2 mM $MgCl_2$, $6H_2O$; 10 glucose. Anatomically matched slices (400 µm) containing the AVPV (plate 30 of the Mouse Brain Atlas of Paxinos et Franklin) or ARC (plate 46) were selected and transferred to a thermostated (32–34 °C) and oxygenated (95% O_2/5% CO_2) brain slice chamber system allowing a continuous flow of fluid throughout the incubation period (2 mL/min). Slices were allowed to equilibrate for 1 h in aCSF before being exposed to 100 nM Dex (dissolved in aCSF with 0.01% ascorbic acid) or ethanol (vehicle, 1:1000 dilution in aCSF with 0.01% ascorbic acid) treatment for 6 h. After incubation, punches were recovered through the AVPV and ARC with a 1 mm diameter canula. Total RNA was extracted using the PicoPure RNA isolation kit from Arcturus (Excilone, Élancourt, France). Total RNAs were reverse transcribed using the Promega kit (Charbonnières-les-Bains, France).

2.3. Cell line culture and hormonal treatment

GT1-7 cells (kindly provided by Dr. Pamela L. Mellon, UCSD, USA) were cultured in DMEM (Dulbecco's Modified Minimum Essential Medium)-glutamax without red-phenol and with 4.5 g/L glucose (Life Technologies, Saint-Aubin, France), containing 10% Fetal calf serum (FCS) (Biowest, Nuaillé, France), and supplemented with 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), at 37 °C in 5% CO_2. For gene expression experiments, cells at 0.4×10^6 cells/well were seeded in 6-well plates, whereas for gene recruitment study, cells were seeded at 3×10^5 cells in T-175 flasks. Twenty-four h later, cells were washed with PBS and fed with DMEM containing 10% hormone-depleted, charcoal-stripped FCS. After overnight steroid starvation, cells were pretreated for 24 h with either ethanol, or either 0.1 nM or 100 nM 17-β estradiol (E2) (Sigma-Aldrich). Twenty-four h after E2 priming, cells were treated with ethanol (Vehicle, V), Dexamethasone (Dex) or different drugs (RU486, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or Actinomycin D (Sigma-Aldrich)) at different concentrations for various periods of time, as indicated in the corresponding figure legends.

2.4. Primer design for genomic amplification and RT-qPCR assays

Primer pairs were designed by using NCBI's software Primer BLAST (http://www-ncbi-nlm-nih-gov.gate2.inist.fr/tools/primer-blast/). Primers were synthesized from Eurogentec (Ougrée, Belgium), purified by the selective precipitation optimized process (SePOP), desalted, and

delivered at 100 μM concentration in water. To determine the expression of endogenous *Esr1*, *Esr2*, *Nr3c1*, *Pdyn*, *Gnrh* and *Hsd11b2* in GT1-7 cells, one μg of total RNA samples was first treated with DNase I (New England Biolabs, Évry, France) and then reverse-transcribed into cDNA with the High Capacity cDNA reverse transcription kit RT-qPCR. cDNA was amplified for 35 cycles (95 °C for 45 s, 56–65 °C for 1 min, and 72 °C for 45 s) in 1X buffer with 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.06 μM (initial concentration) concentrations of each primer (sequences provided in Supplemental Tables), 100 ng of DNA, and 1.25 U of Dream Taq (ThermoFisher Scientific, Villebon-sur-Yevette, France) in a total volume of 50 μL. The amplified samples were subjected to agarose gel electrophoresis to follow gene expression within GT1-7 cells.

2.5. Construction of recombinant plasmid

The pGL4.16 vector (Promega) containing the gene for Firefly luciferase was used in this study. To create the pGL4.16-*Pdyn* promoter-F-Luc construct containing the 1.7-kb promoter in the upstream of the Firefly luciferase gene, the *Pdyn* promoter region was amplified by PCR from mouse genomic DNA by using the primers forward and reverse including a Xho1 and HindIII restriction sites at their 5' ends

(Forward: 5'-CGCCCTCGAGTACTACACCACACTCCACAG-3';

Reverse: 5'-CGCTAAGCTTGATCCAAGATTCAGAAGGTA-3').

PCR product was subcloned into pGL4.16 vector at the Xho1 and HindIII sites. The construct was verified by DNA sequencing (Eurofins, Ivry-sur-Seine, France).

2.6. Transient transfection of GT1-7 cells and Dual® Luciferase reporter assays

The reporter plasmids used in these studies, *Pdyn* promoter, contains the *Pdyn* promoter region which drives the expression of the Firefly luciferase gene. The control plasmid pRL-TK, carrying the *Renilla* luciferase gene under the control of the TK promoter (Promega) was co-transfected to correct for variations in transfection efficiency. For transient transfection assays, GT1-7 cells were plated in 96-well plates at 20×10^3 cells/well in DMEM-glutamax containing 10% FCS for 24 h. The reporter (*Pdyn*-promoter or pGL-vector at 125 ng/well) and the control (Renilla at 20 ng/well) plasmids were transfected using Lipofectamine 2000 reagent (Life Technologies) in OptiMEM (Life Technologies) according to the manufacturer's protocol for 6 h at 37 °C in a 5% CO_2 atmosphere. Then, the media was replaced with DMEM containing 10% hormone-depleted, charcoal-stripped FCS. Following 24 h starvation, cells were treated with either ethanol (V, 1/1000 dilution) or different drug combinations (100 nM E2, 100 nM Dex, 10 μM RU486) for another 24 h. At the end of the experiment, cultures were harvested and lysates prepared in passive lysis buffer (Promega). Luciferase activities were determined using the Dual® Luciferase reporter assays system (Promega). Potency of the *Pdyn* promoter to drive the reporter gene expression was determined by the activity of the Firefly luciferase normalized to that of the Renilla luciferase in each sample.

2.7. RT-qPCR

To evaluate GC-induced expression of endogenous prodynorphin (*Pdyn)*, kisspeptin (*Kiss1)* and actin beta *(Actb)*, GT1-7 cells were pretreated or not with E2 (0.1 nM or 100 nM) for 24 h, and then treated or not (V) with 100 nM Dex for 1.5 h, 6 h or 24 h. Co-treatment with DRB (10 μg/mL), ActD (1 μM) or RU486 (10 μM) was also performed. cDNA samples obtained from cells and tissues RNA extracts were prepared as described above. These samples were analyzed with TaqMan® Universal Master Mix Reagent (Life Technologies), with specific TaqMan Gene expression Assays (*Kiss1*: Mm03058560_m1; *Pdyn*: Mm00457573_m1; *Actb*: Mm02619580_g1) and the QuantStudio™ 6 Flex Real-Time PCR System. A standard curve consisted of four 1:10 serial dilutions of hypothalamus cDNA was performed for each set of primers. All samples were tested in triplicate within a single run. Quantification of the amount of *Pdyn* or *Kiss1* mRNA was calculated relative to that of the *Actb* normalizer gene and expressed as relative units. Experiences were performed at least three times, and the median ± interquartile range was calculated using the Prism curve-fitting program.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with the iDeal ChIP-Seq kit for Transcription Factors (Diagenode, Seraing, Belgium), according to the manufacturer's recommendations. Rabbit monoclonal anti-GR D6H2L (5 μg, Cell Signaling Technology) or polyclonal anti-Jun (7 μg, sc-44, Santa Cruz Biotechnology, CA, USA) antibodies were used for immunoprecipitation of endogenous GR or Jun proteins, respectively. Rabbit control IgG, provided in the kit, was used as negative immunoprecipitation control. ChIP-resulting DNA samples were amplified by qPCR, by using the primer sets (Supplemental Tables) targeted to different regions of mouse *Pdyn*, *Ucp1* or *Trh* genes.

2.9. qPCR

ChIP-resulting DNA samples were amplified by qPCR, by using the QuantStudio™ 6 Flex System (Life Technologies), with a 5 μL DNA sample, 6 μL Fast SYBR Green Master Mix, and 0.5 μL forward and reverse primers (300 nM final concentrations) (primer sequences are provided in Supplemental Tables). Each DNA sample was analyzed in triplicate. All results are expressed as fold recruitment normalized to input samples (Percent Input Method, ChIP analysis; Life Technologies) and expressed as medians ± interquartile range of 3 independent experiments.

2.10. Protein extraction and Western Blot analysis

To follow GR expression, E2 pretreated GT1-7 cells were scraped in RIPA (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton, 10% Glycerol, protease inhibitors cocktail (Sigma-Aldrich) and phosphatase inhibitors (Sigma-Aldrich)). After centrifugation (14,000 × *g*, 4 °C, 15 min), protein concentration was determined by bicinchoninic acid assay (ThermoFisher Scientific). Equal protein amounts (50 μg) were resolved on 7.5% SDS-PAGE and then transferred to nitrocellulose membrane (Whatman Schleicher & Schuell, Dassel, Germany). Blots were incubated for 1 h at room temperature (RT) in a blocking buffer (5% BSA in 0.2% Tween 20 Tris-buffered saline (TBS-T)), before an overnight incubation at 4 °C with primary monoclonal anti-GR (D6H2L, Cell Signaling Technology) or monoclonal anti-α-tubulin (Sigma-Aldrich) antibodies as indicated. After extensive washes with TBS-T, blots were incubated with an IRDye 800-conjugated affinity purified anti-rabbit IgG secondary antibody (Perbio Science, Villebon-sur-Yvette, France) and an IRDye 680-conjugated affinity purified anti-mouse IgG secondary antibody (Perbio Science) for 1 h at RT. After washes, proteins were visualized with an Odyssey-Fc apparatus (Li-Cor, Lincoln, NE, USA). Specific signals for different proteins were normalized by the infrared fluorescence of α-tubulin as determined by densitometry using the Image Studio software (Li-Cor). All Western blots shown are representative of what was observed in at least three independent experiments.

2.11. Statistical analyses

Data are median ± interquartile range (IQR) of n experiments. Non-parametric ANOVA Kruskal-Wallis tests followed by post-test of Dunn or Mann–Whitney U-tests were used when appropriate to determine significant differences (GraphPad Prism software). *P* value of 0.05 was considered as statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

3. Results

3.1. Priming with high levels of E2 allows Pdyn up-regulation by dexamethasone in GT1-7 cells

Stress induces suppression of LH release through down-regulation of kisspeptin expression and release (Kinsey-Jones et al., 2009). To investigate the potential role of the inhibitory neuropeptide dynorphin in these processes, we examined the effects of GC on *Pdyn* (dynorphin precursor gene) mRNA levels in the hypothalamus.

Because of the absence of a characterized kisspeptin cell model, we used a hypothalamic cell line (GT1-7 cells) that expresses dynorphin (*Pdyn*) as well as GR (*Nr3c1*), estrogen receptors ER alpha and beta (*Esr1 and Esr2*), and the metabolizing enzyme 11β hydroxysteroid-dehydrogenase type 2 (*Hsd11b2*) (Supplemental Fig. 1) that inactivates corticosterone into 11-dehydrocorticosterone. To ensure optimal effects of GC on gene regulation, we treated cells with a synthetic and non-metabolizing GR agonist, dexamethasone (Dex), at a stress-like concentration (100 nM, (Dominguez et al., 2014; Kainuma et al., 2009; Tronche et al., 2010)) that has the same potency to induce GR transactivation than corticosterone (Hellal-Levy et al., 1999). Moreover, to mimic the variation of E2 levels during ovulatory cycle, we pretreated GT1-7 cells for 24 h with two different E2 concentrations, one mimicking high E2 levels (100 nM) prevailing at the end of the prooestrus phase, and the second one corresponding to low E2 concentrations (0.1 nM) observed during the dioestrus phase (Chu et al., 2009). Cells were then treated with ethanol (V) or 100 nM Dex for 1.5 h, 6 h or 24 h in order to follow *Pdyn* expression by RT-qPCR. Fig. 1 shows that, in the presence of 100 nM E2, Dex significantly increases by 1.4-fold *Pdyn* expression (non-parametric ANOVA, $P \leq 0.05$) after 1.5 h treatment (Fig. 1). Dex-induced effect is even stronger after 6 h treatment and shows an up-regulation by 2.7-fold of *Pdyn* mRNA levels, only in the presence of high levels of E2 (non-parametric ANOVA, $P \leq 0.001$) (Fig. 1). However, *Pdyn* mRNA return to basal levels after 24 h Dex treatment (Fig. 1). Dex alone or in the presence of 0.1 nM E2 does not modify the *Pdyn* mRNA expression, demonstrating that only priming with high E2 concentrations enables Dex to stimulate *Pdyn* expression.

To examine the importance of GC concentrations and to assess the specificity of this regulation, we pretreated GT1-7 cells with high levels of E2 before Dex treatment at 10 nM or 100 nM for 6 h. As shown in Fig. 2A, in the presence of 100 nM E2, only 100 nM Dex significantly induces *Pdyn* expression by 2.7-fold (non-parametric ANOVA, $P \leq 0.001$). Remarkably, 10 nM Dex treatment, even with 100 nM E2 pretreatment, is unable to modify *Pdyn* mRNA levels, suggesting that threshold GC concentrations are necessary for controlling *Pdyn* expression. Dex induced-*Pdyn* mRNA up-regulation is prevented by a GR antagonist (RU486) co-treatment (Fig. 2B), demonstrating that Dex effects are mediated by GR. To examine whether E2 effects are mediated by its specific receptor, we co-treated GT1-7 cells previously exposed to 100 nM E2 with Dex and ER antagonists (tamoxifen or fulvestrant) but unfortunately these two antagonists alone increase the basal levels of *Pdyn* mRNA preventing any definite conclusion to be drawn (data not shown).

Lastly, we evaluated whether Dex-induced *Pdyn* up-regulation could be the result of transcriptional activation by using two transcription inhibitors, DRB or ActD. Fig. 2C shows that 6 h treatment with 100 nM Dex induces up-regulation of *Pdyn* mRNA of GT1-7 pretreated with 100 nM E2 (about 2.4-fold, non-parametric ANOVA, $P \leq 0.001$). Remarkably, this up-regulation is prevented by co-incubation with either DRB or ActD, without affecting basal levels of *Pdyn* mRNA (Fig. 2C).

Altogether, these data provide evidence that only priming with high concentrations of E2 allows Dex to up-regulate *Pdyn* mRNA transcription, suggesting that GC solely act during a specific window of the ovulatory cycle. Besides, Dex stimulates *Pdyn* transcription exclusively at stress-like concentrations, indicating that these regulations only occur during very specific conditions.

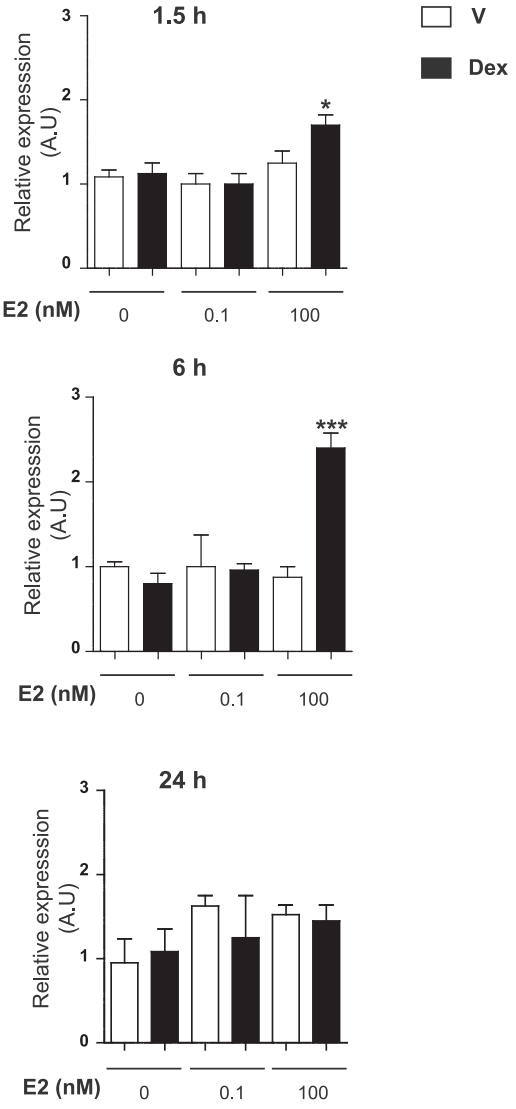


Fig. 1. Dex up-regulates *Pdyn* expression in GT1-7 cells upon pretreatment with high E2 concentrations.
GT1-7 cells were pretreated or not with 0.1 nM or 100 nM E2 for 24 h. Cells were then exposed to vehicle (V) or 100 nM Dex for 1.5, 6 h or 24 h. Relative transcript expression of *Pdyn* was normalized to *Actb* expression, arbitrarily set at 1 for vehicle condition. Data are expressed as medians ± IQR from four independent experiments performed in triplicates. Non-parametric ANOVA Kruskal-Wallis tests followed by post-test of Dunn, $*P \leq 0.05$; $***P \leq 0.001$ compared to vehicle.

3.2. GC regulate Pdyn expression in ovariectomized and E2-supplemented mice

We wondered whether GC could also control *Pdyn* expression *ex vivo* with an estrogenic exposure alike the one observed at the end of the prooestrus phase. To this aim, adult female mice were ovariectomized and E2-supplemented to mimic the ovulatory surge of LH and induce female receptivity (Naule et al., 2015; Raskin et al., 2009). Three weeks after surgery, mice presented comparable hormonal impregnation as shown in Material and Methods. They were euthanized and brain slices containing either AVPV or ARC were incubated with the vehicle or 100 nM Dex for 6 h (Fig. 3A). As depicted in Fig. 3B, *Pdyn* levels are more than 3-fold higher in ARC than in the AVPV under basal conditions reflecting the differential E2 regulation on *Pdyn* expression previously described within these two hypothalamic areas (Kanaya et al., 2017; Simerly et al., 1996). Interestingly, Dex induces a significant

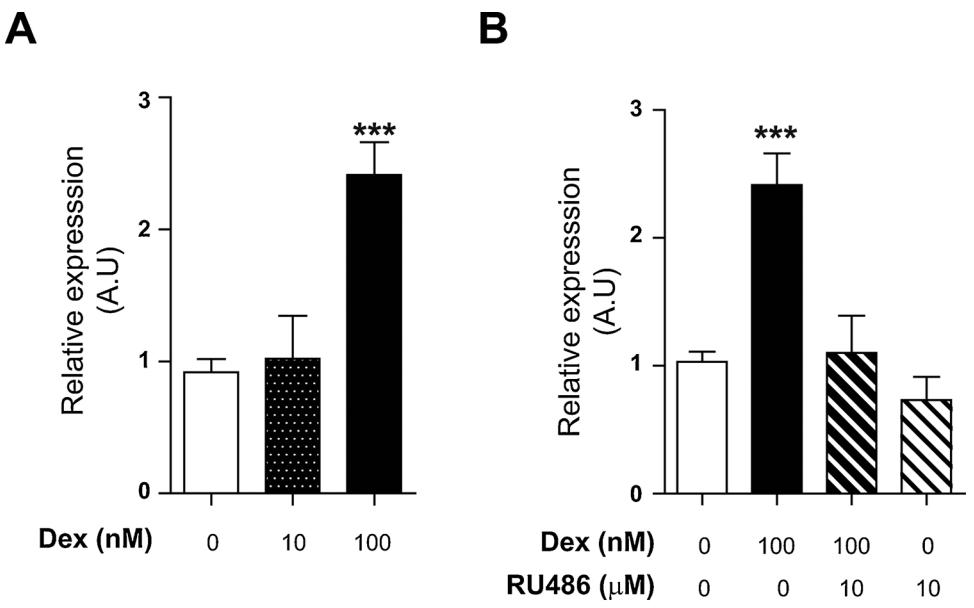
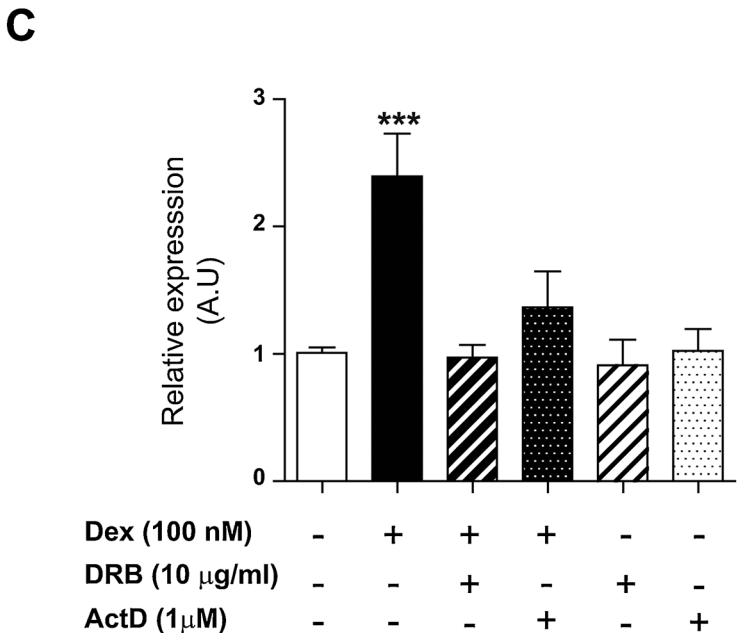


Fig. 2. *Pdyn* transcription is activated *via* GR in GT1-7 cells upon high Dex concentrations.
A, GT1-7 cells were pretreated with 100 nM E2 for 24 h before exposure to vehicle (V), 10 or 100 nM Dex for 6 h. B and C, After 100 nM E2 pretreatment, GT1-7 cells were treated with 100 nM Dex in the absence or in the presence of RU486, a GR antagonist (B), DRB or Actinomycin D (ActD), two transcription inhibitors (C) for 6 h. Relative *Pdyn* transcript expression was normalized to the one of *Actb,* and arbitrarily set at 1 for vehicle condition. Data are expressed as medians ± IQR from three independent experiments performed in triplicates. Non-parametric ANOVA Kruskal-Wallis tests followed by post-test of Dunn, ***$P \leq 0.001$ compared to vehicle condition.



1.55-fold increase in *Pdyn* expression (Mann-Whitney *U*-tests, $P \leq 0.05$) in the AVPV as compared to the vehicle. No significant difference was observed in *Pdyn* expression in the ARC between Dex and vehicle treatment (Fig. 3B). In agreement with previous studies (Dubois et al., 2015; Gonzalez-Martinez et al., 2008), *Kiss1* expression in vehicle samples is more than 20-fold higher in AVPV than in ARC of OVX + E2 mice (Fig. 3C). As expected, Dex treatment significantly decreases *Kiss1* mRNA both in the AVPV and in the ARC (by about 9.2- and 3.2-times less than the control respectively, Mann-Whitney *U*-tests, $P \leq 0.05$) (Fig. 3C). These results reveal that in the presence of high E2 levels, Dex is able to induce *Pdyn* expression in the mouse AVPV. Up-regulation of *Pdyn* would be an important inhibitory signal to kisspeptin neurons present within the AVPV, as well as an important negative input on GnRH neurons to reduce GnRH release.

3.3. E2 does not modify GR expression in GT1-7 cells

To further explore the molecular crosstalk between E2 and GC signaling pathways, we examined whether E2 priming could increase GR mRNA expression and/or protein stability and activity (through GR phosphorylation) to promote GC actions. As shown in Supplemental Fig. 2, 100 nM E2 treatment does not modify *Nr3c1* mRNA (Supplemental Fig. 2A) nor GR protein expression (Supplemental Fig. 2B) levels when compared to *36b4* mRNA or α-tubulin protein levels, respectively.

3.4. The 1.7-kb Pdyn promoter is activated by GR in GT1-7 cells

Given that GC modulate transcriptional activation by binding to glucocorticoid response elements (GREs) in DNA regulatory regions or interacting with other transcription factors (*e.g.* NFκB and AP-1) to

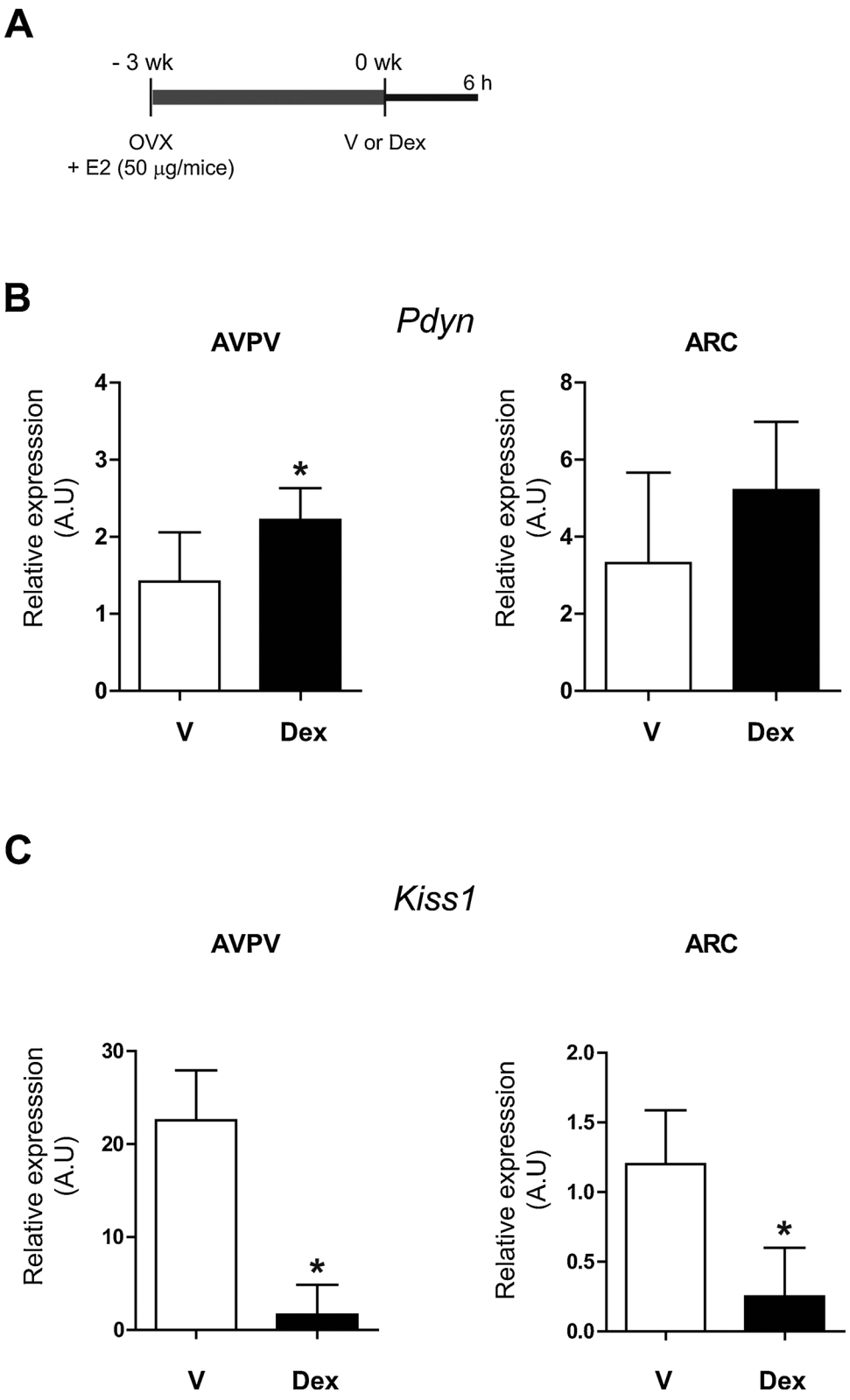


Fig. 3. Dex induces up-regulation of *Pdyn* expression within AVPV of ovariectomized + E2 mice.
A, Ovariectomized mice were implanted with E2. Mice were sacrificed after 3 weeks and brain slices were incubated for 6 h with either the vehicle (V) or 100 nM Dex. Total RNAs extracted from punches of the AVPV or ARC were isolated as described in Material and Methods. B and C, Relative expression levels of mRNA for *Pdyn* (B) or *Kiss1* (C) were determined by RT-qPCR analysis of AVPV or ARC samples from individual mouse. Transcript levels were normalized to *Actb* transcripts abundance. Medians ± IQR (n = 5–7 mice per group) mRNA expression levels are presented. Results are from one experiment performed in triplicates. Statistical significance of difference between Dex and vehicle for ARC or AVPV was analyzed by using the Mann-Whitney *U*-tests. * $P < 0.05$.

regulate gene expression (Uht et al., 1997; Vandevyver et al., 2014), we analyzed *in silico* 1.7-kb 5'-flanking region of Exon I of mouse *Pdyn* (Supplemental Fig. 3) using Jaspar database (URL: http://jaspar.genereg.net/cgi-bin/jaspar_db.pl). Within the proximal *Pdyn* promoter, we identify three putative GREs (5′-AGAACAnnnTGTTCT-3′) (positions: −638, −1490 and −1535 bp) and one potential AP-1 binding site (5′-TGACTCA-3′, positions −1248 bp) (Supplemental Fig. 3). Nine potential hemi-sites for GR (5'-AGAACA-3') are also detected.

To assess whether GR may specifically transactivate *Pdyn* gene, the 1.7-kb mouse Pdyn promoter was subcloned into the promoter-less pGL.4.16 vector, generating the pGL4.16-*Pdyn* promoter-F-Luc plasmid (Fig. 4), in which firefly luciferase (F-Luc) gene expression was driven by the cloned promoter. Twenty-four h after transfection, cells were

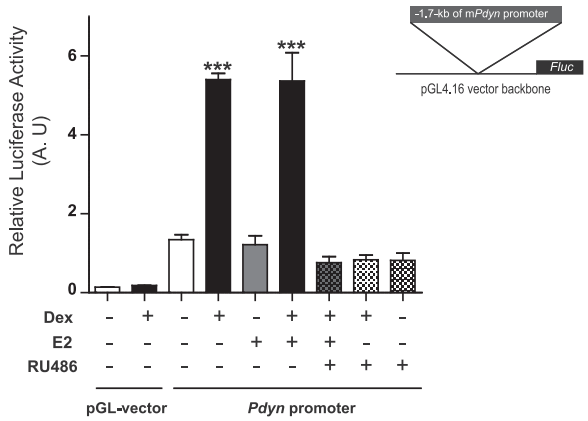


Fig. 4. Dex transactivates *Pdyn* proximal promoter.
1.7 kb mouse *Pdyn* promoter was inserted upstream of the *Firefly* luciferase gene in pGL4.16 vector. *Pdyn* promoter or pGL4.16 vector (pGL-vector) was transfected in GT1-7 cells together with an internal control vector containing *Renilla* luciferase. Twenty-four h post-transfection, cells were treated either with ethanol, 100 nM Dex or 100 nM E2 in the presence or absence of 10 μM RU486, a GR antagonist. Firefly luciferase activity was assessed 24 h after treatment and normalized relative to *Renilla* luciferase activity, arbitrarily set at 1 for vehicle condition. Results are medians ± IQR of 3 independent experiments performed in six replicates (non-parametric ANOVA Kruskal-Wallis tests followed by post-test of Dunn, ***P < 0.001).

exposed to hormonal treatment for another 24 h. Data demonstrate that the promoter-less pGL.4.16 vector (pGL-vector) is not activated by Dex (Fig. 4). Interestingly, the *Pdyn* promoter containing all three GREs significantly responds to Dex, with a 5.2-fold higher Firefly luciferase activity compared to the vehicle treatment (non-parametric ANOVA, $P \leq 0.01$). Co-treatment with RU486 completely abolishes Dex-induced increase in *Pdyn* promoter activity, indicating that Dex controls *Pdyn* promoter activity through GR activation. Since we observed a permissive effect of E2 on Dex-induced *Pdyn* expression, we examined whether Dex and E2 co-treatment would exert a stronger effect on 1.7-kb *Pdyn* promoter activity. *In silico* analysis of the 1.7-kb *Pdyn* promoter does not disclose any potential estrogen response element (ERE). Alternatively, E2 may act indirectly through AP-1 site (Uht et al., 1997) that was identified on the 1.7-kb *Pdyn* promoter (Supplemental Fig. 3). Surprisingly, Fig. 4 shows that E2 treatment alone or together with Dex does neither stimulate nor potentiate Dex-induced *Pdyn* promoter activity (about 5.57 *vs* 5.2-fold induction of Firefly luciferase activity for Dex or Dex + E2 respectively).

3.5. E2 enables GR recruitment upon Pdyn promoter

We next examine whether GR stimulates *Pdyn* expression through either direct GR recruitment on GREs or indirect interaction with AP-1 complex (heterodimer Jun/Fos) by tethering mechanisms. For that, GT1-7 cells (pre-exposed or not with 100 nM E2) were treated with ethanol (V) or 100 nM Dex for 1 h before performing chromatin immunoprecipitation (ChIP). The endogenous GT1-7 promoter region *Pdyn* was immunoprecipitated with anti-GR antibody, anti-Jun or control IgG antibody, and the isolated DNA was quantified by qPCR, with primers encompassing putative GREs (from −1.555 kb to −1.475 kb) (Fig. 5) or the AP-1 site (Supplemental Fig. 4).

Remarkably, GR recruitment on DNA regions only occurs in the presence of both Dex and E2 (Fig. 5). E2 pretreatment enables Dex to induce a significant 12.6-fold (non-parametric ANOVA, $P \leq 0.01$) GR recruitment on *Pdyn* promoter region. Uncoupling protein 1 *Ucp1* promoter region was used as a negative control sequence (Supplemental Fig. 4A). As expected, no significant GR recruitment was observed on the target region of *Ucp1* promoter, independently of the presence of E2 and Dex (Supplemental Fig. 4A), clearly demonstrating that GR recruitment is gene-specific.

Concerning the AP-1 site, ChIP analysis with anti-Jun antibodies does not show any recruitment of Jun on the DNA region encompassing the potential AP-1 site (Supplemental Fig. 4B). As a positive control, we measured the recruitment of Jun to a GR target Thyrotropin Releasing Hormone gene *Trh* (Diaz-Gallardo et al., 2010) that contains two AP-1 sites within its promoter. As shown in Supplemental Fig. 4B, Dex is able to induce a significant Jun recruitment on this *Trh* specific DNA region (by about 3-fold, non-parametric ANOVA, $P \leq 0.05$).

Altogether, our data demonstrate that Dex stimulates 1.7-kb mouse *Pdyn* promoter activity very likely through GR interaction with GREs cis-elements. Though, at genome-wide level, only 100 nM E2 priming enables Dex to promote *Pdyn* transcription.

4. Discussion

In the present study, we identify dynorphin as a novel important transcriptional target for GC that would strengthen GC inhibitory impacts on HPG axis during stress. In a mouse hypothalamic cell line (GT1-7 cells), we demonstrate that only priming with high concentrations of E2 enables stress-like GC levels to specifically up-regulate *Pdyn* expression through ligand- and GR-dependent transcriptional mechanisms. Accordingly, ovariectomized mice with a strong and sustained estrogenic exposure give convergent data on *Pdyn* mRNA levels that were significantly increased in the AVPV upon GC treatment. These data are of high interest since AVPV contains kisspeptin neurons which are the major sensors of E2 positive feedback signals that trigger the preovulatory GnRH/LH surge. During stress, *Pdyn* up-regulation could play a direct or an indirect role in blunting GnRH release. Since specific dynorphin receptors (KOR) are expressed in ARC kisspeptin neurons as well as in GnRH neurons (Weems et al., 2016), one could speculate that during stress, dynorphin would down-regulate kisspeptin as well as GnRH secretion. In accordance with this hypothesis, recent data show that microinjection of a selective KOR antagonist directly into the AVPV area of OVX + E2 rats increases the magnitude of the LH surge (Helena et al., 2015). This effect is similar as the one observed on the same rats with partial ablation of ARC kisspeptin neurons (Helena et al., 2015). Helena and colleagues demonstrate the existence of E2-dependent mechanisms that promote ARC dynorphin producing neurons to act indirectly on AVPV kisspeptin to reduce LH surge (Helena et al., 2015). Fibers from ARC kisspeptin neurons are described to come into direct contact with kisspeptin neurons of the AVPV (Yeo et al., 2016; Yip et al., 2015). Although ARC kisspeptin neurons express KOR (Weems et al., 2016), it is yet to be determined whether AVPV kisspeptin neurons do so. Answering to this question will help to determine whether dynorphin could act directly on AVPV kisspeptin neurons.

KOR is a membrane receptor coupled to inhibitory G-proteins that inhibits cyclic adenosine monophosphate (cAMP) production. This receptor also activates mitogen-activated protein kinases which in turn activates transcription factors and alters gene expression (Bruchas et al., 2010). KOR activation decreases cell firing and neurotransmitter release (Bruchas et al., 2010). We analyzed *in silico* the −1.4 kb 5'-flanking region of mouse *Kiss1* promoter using Jaspar database and detected three potential cAMP response elements (5'TGACGTCA3') at -687, -717 and -1138 bp from exon I. Since KOR activation decreases cAMP production, one may speculate that dynorphin could decrease cAMP-induced, CRE-mediated *Kiss1* promoter activity and therefore induces down-regulation of *Kiss1* expression. Our results suggest then that at the end of the prooestrus phase, stress-related GC levels would stimulate *Pdyn* expression leading to lower *Kiss1* secretion and prevent the preovulatory GnRH/LH surge.

E2 and GC crosstalk has already been reported with one receptor-mediated signaling pathway impeding the other one (Cvoro et al., 2011; Karmakar et al., 2013). For instance, GR interacts with ER to destabilize the ER-Steroid Receptor Coactivator-3 complex leading to ER activity repression in breast cancer cells (Karmakar et al., 2013). Conversely, an

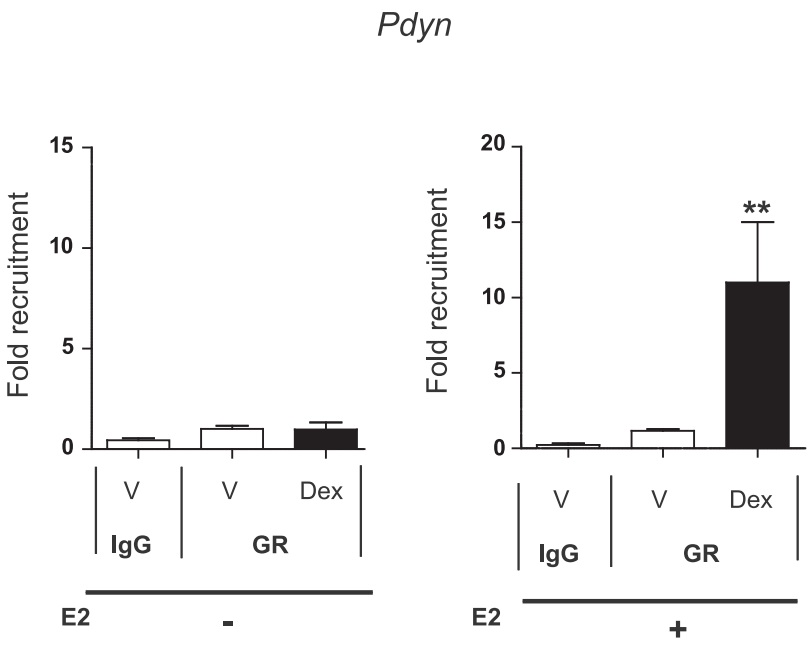


Fig. 5. E2 priming is required for Dex-induced GR recruitment on *Pdyn* promoter.

GT1-7 cells, pretreated or not with 100 nM E2, were treated for 1 h with 100 nM Dex or ethanol (V), fixed and lysed. ChIP assays were performed on sheared chromatin extracts using GR antibody, or unrelated rabbit IgG antibody as a negative control. Immunoprecipitated and eluted DNA fragments were analyzed by qPCR using primer pair encompassing genomic sequence containing GREs within the proximal Pdyn gene (see sequences in Supplemental Tables). Histograms represent the fold induction of GR enrichment compared to vehicle condition, arbitrarily set at 1, and are medians ± IQR of three independent experiments performed in triplicates. Statistical difference is indicated as compared to vehicle condition for Dex-induced cells treated (non-parametric ANOVA Kruskal-Wallis tests followed by post-test of Dunn, $^{**}P < 0.01$).

inhibitory effect of ER was also described on GR repressing proinflammatory genes expression in human osteosarcoma cells (Cvoro et al., 2011). In our study, we observe a positive effect of E2 on GR signaling in GT1-7 cells that occurs under specific conditions, notably the requirement of high E2 concentrations. Indeed, 0.1 nM E2 pretreatment (corresponding to the low E2 concentration prevailing during the luteal phase (Chu et al., 2009)) is inefficient in enabling GC-induced *Pdyn* expression, indicating that GC may stimulate *Pdyn* expression at a specific period of the ovulatory cycle. Besides, upon priming with high doses of E2, up-regulation of *Pdyn* expression was solely observed at a threshold GC concentration only found under specific situations *e.g.* stress or pathological hypercorticism.

E2 exposure does not affect GR mRNA or protein levels in GT1-7 cells. Unliganded GR activation has been already described (Galliher-Beckley et al., 2011), therefore one could speculate that E2 could induce GR phosphorylation and then promotes GR activation. Nonetheless, as shown in Fig. 1, basal GR activity observed on basal *Pdyn* levels remains unchanged independently of the presence of E2. The lack of effect of E2 on GR expression and activity was also described in female rat hippocampus during stress (Green et al., 2018). We went further to understand the molecular mechanism of ER and GR crosstalk by generating a 1.7-kb mouse *Pdyn* promoter construct. This proximal promoter contains three GREs and one AP-1 site on which GR could functionally interact with. Similarly, ER binds to consensus ERE or indirectly binds to chromatin through tethering to other transcription factors like AP-1. By using Jaspar database, we identify an ERE site far away from the transcription start site at -5.2-kb upstream of the first exon of mouse *Pdyn*. However, ER could potentially bind to the AP-1 site present within the proximal *Pdyn* promoter. We demonstrate that, in contrast to E2, GC are able to transactivate the proximal *Pdyn* promoter (luciferase assays), suggesting that GR may bind to *Pdyn* promoter on either GRE or/and AP-1 sites. ChIP experiments validate that GR is indeed recruited on *Pdyn* proximal promoter and especially on GRE containing regions. ChIP data also confirm that GR recruitment on *Pdyn* promoter solely occurs in presence of 100 nM E2, suggesting that E2 may facilitate GR DNA accessibility, probably after chromatin remodeling as already proposed for GR transcriptional regulations (Miranda et al., 2013; Voss et al., 2011). In these studies, GR was described to interact with ATP-dependent chromatin remodelers (SWI/SNF complexes) to trigger chromatin remodeling at specific sites, priming the chromatin landscape, facilitating accessibility of a second binding factor (Voss et al., 2011). Hence, the relative discrepancy between the significant positive effect of Dex alone on *Pdyn* promoter-driven luciferase activity and the E2 requirement for Dex-induced GR recruitment on *Pdyn* promoter (ChIP experiments) reveals that E2 may promote chromatin remodeling to increase the odds of GR to bind GRE. ER has been shown to recruit more than one remodeling complex to response elements upon activation (Miranda et al., 2013) and therefore could also dictate GR binding. In addition, genome wide sequencing and analysis of global changes in chromatin structure have shown that GR binding may be determined by an “assisted loading” mechanisms (Miranda et al., 2013) which lead to rapid reprogramming of the chromatin structure during a transient window by which GR could access to GRE previously unavailable (Miranda et al., 2013). Understanding how E2 modulates cellular responses of GC should give general insights into functional steroid receptor crosstalk.

5. Conclusions

We identify dynorphin as an important inhibitory neuropeptide induced by hypercorticism. Using an *ex vivo* mouse brain model and a hypothalamic cell line, we demonstrate that GC induce *Pdyn* up-regulation, exclusively after E2 priming. Our work converges with others to propose that HPG axis is specifically receptive to stress at the end of the prooestrus phase when positive E2 feedback signals induce GnRH/LH surge. During this critical period, elevated GC levels would stimulate *Pdyn* expression which in turn leads to *Kiss1* down-regulation and GnRH/LH release suppression. At the molecular level, we describe a novel crosstalk between E2 and GC signaling pathways where E2 exposure may unmask GRE binding sites within *Pdyn* promoter to enable GR DNA accessibility. Future challenges should clarify the exact role of dynorphin on preovulatory GnRH/LH surge and investigate its potential implication in other reproductive dysfunctions (sexual behavior, pregnancy rate and litter size) occurring during stress. A better understanding of molecular mechanisms by which GC modulate HHG axis will facilitate management of stress-related infertility.

Declarations of interest

The authors hereby affirm that they have no competing or conflicts of interests.

Author contributions

S. Mhaouty-Kodja, M. Lombès and S. Chauvin designed research; M. Ayrout, F. Le Billan, M. Lombès and S. Chauvin analyzed data; M. Ayrout, F. Le Billan, V. Grange-Messent, S. Mhaouty-Kodja and S. Chauvin performed research; M. Ayrout, M. Lombès and S. Chauvin wrote the paper; all authors corrected and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.08.034.

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