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

Mohsen Ayrout[a], Florian Le Billan[a], Valérie Grange-Messent[b], Sakina Mhaouty-Kodja[b], Marc Lombès[a], Stéphanie Chauvin[a,*]

[a] INSERM UMR_S1185, Fac Med Paris Sud, Université Paris-Saclay, 94276 Le Kremlin Bicêtre, France
[b] Sorbonne Université, CNRS, INSERM, Neuroscience Paris Seine – Institut de Biologie Paris Seine, 75005 Paris, France

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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

* Corresponding author: INSERM U1185 – Faculté de Médecine Paris Sud, 63, rue Gabriel Péri, 94276 Le Kremlin-Bicêtre Cedex, France.
E-mail address: stephanie.chauvin@inserm.fr (S. Chauvin).

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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