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Transcriptional interaction between cFOS and the homeodomain-binding transcription factor VAX1 on the GnRH promoter controls *Gnrh1* expression levels in a GnRH neuron maturation specific manner

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is required for pubertal onset and reproduction, thus the control of GnRH transcription is tightly regulated during development and adulthood. GnRH neuron development depends on transcription factors of the homeodomain family. For example, Ventral anterior homeobox 1 (*Vax1*) is necessary to maintain GnRH expression after embryonic day 13 in the mouse. To further our understanding of the mechanisms by which VAX1 regulates GnRH gene expression, we asked whether VAX1 interacts with other transcription factors to modify GnRH expression levels. Using the GnRH cell lines, GN11 and GT1-7, we found that activation of PKC enhances expression of the immediate early gene *cFos* in both GN11, and GT1-7, and represses expression of *Vax1* in GT1-7. Further, VAX1 interacts with cFOS while bound to the GnRH promoter. In immature GN11 cells, VAX1 and cFOS enhance GnRH expression, whereas VAX1 and cFOS have a repressive role in the mature GT1-7 cells.

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1. Introduction

Pubertal onset and fertility both depend on proper developmental migration and maturation of gonadotropin-releasing hormone (GnRH) neurons, with improper GnRH neuron development or regulation impairing pubertal onset and fertility (Herbison et al., 2008; Mason et al., 1986). Despite the critical role of GnRH in reproduction, we still have gaps in our understanding of the transcriptional program involved in GnRH neuron function (Balasubramanian et al., 2010; Stamou et al., 2015). The slow advances in the field are in great part due to challenges associated with the *in vivo* study of GnRH neurons, including a long GnRH neuron migration path, the scattered localization of GnRH neurons throughout the anterior hypothalamic area, the sparsity of this cell population (represented by approximately 800 GnRH neurons in the mouse), and the long projections GnRH neurons send from the preoptic area to the median eminence, where they release GnRH in a pulsatile fashion (Fueshko and Wray, 1994; Messina et al., 2016; Tarozzo et al., 1994).

Most of our current knowledge of GnRH neuron development and control of *Gnrh1* expression comes from the use of well-established immortalized mouse GnRH neuron cell lines, represented by the immature, migratory GN11 cells (Radovick et al., 1990), and the mature, non-migratory GT1-7 cells (Mellon et al., 1990). Indeed, these cell lines have helped identify novel transcription factors of the homeodomain binding family that are required for normal GnRH neuron development and GnRH expression, including Orthodenticle homeobox 2 (*Otx2*) (Diaczok et al., 2011; Kelley et al., 2000; Larder et al., 2013), Ventral anterior homeobox 1 (*Vax1*) (Hoffmann et al., 2014, 2016), Sine oculis-related homeobox (*Six*) 3 (unpublished data) and *Six6* (Larder et al., 2011). In addition, GN11 and GT1-7 cell lines have repeatedly proven to be a valid *in vitro* model recapitulating many characteristics of immature and mature GnRH neurons respectively, allowing for advances in our understanding of factors important for GnRH neuron maturation, and *Gnrh1* expression (Cariboni et al., 2007; Givens et al., 2005; Glidewell-Kenney et al., 2013; Magni et al., 2007; Tang et al., 2005).

Despite the great advances in the field of GnRH neuron development, it is still unclear what combination of transcription factors and input from the surrounding environment are required to specify and fine-tune *Gnrh1* expression. The significance of

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Abbreviations

AP1	Activator protein-1
BSA	Bovine serum albumin
cFos	FBJ osteosarcoma oncogene
cJun	jun proto-oncogene
Dlx	Distal-less homeobox
DMSO	Dimethylsulfoxide
E	Embryonic day
Fgf	Fibroblast growth factor
FGFR	Fgf receptor
GnRH	Gonadotropin-releasing hormone
H2afz	H2A histone family, member Z
IEG	Immediate early gene
KO	knock-out
LH	Luteinizing hormone
Otx2	Orthodenticle homeobox 2
PKC	protein kinase C
Ppia	peptidylprolyl isomerase A
qRT-PCR	Quantitative real-time PCR
SRF	serum response factor
Six	Sine oculis-related homeobox
TAC	Tachykinin receptor
TPA	Phorbol 12-myristate 13-acetate
Vax1	Ventral anterior homeobox 1

homeodomain transcription factors in fertility is starting to be appreciated, where knock-out (KO) of *Six6* leads to loss of GnRH neurons during late development (Larder et al., 2011), and loss of *Six3*, a SIX homeodomain transcription factor related to SIX6, is required for the development of the olfactory system and proper GnRH neuron migration (unpublished). In addition, *Vax1*, a transcription factor not detected in the immature GN11 cells and highly expressed in the mature GT1-7 cells, is necessary to maintain GnRH expression after embryonic day 13 (E13) in the mouse (Hoffmann and Mellon, 2016; Hoffmann et al., 2016). This suggests that the onset of expression of *Vax1* in GnRH neurons during development is critical in GnRH transcription, and that the transcriptional program of VAX1 is required for appropriately adjusting GnRH expression during development.

Interestingly, a physical interaction between developmental homeodomain transcription factors and immediate early genes (IEG), such as members of the activating protein-1 (AP1) family, has been found to regulate transcriptional activity by either enhancing or silencing transcriptional activity of these IEG-homeodomain transcription factor complexes (Jeong et al., 2004; Kessel et al., 1988; Schaefer et al., 2001). The idea that IEGs and homeodomain transcription factors interact and form transcriptionally active complexes could be important from a developmental and cellular maturation standpoint, allowing specific expression of key genes to unique cell populations (Jeong et al., 2004). Indeed, during development, the level, duration and timing of transcription factors are imperative for cellular specification and embryogenesis (Briscoe and Small, 2015; Kicheva and Briscoe, 2015; Lewis, 2008). The rapid expression of an IEG, such as FBJ osteosarcoma oncogene (cFOS) or Jun proto-oncogene (cJUN), could thus regulate transcriptional activity of homeodomain transcription factors during a very short developmental time-frame. Further, cFOS is strongly induced in mature GnRH neurons in response to the phorbol esther, phorbol 12-myristate 13-acetate (TPA), which activates protein kinase C (PKC), leading to recruitment of cFOS to the GnRH regulatory region, and repression of *Gnrh1* expression (Glidewell-Kenney et al., 2013, 2014). The neurokinin 3 receptor, a receptor critical in fertility (Balasubramanian et al., 2010; Topaloglu et al., 2009; Yang and Seminara, 2012), also represses *Gnrh1* expression through cFOS. The pathway induced by activation of the neurokinin 3 receptor leads to induction of PKC, and recruitment of transcription factors, including serum response factor (SRF), and Elk-1, which in their turn regulate *cFos*, leading to a repression of *Gnrh1* expression. Based on the importance of IEG's, and particularly cFOS in the regulation of *Gnrh1*, we asked whether cFOS could modulate the level of GnRH expression during GnRH neuron maturation, through interaction with homeodomain transcription factors.

2. Material and methods

2.1. Cell culture

GT1-7 (Mellon et al., 1990), GN11 (kindly provided by Sally Radovick), and αT3-1 (Windle et al., 1990) cell lines were cultured in DMEM (Mediatech Inc., Herndon, VA), containing, 10% fetal bovine serum (Gemini Bio, West Sacramento, CA), and 1x penicillin-streptomycin (Life Technologies, Inc./Invitrogen, Grand Island, NY) in a humidified 5% CO_2 incubator at 37 °C. For luciferase assays GN11 and GT1-7 cells were seeded into 24-well plates (Nunc, Roskilde, Denmark) at 75,000 and 200,000 per well, respectively. Cells transfected for quantitative real time PCR (qRT-PCR) were plated into 10 cm dishes (Nunc) at 3 million (GT1-7) and 1 million (GN11, NIH3T3) cells per dish. O/N transfection of cells was done approximately 21 h after plating. At the time of phorbol 12-myristate13-acetate 100 nM (TPA, Tocris Bioscience, dissolved in dimethylsulfoxide) or DMSO (1/2000; Sigma) treatment, media was changed to DMEM containing 0.1% BSA. To determine the impact of the 21 h TPA 100 nM treatment on cell morphology, live GN11 and GT1-7 cells were visualized using the Keyence BZ-X700 Microscope (Keyence, Laguna Hills, CA). To increase the visibility of the cells, adjustments of brightness, contrast, and color balance were done with Image J (National Institutes of Health, Bethesda) and applied to the entire image.

2.2. Quantitative real-time PCR

Untreated cells, or cells treated with DMSO or TPA 100 nM were harvested in TRIzol® (Invitrogen) and total RNA from GT1-7, GN11 and αT3 cells extracted according to manufacturer's recommendations. DNA was eliminated by the use of the DNA-free™ kit (Applied Biosystems, Foster City, CA), whereas cDNA was obtained by reverse transcription of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA products were detected using iQ SYBR Green Supermix (BioRad) on the CFX Connect real-time detection system (Bio-Rad). Primers to the coding sequences for mouse *Otx2* (F: TCGAAGAGCTAAGTGCCGCC, R: GCAATGGTTGGGACTGAGG), *cJun* (F: GTCCCCTATCGACATGGAGTCT, R: GAGTTTTGCGCTTTCAAGGTTT), *cFos* (F GGCAAAGTAGAGCAGCTATCTCCT, R: TCAGCTCCCTCCTCCGATTC), *Six6* (F: TCGATGTTCCAGCTGCCCAT, R: TGGAAAGCCACGATGGCTCT), *Gnrh1* (F: TGCTGACTGTGTGTTTGGAAGGCT, R: TTTGATCCACCTCCTTGCGACTCA), *Vax1* (F: CCGGATCCTAGTCCGAGATGCC, R: TCTCCCGGCCCACCACGTAT), *Ppia* (F: AAGTTCCAAAGACAGCAGAAAAC, R: CTCAAATTTCTCTCCGTAGATGG), *H2afz* (F: TCACCGCAGAGGTACTTGAG, R: GATGTGTGGGATGACACCA) synthesized by

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