Neuroscience 303 (2015) 312–322

MICROARRAY ANALYSIS OF NEONATAL RAT ANTEROVENTRAL PERIVENTRICULAR TRANSCRIPTOMES IDENTIFIES THE PROAPOPTOTIC CUGBP2 GENE AS SEX-SPECIFIC AND REGULATED BY ESTRADIOL

J. DEL PINO SANS,[a†] S. KRISHNAN,[a] L. K. AGGISON,[a] H. L. ADAMS,[c] M. M. SHRIKANT,[b] F. LÓPEZ-GIRÁLDEZ[b] AND S. L. PETERSEN[a*]

[a] *Department of Veterinary and Animal Sciences, 661 North Pleasant Street, University of Massachusetts Amherst, Amherst, MA 01003, United States*

[b] *Yale Center for Genome Analysis, Yale University, Orange, CT, United States*

[c] *Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Amherst, MA 01003, United States*

Abstract—Sexually dimorphic neural structures regulate numerous gender-specific functions including luteinizing hormone (LH) release patterns. The female cyclic surge pattern of release is controlled by the anteroventral periventricular nucleus (AVPV), a preoptic area (POA) region that is significantly smaller in males. The prevailing hypothesis used to explain these differences in structure and function is that a ''default'' feminine AVPV is defeminized by exposure to estradiol (E_2), a metabolite of testosterone (T) produced by the perinatal testes. E_2 exposure then culminates in apoptosis in the male AVPV, but the upstream pathways are poorly understood. To address this issue, we compared AVPV transcriptomes of postnatal day 2 (PND2) males and females with those of females treated with E_2 or vehicle. Only six of 89 sex-specific genes were also regulated by E_2 in the PND2 AVPV and E_2 regulated over 280 genes not found to be sex-specific. Of targets that changed similarly in males and E_2-treated females, the gene encoding CUG triplet repeat, RNA-binding protein 2 (Cugbp2), a proapoptotic protein, showed the highest fold-changes. Quantitative polymerase chain reaction (QPCR) studies confirmed higher mRNA levels in PND2 male and E_2-treated female AVPVs wherein E_2 induces apoptosis. POA mapping studies detected Cugbp2 mRNA in the AVPV and in the sexually dimorphic nucleus of the POA (SDN–POA); however, sex differences and E_2 effects occurred only in the AVPV. Combined with evidence that Cugbp2 regulates splicing and translation of mRNAs linked to sexual differentiation, we propose that this gene mediates E_2-dependent effects on AVPV defeminization.

Key words: sexual differentiation, anteroventral periventricular nucleus, estradiol, transcriptome, luteinizing hormone.

INTRODUCTION

Sex-dependent neural circuits control a wide range of physiological functions that can be disrupted by inappropriate activation or inhibition of androgen receptors (ARs) or estrogen receptors (ERs) during development. The neural control of luteinizing hormone (LH) release is one such sex-specific function and is critical for fertility. Ovulation requires a cyclic release pattern that culminates in the preovulatory surge of LH release, whereas testicular function is maintained by a tonic release pattern characterized by a higher LH pulse frequency than is seen in females (Foecking et al., 2005). If female rodents, sheep or non-human primates are exposed perinatally to testosterone (T), they develop polycystic ovaries and fail to ovulate (Barraclough, 1961; Herbosa et al., 1996; Masek et al., 1999). Moreover, perinatal androgen exposure in females increases LH pulse frequency in adulthood (Foecking and Levine, 2005). Women exposed to elevated levels of T during development may also have an increased risk of polycystic ovary syndrome (PCOS) (Abbott et al., 2005). Similarly, it has been suggested that developmental exposure to certain environmental chemicals may predispose women to the disease (Fernandez et al., 2010). However, it is difficult to unequivocally link PCOS with developmental exposure to T or environmental contaminants without understanding the mechanisms underlying defeminization of LH release patterns.

The neural site in which T acts to defeminize LH release patterns in rodent models is likely the anteroventral periventricular nucleus (AVPV), a preoptic cell group in which estradiol (E_2) acts to trigger LH

*Corresponding author. Address: Department of Veterinary and Animal Sciences, Life Science Laboratories N231, University of Massachusetts Amherst, Amherst, MA 01003, United States.
E-mail address: spetersen@vasci.umass.edu (S. L. Petersen).
† Current address: Toxicology and Pharmacology Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, Madrid 28040, Spain.

Abbreviations: AR, androgen receptor; AVPV, anteroventral periventricular nucleus; Cox2, cyclooxygenase 2; Cugbp2, CUG triplet repeat, RNA-binding protein 2; DAVID, Database for Annotation, Visualization and Integrated Discovery; E_2, estradiol; ER, estrogen receptor; ISHH, *In situ* hybridization histochemical; LH, luteinizing hormone; Myh7B, myosin heavy chain 7B; Nr1, NMDA receptor subunit 1; PCOS, polycystic ovary syndrome; PGE2, prostaglandin E2; PND2, postnatal day 2; POA, preoptic area; QPCR, quantitative polymerase chain reaction; SDN, sexually dimorphic nucleus; T, testosterone.

http://dx.doi.org/10.1016/j.neuroscience.2015.07.008
0306-4522/

surge release (Wiegand et al., 1980; Wiegand and Terasawa, 1982; Ronnekleiv and Kelly, 1986; Petersen and Barraclough, 1989). The AVPV is an ER-rich structure with nearly three times as many cells in females as in males (Forger, 2009). In females, most of these cells are dual-phenotype GABA/glutamate neurons that contain virtually all ER in the AVPV (Ottem et al., 2004). Some AVPV GABAergic neurons also express kisspeptin 1 (Petersen et al., 2012) and these neurons directly regulate GnRH neuronal excitability important for the LH surge (Liu et al., 2011). Importantly, perinatal T exposure that produces anovulatory syndrome also decreases the number of AVPV GABA/glutamate neurons in adult females (Petersen et al., 2012).

It is generally held that sexual differentiation of the brain requires local conversion of T to E_2 by aromatase, and it is E_2 that masculinizes and defeminizes neuroendocrine structures (Roselli et al., 2009; Semaan and Kauffman, 2010). This idea is consistent with evidence that developmental exposure to E_2 upregulates *bax* (proapoptotic molecule) and downregulates *bcl2* (anti-apoptotic molecule) to defeminize the male AVPV (Tsukahara et al., 2008; Forger, 2009). Consequently, the male AVPV is significantly smaller than that of the female (Forger et al., 2004) and cannot support the cyclic surge pattern of LH release necessary for ovulation (Petersen et al., 2012). Although it has been clear for decades that early exposure to gonadal hormones produces sexual dimorphism of AVPV and determines adult LH release patterns (Petersen et al., 2012), the underlying mechanisms remain poorly understood.

To address this issue, we used whole genome arrays to identify sex-specific genes and pathways that were regulated by E_2 in a male-specific expression pattern. We reasoned that if the male AVPV forms as a result of E_2-dependent processes, genes important for defeminization of the nucleus would be similarly regulated in males and E_2-treated females. Our bioinformatics analyses identified a small subset of genes that fulfilled this criterion. *CUG triplet repeat, RNA-binding protein 2* (*cugbp2*) emerged as the most compelling target because it regulates splicing or translation of a number of mRNAs that encode proteins previously shown to play a role in sexual differentiation of the brain. The importance of Cugbp2 notwithstanding, most male-specific genes and pathways in the developing AVPV were not similarly regulated by E_2. Conversely, E_2 regulated a number of genes and pathways not identified as sex-dependent. Thus, AVPV defeminization in the male is not likely achieved simply by conversion of a ''default'' feminine nucleus to a masculine nucleus by exposing it to E_2 during development.

EXPERIMENTAL PROCEDURES

Animals

Pregnant Holtzman Sprague–Dawley rats (Harlan Sprague–Dawley, Madison, WI, USA) were individually housed and maintained in a temperature- and lighting-controlled room (14:10 light:dark cycle; lights on 0500 h) with food and water available *ad libitum.* Litters were culled to eight pups/litter on the day of birth (postnatal day 0; PND0).

Microarray identification of sex-specific genes regulated by E_2

Affymetrix microarray analysis. For microarray analysis, experimental groups were: (a) males and females (n = 12/group) treated with sesame oil vehicle, and (b) females treated with 10 ng E_2 benzoate or oil vehicle (n = 12/group). Injection volumes were 0.1 ml and injection sites were sealed immediately with Scotch® Super Glue (3M; St Paul MN). Pups were treated on PND1 and killed on PND2, 18 h after treatment, a time interval that allows detection of both up- and down-regulated targets.

For each treatment group, we used three-four pools of RNA extracted from AVPV microdissections of three animals representing three different litters. The quality of total RNA was evaluated by A260/A280 ratio (found to be at least 1.8 for each sample) using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and by the RNA Integrity Number (RIN > 8.0) using a 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Affymetrix Rat 230 2.0 arrays containing over 31,000 reporters (probe set) (Affymetrix, Santa Clara, CA, USA) were used for gene expression analysis of AVPV tissue pools: four male control samples, four female controls, three vehicle-treated females and three E_2-treated females. Preparation of labeled cRNA for hybridization onto Affymetrix GeneChips followed the recommended Affymetrix protocol.

The Yale Center for the NIH Neuroscience Microarray Consortium carried out microarray analysis. Double-stranded cDNA was synthesized from 1 to 5 μg of total RNA using a Superscript Choice System (Life Technologies, Carlsbad, CA, USA), with an HPLC-purified oligo (dT) primer containing a T7 RNA polymerase promoter sequence at the 5′-end (Proligo LLC, Boulder, CO, USA). The second cDNA strand was synthesized using *E. Coli* DNA polymerase I, RNase H and DNA ligase. Labeled cRNA was generated from cDNA by *in vitro* transcription using a GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA) following the manufacturer's instructions and incorporating biotinylated synthetic analog as pseudouridine reagent, a reaction mediated by MEGAscript T7 RNA Polymerase (Life Technologies, Carlsbad, CA). Biotin-labeled cRNA was purified using GeneChip cleanup module (Affymetrix, Santa Clara, CA) prior to fragmenting to a size of 35–200 bases by incubating at 94 °C for 35 min in fragmentation buffer. Fragmented cRNA was hybridized to the arrays for 16 h at 45 °C.

After hybridization, arrays were washed using an Affymetrix fluidics station and stained with streptavidin–phycoerythrin (10 μg/ml; Life Technologies, Carlsbad, CA). Washed arrays were inspected for hybridization artifacts and then scanned with an Affymetrix GeneChip®Scanner 3000. Images were analyzed using Affymetrix Microarray Suite 5.0, scaling to a target