Molecular and Cellular Endocrinology 419 (2016) 102–112

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Testing the critical window of estradiol replacement on gene expression of vasopressin, oxytocin, and their receptors, in the hypothalamus of aging female rats

Alexandra N. Garcia [a], Christina K. Depena [b], Weiling Yin [b], Andrea C. Gore [a, b, *]

[a] Department of Psychology, The University of Texas at Austin, Austin, TX 78712, USA
[b] Division of Pharmacology and Toxicology, The University of Texas at Austin, Austin, TX 78712, USA

ARTICLE INFO

Article history:
Received 9 June 2015
Received in revised form
2 October 2015
Accepted 2 October 2015
Available online 8 October 2015

Keywords:
Vasopressin
Oxytocin
Menopause
Estradiol
Paraventricular nucleus
Supraoptic nucleus

ABSTRACT

The current study tested the "critical window" hypothesis of menopause that postulates that the timing and duration of hormone treatment determine their potential outcomes. Our focus was genes in the rat hypothalamus involved in social and affiliative behaviors that change with aging and/or estradiol (E_2): *Avp*, *Avpr1a*, *Oxt*, *Oxtr*, and *Esr2* in the paraventricular nucleus (PVN) and supraoptic nucleus (SON). Rats were reproductively mature or aging adults, ovariectomized, given E_2 or vehicle treatment of different durations, with or without a post-ovariectomy delay. Our hypothesis was that age-related changes in gene expression are mitigated by E_2 treatments. Contrary to this, PVN *Oxtr* increased with E_2, and *Avpr1a* increased with age. In the SON, *Avpr1a* increased with age, Oxtr with age and timing, and *Avp* was altered by duration. Thus, chronological age and E_2 have independent actions on gene expression, with the "critical window" hypothesis supported by the observed timing and duration effects.

1. Introduction

Menopause is the physiological cessation of ovarian function during which menstrual cyclicity ceases and concentrations of ovarian hormones such as estrogens and progesterone decline. Hormone replacement therapy (HRT) is currently the most commonly used therapy to treat menopausal symptoms. However, since the termination of the Women's Health Initiative (WHI) study of conjugated equine estrogens and medroxyprogesterone acetate treatment, many women have been left asking questions about whether, when, and for how long to take hormone treatments for menopausal symptoms. The WHI initially reported that there was a small but significant increase in adverse incidents in women taking hormone therapy (Rossouw et al., 2002; Manson et al., 2013). Reanalysis suggested that there might be a critical window post-menopause during which hormone treatment is beneficial to neurobiological and other health-related endpoints in women (Klaiber et al., 2005; Bhupathiraju and Manson, 2014). Therefore, it is necessary to understand the risks and benefits of estrogen treatments in the context of timing and duration for women to be able to make informed decisions and to improve quality of life.

In addition to hot flashes, neurobehavioral changes such as anxiety and depression increase at menopause in humans, which can lead to social isolation and impact the quality of life (Uguz et al., 2011; Deeks and McCabe, 2004; Lanza di Scalea et al., 2012). Oxytocin (*Oxt*) and vasopressin (*Avp*) are modulators of these behaviors in mammalian species [De Kloet et al., 2005 (mice); Klenerova et al., 2009 (rat); Meyer-Lindenberg et al., 2011 (human); Lim and Young, 2006 (voles)]. Both of these neurohypophyseal peptides are synthesized in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus.

Vasopressin and oxytocin neurons are regulated by estrogens, predominantly through the estrogen receptor beta (ERβ) that is the dominant ER in these regions, as demonstrated in knockout mice (Winslow and Insel, 2004) and by gene and protein expression in rats (Hrabovszky et al., 1998). ERβ protein and mRNA are also colocalized with oxytocin- and to a lesser extent with vasopressin–expressing neurons of rats and mice (Hrabovszky et al., 1998; Alves et al., 1998; Patisaul et al., 2003). However, the effects of estradiol on the regulation of vasopressin and oxytocin are mixed, and most studies utilized short-term treatment regimes.

* Corresponding author. Division of Pharmacology and Toxicology, The University of Texas at Austin, 107 W. Dean Keeton, C0875, Austin, TX 78712, USA.
E-mail address: andrea.gore@austin.utexas.edu (A.C. Gore).

http://dx.doi.org/10.1016/j.mce.2015.10.004
0303-7207/

Some studies showed up-regulation [Roy et al., 1999 (mRNA, monkey); Patisaul et al., 2003 (mRNA & protein, mouse)], others down-regulation [Shughrue et al., 2002 (protein, rat); Van Tol et al., 1988 (mRNA, rat); Nomura et al., 2002 (mRNA & protein, mouse)], and still others no effect [Peter et al., 1990 (mRNA, rat); Rhodes et al., 1981 (protein, rat); Akaishi and Sakuma, 1985 (protein, rat)] of estradiol treatment. This inconsistency in results was part of our motivation for conducting the work, and to hypothesize that the effects of estrogen would be influenced by different timings and durations of treatment.

Along with oxytocin and vasopressin, we also selected *Oxtr* and *Avpr1a* gene expression as endpoints because of their roles in mediating effects of their respective nonapeptides on social behaviors such as anxiety and depression (Young, 1999; Bielsky et al., 2004; Sala et al., 2011). Previous studies using RT-PCR, *in situ* hybridization, immunohistochemistry and electrophysiology have shown that mRNA and protein of the vasopressin receptor 1a and the oxytocin receptor are expressed in the SON and PVN of rats (Hurbin et al., 1998, 2002; Gouzènes et al., 1999; Yoshimura et al., 1993). This study was designed to help fill the gap in research and to gain mechanistic insight into the molecular changes that occur during aging and in response to differential modes of treatment to assess the effects of timing and duration of E_2. By studying outcomes of *Avp*, *Avpr1a*, *Oxt*, *Oxtr* and *Esr2* in the PVN and SON of the hypothalamus, we can have a better understanding of the neural substrates involved in social behavior as a basis for future work testing the "critical window" hypothesis on the behaviors themselves.

2. Materials and methods

2.1. Animals and husbandry

All animal procedures were conducted in accordance with The Guide for the Care and Use of Experimental Animals following protocols approved by The University of Austin IACUC committee and NIH standards. Female Sprague Dawley rats (Harlan) were purchased as reproductively mature adults (MAT, ~3 months, sexually naïve) or aging adults (AG, ~11 months, retired breeders). These animals are the same as those used in a separate study (Yin et al., 2015) for analyses of different brain regions. Upon arrival, rats were pair housed on a 12-h light, 12-h dark cycle (lights on at 0700) and received water and food ad libitum. They were allowed to acclimate to the room for two weeks prior to surgery during which time estrous cyclicity was monitored daily by vaginal lavage of sterile saline. Only females with regular 4–5 day cycles were used. All rats received ovariectomy surgery under isofluorane inhalation anesthesia. A single injection of Rimadyl (5 mg/kg) was given at the start of surgery. Bilateral dorsolateral incisions were made through the skin, muscle, and peritoneum, and the ovaries were ligated and removed. Muscles were sutured and wound clips used to close the skin.

Animals were randomly assigned to one of eight treatment groups as illustrated in Fig. 1 to test different timings and durations of hormone treatment based on those used in the WHI, in which the women experienced an average 12-year delay in treatment relative to the last menstrual period. We calculated based upon the rats' life cycle compared to humans, that 3 months to an adult rat is equal to about 5 years in a woman (Sengupta, 2013; Quinn, 2005). Capsules containing either 100% cholesterol (Veh) or 5% 17β-estradiol/95% cholesterol (E_2) were implanted subcutaneously between the shoulder blades at the time of surgery. Because the aim of this study was to address and reevaluate limitations of the WHI, we chose to use continuous exposure to E_2 at a physiologically relevant dosage to mimic the type of treatment given in the Estrogen-alone WHI studies (The Women's Health Initiative Study Group, 1998). This treatment regime is clinically relevant, as continuous E2 treatments are still in common use for subsets of postmenopausal women.

2.2. Tissue processing

Rats were euthanized 3 or 6 months after OVX and hormone treatment, when mature adults (MAT) were ~7 months, and aging adults (AG) were ~14 or ~17 months of age. All animals were weighed and euthanized by rapid decapitation starting at 1330 h with the last animal killed not later than 1600 h; therefore they were all killed during the lights on period. Although there is a diurnal rhythm of vasopressin gene expression in the SCN, previous studies have shown that within the SON and PVN, the areas used in the present study, there is no diurnal change in vasopressin or oxytocin gene expression in rats (Uhl and Reppert, 1986; Burbach et al., 1988). In addition, when we used time of euthanasia as a covariate in statistical analyses, no time of day effect was found. Brains were removed and sectioned in 1-mm coronal sections using an ice-cold stainless steel brain matrix. Sections were placed into cryogenic storage vials that contained RNAlater (Life technologies, Grand Island, NY). Tubes were stored at 4 °C overnight, then mounted onto chilled slides and placed in a −20 °C freezer for storage. Using Palkovits punches and the Paxinos and Watson (2009) rat brain atlas (all coordinates are based on that atlas), bilateral micropunches were taken under a dissecting microscope. The PVN punch (1.22 mm diameter) began rostrally at ~Bregma = −0.84 mm, and extended caudally 1 mm. The SON punches (0.96 mm diameter) started rostrally at ~Bregma = −0.60 mm and extended caudally for 1 mm. The choice of sampling areas was based on previous studies showing large concentrations of magnocellular neurons containing both vasopressin and oxytocin protein in rats at this range of coordinates (Rhodes et al., 1981; Swanson and Sawchenko, 1983). The PVN has several subdivisions (Swanson and Sawchenko, 1983), and the location of our punches includes the part that lies ventromedial to the descending column of the fornix between Bregma = −1.44 to −1.56, and the lateral magnocellar subdivision (Krieg, 1932; Swanson and Sawchenko, 1983) from Bregma = −1.72 to −1.92. Punches for each region were placed in a frozen Eppendorf tube for storage at −80 °C. At the time of decapitation trunk blood was collected and allowed to clot, serum was separated and centrifuged (2300× g for 5 min) then stored in Eppendorf tubes at −80 °C.

2.3. Real-time PCR assays

RNA was extracted from frozen PVN and SON punches using an Allprep RNeasy mini kit (Qiagen, Valencia, California), according to the manufacturer's protocol. Although we started with n = 10 rats per group, loss of some tissues during freezer storage caused some attrition (a subset of samples were frozen with too much liquid RNAlater that made it difficult to visualize brain landmarks; only samples were included with which we were confident in the landmarks), resulting in n = 7 for most gene assays. After extraction the quality of RNA was checked on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and all RIN values fell in the range of 8.6–9.9. The quantity of RNA was measured using a GloMax-Multi Detection System (Promega, Madison, WI), and ranged from 71 to 741 ng/μl in SON, and from 207 to 1550 ng/μl in the PVN. Using a high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY), mRNA (PVN 200 ng, SON 150 ng) was converted to single-stranded cDNA. Samples were stored at −20 °C until use. Quantitative real-time PCR was used to analyze 5 genes in the PVN and 4 genes in the SON: *Avp*, *Oxt*, *Avpr1a*, *Oxtr*, *Esr2* (FAM) in tandem with *Gapdh* (VIC). *Esr2* could not be run in SON due to the smaller