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SEX- AND AGE-SPECIFIC DIFFERENCES IN RELAXIN FAMILY PEPTIDE RECEPTOR EXPRESSION WITHIN THE HIPPOCAMPUS AND AMYGDALA IN RATS

K. L. MEADOWS AND E. M. BYRNES *

Tufts University Cummings School of Veterinary Medicine, 200 Westboro Road, Grafton, MA 01536, United States

Abstract—Relaxin is an essential pregnancy-related hormone with broad peripheral effects mediated by activation of relaxin-like family peptide 1 receptors (RXFP1). More recent studies suggest an additional role for relaxin as a neuropeptide, with RXFP1 receptors expressed in numerous brain regions. Neurons in an area of the brainstem known as the nucleus incertus (NI) produce relaxin 3 (RLN3), the most recently identified neuropeptide in the relaxin family. RLN3 has been shown to activate both RXFP1 and relaxin-like family peptide receptor 3 (RXFP3) receptor subtypes. Studies suggest wide-ranging neuromodulatory effects of both RXFP1 and RXFP3 activation, although to date the majority of studies have been conducted in young males. In the current study, we examined potential sex- and age-related changes in RLN3 gene expression in the NI as well as RXFP1 and RXFP3 gene expression in the dorsal hippocampus (HI), ventral hippocampus (vHI) and amygdala (AMYG) using young adult (9–12 weeks) and middle-aged (9–12 months) male and female rats. In addition, regional changes in RXFP1 and RXFP3 protein expression were examined in the CA1, CA2/CA3 and dentate gyrus (DG) as well as within basolateral (BLA), central (CeA), and medial (MeA) amygdaloid nuclei. In the NI, RLN3 showed an age-related decrease in males. In the HI, only the RXFP3 receptor showed an age-related change in gene expression, however, both receptor subtypes showed age-related changes in protein expression that were region specific. Additionally, while gene and protein expression of both receptors increased with age in AMYG, these effects were both region- and sex-specific. Finally, overall males displayed a greater number of cells that express the RXFP3 protein in all of the amygdaloid nuclei examined. Cognitive and emotional processes regulated by activity within the HI and AMYG are modulated by both sex and age. The vast majority of studies exploring the influence of sex on age-related changes in the HI and AMYG have focused on sex hormones, with few studies examining the role of neuropeptides. The current findings suggest that changes in relaxin family peptides may contribute to the significant sex differences observed in these brain regions as a function of aging.

Key words: aging, sex differences, relaxin, relaxin 3, amygdala, hippocampus.

INTRODUCTION

The relaxin-3 (RLN3) peptide is a highly conserved member of the insulin superfamily that is primarily expressed in the brain (Ma and Gundlach, 2007). Discovered in 2001, the largest cluster of RLN3 cell bodies are found in the nucleus incertus (NI) of the brainstem and the peptide is co-expressed in GABA neurons that project widely to the forebrain (Ma et al., 2007). The effects of RLN3 are mediated, at least in part, by activation at the relaxin-like family peptide receptor 3 (RXFP3) which is a G_i-protein-coupled receptor (Smith et al., 2011). Findings suggest that RLN3 can also activate relaxin-like family peptide receptor 1 (RXFP1) which is a G_s-protein-coupled receptor (Gundlach et al., 2009; Hossain et al., 2011). Histological studies indicate that both receptors are broadly expressed throughout the forebrain, including significant expression in both the hippocampus (HI) and amygdala (AMYG) (Tanaka, 2010). Functionally, a number of recent studies suggest that RLN3 may modulate a wide array of neural processes and behavioral outputs, particularly in response to changing environmental conditions (Bathgate et al., 2013). Indeed, it has been suggested that RLN3 may be part of a broad arousal network that serves to modulate/integrate physiological and behavioral responses to maintain homeostasis in the face of external demands (Smith et al., 2014b).

A number of homeostatic mechanisms become less effective with age (McEwen, 2000). Moreover, both sex and the experience of stress influence how aging impacts neural regulation of homeostasis (Heuser et al., 1994; McEwen, 2002). Both the HI and AMYG play an important role in maintaining homeostasis when an organism experiences stress (Tsigos and Chrousos, 2002). Interestingly both of these regions are particularly vulnerable to

*Corresponding author. Address: Department of Biomedical Sciences, Cummings School of Veterinary Medicine, Tufts University, 200 Westboro Road, North Grafton, MA 01536, United States. Tel: +1-508-839-7970.

E-mail addresses: Kristy.Meadows@tufts.edu (K. L. Meadows), elizabeth.byrnes@tufts.edu (E. M. Byrnes).

Abbreviations: AMYG, amygdala; ANOVA, analysis of variance; BLA, basolateral amygdaloid nucleus; BSA, bovine serum albumin; CeA, central amygdaloid nucleus; DG, dentate gyrus; dHI, dorsal HI; HI, hippocampus; HPA, hypothalamic–pituitary–adrenal; IgG, immunoglobulin G; MeA, medial amygdaloid nucleus; NI, nucleus incertus; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; RLN3, relaxin 3; RXFP1, relaxin-like family peptide 1 receptors; RXFP3, relaxin-like family peptide receptor 3; TBST, Tris-Buffered Saline and Tween 20; vHI, ventral hippocampus.

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age-related pathologies that impact cognitive and emotional processes (Garrido, 2011). Functional sex differences in the adult HI and AMYG have been well-documented across a variety of species, including humans. Indeed, the impact of sex on age-related changes in these brain regions may contribute to the emergence of significant sex differences in affective and cognitive processes with advancing age (Gur and Gur, 2002; Sutin et al., 2009; Miche et al., 2014).

The vast majority of studies examining sex differences in the aging brain have focused on changes associated with circulating sex hormones and their associated receptors (Markham and Juraska, 2002; Norbury et al., 2003; Sherwin and Henry, 2008). One key target for these studies is the regulation of the hypothalamic–pituitary–adrenal (HPA) axis which is significantly modified during aging and demonstrates significant sex differences (McEwen, 2002). Within that context, sex differences associated with neuropeptides, such as vasopressin and oxytocin, have also been considered (Ishunina et al., 2000; Keck et al., 2000; Swaab et al., 2005). Outside of the hypothalamus, however, the role of neuropeptides in aging has received less attention. This gap in our knowledge is unfortunate as neuropeptides and their receptors may prove more selective targets for novel drug therapies, but such therapies may be sex-specific.

Recently, a significant sex difference in the expression of RLN3 mRNA in the NI was reported in rats, with chronically stressed females displaying higher RLN3 mRNA expression when compared to males but only following food restriction (Lenglos et al., 2013). These findings suggest that modifications in the RLN3 system in response to particular environmental demands may be sex-specific. Few studies have examined RLN3 or RXFP3 expression in females and there are no data reported on aged females. The lack of data on age- and sex-dependent modifications in this particular neuropeptide is unfortunate given its diverse neuromodulatory effects. Moreover, RXFP1 and RXFP3, both of which can be activated by RLN3, are expressed in brain regions critical for maintaining homeostasis and in which significant sex-specific aging effects have been documented (McGowan et al., 2009). Thus, RLN3 system is well-positioned to serve as an interface between the changing hormonal milieu and alterations in neural function during the aging process.

In the current study, we examine age- and sex-related expression of RLN3 in the NI as well as relaxin receptor expression in dorsal HI (dHI), ventral HI (vHI) and AMYG using young adult (9–12 weeks) and middle-aged (9–12 months) rats. Specifically, experiment one examined sex and age-associated changes in gene expression of RLN3, RXFP1 and RXFP3 using quantitative polymerase chain reaction (qPCR). Experiment two examined sex and age-associated changes using fluorescent immunohistochemistry to determine if changes in gene expression translate into protein expression. Both the HI and AMYG contain functionally and anatomically distinct subregions (Hoge and Kesner, 2007; Ball et al., 2007; Goodrich-Hunsaker et al., 2008; Fanselow and Dong, 2010; Kim et al., 2012). Thus, in the current study age- and sex-specific modifications were examined in CA1, CA2/CA3 and DG of the dHI and vHI as well as in basolateral amygdaloid (BLA), central amygdaloid (CeA), and medial amygdaloid (MeA) nuclei. The findings indicate that both sex and age influence the expression of relaxin family receptor subtypes in brain regions that play a critical role in both emotional and cognitive processes.

EXPERIMENTAL PROCEDURES

Experiment 1 – gene expression

Experimental animals. Twelve young (9–12 weeks) virgin male, 10 middle-aged (9–12 months) virgin male, 20 middle-aged (9–12 months) reproductively experienced male, 12 young (9–12 weeks) virgin female, 10 middle-aged (9–12 months) virgin female, and 22 middle-aged (9–12 months) reproductively experienced female Sprague-Dawley rats were obtained from Charles River Laboratory. For 1 week prior to testing, the rats were acclimated to a holding facility to reduce nonspecific stress. All animals were group-housed in light- (on 0700–1900 h) and temperature- (21–24 °C) controlled rooms and provided with food and water *ad libitum*. Males and females were kept in the same animal holding room. Following the acclimation period, vaginal smears were obtained from female rats daily between 0800 and 1100 h for two full cycles to determine the stage of the estrous cycle. All females were utilized during diestrus to minimize the influence of hormonal fluctuations. All animals were maintained in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals, and all animal procedures were approved by the Institutional Animals Care and Use Committee of Tufts University.

Tissue collection and preparation. For all subjects, tissue was collected between 0900 and 1100 h. Animals were briefly exposed to CO_2 (60 s) and decapitated. Brains were rapidly removed, frozen in methyl butane, and stored at −80 °C. At the time of dissection, two, one millimeter micropunches were collected bilaterally from the dHI (AP: −2.16 mm from bregma; M/L: 5.0 mm from midline; D/V: 8.5 mm from the skull surface),the vHI (AP: −5.40 mm from bregma; M/L: 4.5 mm from midline; D/V: 4.5–7 mm from the skull surface), two millimeter micropunches were collected bilaterally from the AMYG (AP: −2.16 mm from bregma; M/L: 5.0 mm from midline; D/V: 8.5 mm from the skull surface), and a single two millimeter midline punch was collected from the NI (AP: −9.0 mm from bregma; M/L: 0.0 mm from midline; D/V: 7.5 mm from the skull surface). All coordinates were determined from the atlas of Paxinos and Watson (Paxinos and Watson, 2007). Micropunches were placed into RNase-free tubes and stored at −80 °C prior to processing for qPCR.

RNA extraction and cDNA synthesis. Total RNA was isolated from micropunches using the RNeasy Mini Kit®