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Increased oxytocin-monomeric red fluorescent protein 1 fluorescent intensity with urocortin-like immunoreactivity in the hypothalamo-neurohypophysial system of aged transgenic rats

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ABSTRACT

To visualize oxytocin in the hypothalamo-neurohypophysial system, we generated a transgenic rat that expresses the oxytocin-monomeric red fluorescent protein 1 (mRFP1) fusion gene. In the present study, we examined the age-related changes of oxytocin-mRFP1 fluorescent intensity in the posterior pituitary (PP), the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of transgenic rats. The mRFP1 fluorescent intensities were significantly increased in the PP, the SON and the PVN of 12-, 18- and 24-month-old transgenic rats in comparison with 3-month-old transgenic rats. Immunohistochemical staining for urocortin, which belongs to the family of corticotropin-releasing factor family, revealed that the numbers of urocortin-like immunoreactive (LI) cells in the SON and the PVN were significantly increased in 12-, 18- and 24-month-old transgenic rats in comparison with 3-month-old transgenic rats. Almost all of urocortin-LI cells co-exist mRFP1-expressing cells in the SON and the PVN of aged transgenic rats. These results suggest that oxytocin content of the hypothalamo-neurohypophysial system may be modulated by age-related regulation. The physiological role of the co-existence of oxytocin and urocortin in the SON and PVN of aged rats remains unclear.

1. Introduction

The neurohypophysial hormones, oxytocin and arginine vasopressin (AVP) are synthesized in the magnocellular neurosecretory cells (MNCs), which are mainly localized in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus (Brownstein et al., 1980). The MNCs project their axon terminals to the posterior pituitary (PP) and secrete oxytocin and AVP into the systemic circulation. Oxytocin is involved in not only parturition and the milk ejection reflex but also various functions such as regulation of anti-nociception, anxiety, feeding, stress responses and social recognition (Meyer-Lindenberg et al., 2011). On the other hand, AVP (classified as an anti-diuretic hormone) maintains body fluid homeostasis by acting on the kidney to promote water reabsorption.

Aging affects the whole body, including the neuroendocrine responses to stress (Rehman and Masson, 2001; Robert and Labat-Robert, 2015). Previous studies suggest that aging alters the hypothalamo-neurohypophysial functions in experimental animals and human (Arsenijevic et al., 1995; Calza et al., 1997; Cowen et al., 2013; Huffmeijer et al., 2013; Keck et al., 2000; Terwel et al., 1992; Wierda et al., 1991). Recently, Elabd et al. (2014) demonstrated that oxytocin is an age-specific circulating hormone to

Abbreviations: AVP, arginine vasopressin; CRF, corticotropin releasing factor; CRFR, corticotropin-releasing factor receptor; dm, dorsolateral magnocellular; dp, dorsal parvocellular; eGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; LI, like immunoreactive; MAPK, mitogen-activated protein kinase; MNCs, magnocellular neurosecretory cells; mp, medial parvocellular; mRFP1, monomeric red fluorescent protein 1; PB, phosphate buffer; PFA, paraformaldehyde; PVN, paraventricular nucleus; PP, posterior pituitary; SON, supraoptic nucleus.

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maintain skeletal muscle in mice (Elabd et al., 2014). Oxytocin may be an important hormone in the aging process. However, knowledge is still scarce regarding the relationship between aging and neurohypophysial hormones, in particular, oxytocin.

In the present study, *first* we examined the age-related changes of oxytocin in the PP, SON and PVN, using a transgenic rat line that expresses the oxytocin-monomeric red fluorescent protein 1 (mRFP1) fusion gene. The oxytocin-mRFP1 transgenic rats showed significant increases in mRFP1 fluorescent intensity in the PP, SON and PVN after chronic salt loading (Katoh et al., 2011) and both acute and chronic nociceptive stimuli (Matsuura et al., 2015; Matsuura et al., 2016).

Second, we examined whether urocortin-like immunoreactive (urocortin-LI) cells co-exist with mRFP1 in the SON and PVN of aged oxytocin-mRFP1 transgenic rats because we reported previously that the numbers of urocortin-LI cells in the SON and the PVN were significantly increased in aged rats (Hara et al., 2000) as well as salt-loaded and dehydrated rats (Hara et al., 1997a; Hara et al., 1997b). Urocortin was discovered as a member of the corticotropin-releasing factor (CRF) family (Vaughan et al., 1995). Immunohistochemical studies demonstrated that urocortin-LI is present in the hypothalamo-neurohypophysial system in rats (Vaughan et al., 1995; Kozicz et al., 1998; Morin et al., 1999). Recently, urocortin 1, 2 and 3 were identified to bind to CRF receptors (CRFR1 and CRFR2), and they are recognized to be involved in stress-induced changes in feeding behavior (Stengel and Tache, 2014).

Finally, we examined the relationship between oxytocin and AVP in aged rats, using immunohistochemistry for AVP in 24-month-old oxytocin-mRFP1 transgenic rats. In addition, the physiological responses to chronic salt loading were examined by using 24-month-old transgenic rats expressing the AVP-enhanced green fluorescent protein (eGFP) fusion gene (Ueta et al., 2005; Fujio et al., 2006). Moreover, we examined the gene expressions of the oxytocin and AVP in aged rats, using *in situ* hybridization histochemistry for 20-month-old AVP-eGFP transgenic rats.

2. Materials and methods

2.1. Animals

We used adult male oxytocin-mRFP1 Wistar transgenic rats (aged 3, 12, 18 and 24 months, weighing 450–1015 g), AVP-eGFP Wistar transgenic rats (aged 20 and 24 months, weighing 613–1200 g), and Wistar rats (aged 3 months, 450–520 g). The rats were bred and housed under normal laboratory conditions (23–25 °C, 12 h light/12 h dark cycle, lights on 0700 h) with free access to food and drinking tap water. All experiments in this study were performed in accordance with the guidelines on the use and care of laboratory animals established by the Physiological Society of Japan and were approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan. Both oxytocin-mRFP1 transgenic rats and AVP-eGFP transgenic rats were screened by polymerase chain reactions using genomic DNA extracted from ear biopsies, as described previously (Katoh et al., 2011; Katoh et al., 2014; Todoroki et al., 2010; Ueta et al., 2005).

2.2. Experimental procedures

2.2.1. Measurements of food and water intake

Oxytocin-mRFP1 transgenic rats were divided into four groups by age (n = 4-8 in each group). Each rat was housed in a plastic cage to measure its daily food and water intake.

AVP-eGFP transgenic rats (24 months old) were allowed free access to tap water and dry food, and their water intake and urine volume were measured for 5 days, using a metabolic cage. These rats were given 2% (w/v) salt solution instead of tap water for next 5 days.

2.2.2. Observation of oxytocin-mRFP1 fluorescence in transgenic rats

Oxytocin-mRFP1 transgenic rats were divided into four groups by age (3-, 12-, 18- and 24 months old). All rats were allowed free access to tap water and dry food. These rats were deeply anesthetized with intraperitoneal (i.p.) administration of sodium pentobarbital (50 mg/kg). They were perfused transcardially with 0.1 M phosphate buffer (PB, pH 7.4) containing heparin (1000 U/L), which was followed by 4% paraformaldehyde (PFA) in 0.1 M PB. The brains and pituitaries were carefully removed, and the brains were divided into three blocks including the hypothalamus.

The blocks were post-fixed with 4% PFA in 0.1 M PB for 48 h at 4 °C as described previously (Matsuura et al., 2015; Katoh et al., 2011; Katoh et al., 2014). The tissues were then cryoprotected in 20% sucrose in 0.1 M PB for 48 h at 4 °C. The fixed tissues were cut at 30-μm thickness with a microtome (REM-700; Yamato Kohki Industrial Co., Ltd., Saitama, Japan) into sections containing the SON and the PVN. The locations of the regions were determined according to coordinates given in a rat brain atlas (Paxinos and Watson, 1998). The sections were rinsed twice with 0.1 M PB and placed on glass slides.

The sections containing the SON and the PVN were examined by fluorescence microscopy [ECLIPSE Ti-E (FL/DIC); Nikon Corporation, Tokyo, Japan] with an mRFP1 filter to examine oxytocin-mRFP1 fluorescence. The images were captured with a digital camera (DS-Qi1Mc, Nikon Corporation, Tokyo, Japan). We used microscopy to confirm that the sections were appropriate when the tissues were cut with a microtome, and we selected three sections of the SON and the PVN per rat. Both sides of the SON and PVN were observed by fluorescence microscopy.

2.2.3. Measurement of mRFP1 fluorescence intensity in the PP, SON and PVN

The intact PP specimens were examined by fluorescence microscopy (ECLIPSE E 600; Nikon Corporation, Tokyo, Japan) with an mRFP1 filter (Nikon Corporation, Tokyo, Japan) to visualize oxytocin-mRFP1 fluorescence. The images were captured with a digital camera (DS-L2, DS-Fi1; Nikon Corporation, Tokyo, Japan).

The average mRFP1 fluorescent intensity values per unit area in the SON and the PVN were quantified with an imaging analysis system. The dorsolateral magnocellular (dm) and dorsal (dp) and medial (mp) parvocellular divisions of the PVN were divided and quantified. Sections containing the SON, the dmPVN, the dpPVN and the mpPVN were examined by a fluorescence microscopy with an RFP filter in order to examine oxytocin-mRFP1 fluorescence. The images were captured with a digital camera. We used an imaging analysis system (NIS-Elements; Nikon Corporation, Tokyo, Japan) to average the mRFP1 fluorescent intensity per unit area in the PP, the SON, the dmPVN, the dpPVN and the mpPVN for each rat.

2.2.4. Immunohistochemistry for urocortin and AVP in the SON and PVN

The sections were rinsed twice with 0.1 M phosphate-buffered saline (PBS) and washed in 0.1 M PBS (pH 7.6) containing 0.3% Triton X-100. The sections were incubated for 4 days at 4 °C with a primary anti-urocortin antibody (Yanaihara Institute Inc., Shizuoka, Japan, #Y360, 1:3000 in PBS) or a primary anti-AVP antibody (ImmunoStar, Hudson, USA, #20069, 1:5000 in PBS) solution. After being washed four times in 0.1 M PBS (pH 7.6) containing 0.3% Triton X-100, the floating sections were treated for 2 h at 4 °C with a

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