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Aging-related changes in RP3V kisspeptin neurons predate the reduced activation of GnRH neurons during the early reproductive decline in female mice

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ABSTRACT

Kisspeptin neurons in the rostral periventricular area of the third ventricle (RP3V) play a key role in relaying the positive feedback effects of estradiol that activate gonadotropin-releasing hormone (GnRH) neurons and drive a surge in the GnRH/luteinizing hormone (LH) level. However, the precise role of kisspeptin neurons during female reproductive senescence remains unclear. Focusing on middle-aged intact female mice with irregular estrous cycles, we found a parallel decline in c-Fos–positive kisspeptin neurons and c-Fos–positive GnRH neurons at the time of the GnRH/LH surge. Furthermore, in kisspeptin neurons, the expression of estrogen receptor α (ERα), but not progesterone receptor (PR), decreased with age. Interestingly, some kisspeptin neurons in the RP3V, but none of the GnRH neurons in the rostral preoptic area (rPOA), had a characteristic cellular senescence in middle-aged mice and old mice. These data suggest that, among the groups of neurons involved in reproductive control, the kisspeptin neurons in the RP3V are likely among the earliest to undergo aging processes and thus participate in initiating the early reproductive decline.

1. Introduction

Age-related female reproductive decline involves deficits at all levels of the hypothalamic–pituitary–ovarian axis. However, accumulating evidence indicates that changes in the hypothalamus initiate reproductive senescence (Downs and Wise, 2009; Lu et al., 1994; Rubin, 2000; Wise, 1993; Wise et al., 2002; Yin and Gore, 2006; Zhang et al., 2013). In hypothalamus, GnRH neurons converge and send the final output from the neuronal network controlling fertility in all mammals (Levine, 1997), and their activation at the time of the GnRH/LH surge has been found to be reduced in middle-aged rats (Le et al., 2001; Lloyd et al., 1994). Because GnRH neurons express estrogen receptor–β (ERβ), but not ERα (Herbison and Pape, 2001; Petersen et al., 2003), their activation by estrogen is relayed by ERα in the interneurons projecting to GnRH neurons (Wintermantel et al., 2006).

Kisspeptin is an excitatory neuropeptide encoded by the *Kiss1* gene; it includes kisspeptin-54 and 3 shorter cleavage fragments (West et al., 1998). In the brain of adult female mice, kisspeptin neurons are localized mainly in the arcuate nucleus (ARC) and dense periventricular continuum of cells within the rostral part of the third ventricle (RP3V) (Clarkson et al., 2009). Recent studies reveal that kisspeptin plays a central role in relaying the positive feedback effects of estradiol on GnRH neurons. First, GnRH neurons express the *Kiss1* receptor (Quaynor et al., 2007) and are innervated and activated by kisspeptin neurons (Clarkson and Herbison, 2006; Dumalska et al., 2008; Pielecka-Fortuna et al., 2008). Second, kisspeptin neurons in the RP3V express ERα and progesterone receptor (PR) (Clarkson et al., 2008). The peak expression of *Kiss1* mRNA in the RP3V coincides with the GnRH/LH surge and the c-Fos induction in kisspeptin neurons (Adachi et al., 2007; Dungan et al., 2007; Smith et al., 2005, 2006; Tomikawa et al., 2012). Third, mice that have been ovariectomized and treated with both estradiol and progesterone show a clear LH surge and c-Fos induction in approximately 50% of their GnRH neurons; however, mice bearing targeted deletions in *Kiss1* or *Kiss1r* appear to lack the capacity (Clarkson et al., 2008; Popa et al., 2013). Furthermore, conditional ablation of ERα in kisspeptin neurons prevents mice from acquiring normal ovulatory cyclicity (Mayer et al., 2010). These findings demonstrate that kisspeptin neurons are critical for the activation of GnRH neurons.

Kisspeptin has been suggested to be associated with reproductive aging (Eghlidi et al., 2010; Ishii et al., 2013; Kim et al., 2009; Lederman et al., 2010; Rometo et al., 2007), but the

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underlying mechanisms remain unknown. In the present study using intact female mice, we first studied the activation of GnRH neurons in rPOA and kisspeptin neurons in the RP3V at the time of the GnRH/LH surge. We then studied the expression of ERα and PR, and the potential senescent characteristics in these neurons at different ages. Convergent results from several senescent markers suggest that kisspeptin neurons are among the earliest to undergo aging process. Accompanied by the lost of ERα expression, the aging of kisspeptin neurons may play a crucial role in initiating reproductive decline in intact female mice.

2. Methods

2.1. Animals

All C57BL/6J mice were purchased from Slack Animal Co (Shanghai, China) and housed (4-5 per cage) under a 12-hour light/12-hour dark lighting schedule (light on at 5:00 h) with ad libitum access to water and food. All protocols and procedures used in these studies were approved by the Institutional Animal Care and Use Committee at Fujian Medical University in compliance with the US National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals." To avoid the effects of pheromone on neuronal activation (Bakker et al., 2010), all female mice used in this study were housed in the same room in the absence of male mice. Young, middle-aged, and old mice were 3 to 4 months, 9 to 10 months, and 18 to 19 months of age, respectively.

Estrous cycles were monitored by daily vaginal smears. Young mice with regular 4- to 5-day cycles, and middle-aged mice with irregular 6- to 7-day cycles were used on proestrus or metestrus (diestrus day 1) corresponding to the highest and lowest estradiol levels, respectively. Acyclic old mice have only estrus and diestrus and were used on diestrus. The proestrus mice were screened by serum LH levels using the commonly used protocol for capturing peak LH levels at 16:00 h, 1 hour before switching to dark cycle, as previously reported (Herbison et al., 2008). Serum LH levels were assayed with Rodent LH ELISA kit (Endocrine Technologies, CA), which had a detection threshold of 0.5 ng/mL. Mice with LH levels greater than 5 ng/mL were used in the studies (Supplementary data, Fig. S1).

2.2. Immunohistochemistry

Animals were anesthetized with chloral hydrate i.p. at 16:00 h of proestrus and metestrus, and blood samples were collected from the heart. The mice were immediately perfused with 0.1 mol/L phosphate-buffered saline solution (PBS; 15 mL) and subsequently with 4% paraformaldhyde (20 mL). Brains were removed and postfixed in 4% paraformaldhyde for 4 to 6 hours and then transferred to 30% sucrose at 4 °C. After the brains sank, they were quickly frozen in liquid nitrogen and stored at −80 °C until sectioning. Brains were sectioned into 30-μm thickness from the level of the medial septum (MS) to the caudal hypothalamus on a freezing microtome (CM1850, Leica, Wetzlar, Germany) and stored in cryoprotectant solution with 30% glycerin, 30% ethylene glycol (Sigma, St. Louis, MO) and 40% 0.1 mol/L PBS at −20 °C. Five or 6 animals were used for each group.

A 1-in-5 series of sections was stereologically selected for immunohistochemistry (IHC). The Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 1997) and another publication (Clarkson et al., 2008) were referenced to determine the position of GnRH neurons and kisspeptin neurons. Briefly, 6 of 30 sections per mouse for GnRH neurons were selected, of which 4 contained the MS and 2 contained the rPOA. Four of 20 sections per mouse containing kisspeptin neurons in the periventricular continuum along the RP3V were selected.

Free-floating, single or dual-label chromogen IHC was conducted according to the published protocol (Clarkson et al., 2008). Sections were treated with 3% hydrogen peroxide (H_2O_2) for 10 minutes to quench endogenous peroxidase and then were washed in Tris-buffered saline (TBS). For the first immunolabeling, sections were blocked for 1 hour at room temperature (RT) in a solution containing 0.3% Triton X-100, 0.25% bovine serum albumin (BSA), and 5% normal goat serum (NGS), followed by a 48-hour incubation at 4 °C in rabbit polyclonal antisera against either ERα, PR, or c-Fos in TBS containing 0.3% Triton X-100, 0.25% BSA, and 2% NGS. Sections were then incubated in biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) at 1:600 dilution for 90 minutes at RT. After being washed in TBS, the sections were incubated in Vector Elite avidin–peroxidase at 1:200 dilution for 60 minutes at RT. Immunoreactivity was revealed using nickel-diaminobenzidine hydrochloride (DABNi), which resulted in a black precipitate within the nucleus of the labeled cell (Hoffman et al., 2008). For the second immunolabeling, sections were treated in 3% H_2O_2, washed in TBS, and then incubated in a polyclonal rabbit anti-GnRH or anti-kisspeptin antiserum in TBS containing 0.3% Triton X-100, 0.25% BSA, and 2% NGS for 48 hours at 4 °C. Sections were then incubated in biotinylated anti-rabbit IgG antibody (Vector Laboratories) at 1:600 dilution for 90 minutes at RT. After being washed in TBS, the sections were incubated in Vector Elite avidin–peroxidase at 1:200 dilution for 60 minutes at RT. Staining was then developed using DAB without Ni to generate a brown precipitate within the cytoplasm. The sections were washed thoroughly in TBS, mounted onto poly-L-lysine-coated glass slides, air dried, dehydrated in ethanol, cleared in xylene, and then coverslipped with permanent mounting medium (Vector Laboratories).

Polyclonal rabbit antisera directed against ERα (1:20,000; Millipore, Billerica, MA, USA), PR (1:2000; Dako, Glostrup, Denmark), c-Fos (1:8000; SC52; Santa Cruz Biotechnology, Santa Cruz, CA), kisspeptin-10 (AC566, 1:20000; gift from A. Caraty, Institut National de la Recherche Agronomique, Paris, France), GnRH (1:20,000; LR1; gift from R.Benoit, McGill University, Montreal General Hospital, QC, Canada), and monoclonal mouse anti-p16 have all been characterized previously (Clarkson and Herbison, 2006; Clarkson et al., 2008; Helena et al., 2006; Krishnamurthy et al., 2004). When the primary antibodies were omitted under the conditions used, no significant immunoreactivity was detected.

2.3. Double immunofluorescence

Free-floating sections were washed thoroughly in TBS, blocked for 1 hour at RT in TBS containing 0.3% Triton X-100 and 5% BSA, and then incubated for 48 hours at 4 °C in the polyclonal rabbit anti–kisspeptin-10 antiserum (1:5000), together with rat monoclonal antibody against ERα (H222, Santa Cruz Biotechnology, 1:100) or mouse monoclonal antibody against p16 (F12, Santa Cruz Biotechnology, 1:500), in TBS containing 0.3% Triton X-100 and 5% BSA. The primary antibody was omitted in control sections. After incubation in the primary antibody, sections were washed 6 times in TBS (10 minutes per wash) and incubated at RT for 1 hour in a mixture of respective secondary antibodies in TBS. Immunoreactive (IR) kisspeptin and ERα were detected with Cy3-conjugated goat anti-rabbit IgG (1:2000; ABCAM, Cambridge, MA) and either Alexa Fluor 488–conjugated donkey anti-rat IgG (1:2000; Invitrogen, Carlsbad, CA) or FITC-conjugated goat anti-mouse IgG (1:500; KPL, Gaithersburg, MD), respectively. Sections were washed, mounted on poly-L-lysine-coated glass slides, and then coverslipped with prolong Gold antifade reagent (Invitrogen). To avoid the