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Age-dependent variation in the RFRP-3 neurons is inversely correlated with gonadal activity of mice

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ABSTRACT

The present study analyzed changes in the expression of RFamide-related peptide-3 (RFRP-3; a mammalian ortholog of avian gonadotropin-inhibitory hormone), in the brain and correlated it with testicular activity of mice of different age groups (day-old, 1-, 3-, 5-, 7-, 9-, 11-, 13-week and 1.5-year-old). Testicular activity after a progressive increase up to 13-week of age declined in the old mice. On the other hand, while immunoreactive (*ir*) RFRP-3 neurons were not seen in the day-old mice, few appeared in 1-week-old mice, their number and size increased drastically at 3-week of age. This condition remained unaltered until 7-week of age followed by a progressive decline up to the age of 13-week and thereafter increased again in the old age. The present findings indicate that hyperactivity of the *ir*-RFRP-3 neurons of dorsomedial nucleus of hypothalamus (DMH) observed in prepubertal mice declines in reproductively active mice and increases again in the old mice having declined reproductive performance. It is concluded that aging mice exhibits inverse correlation of RFRP-3 neurons and gonadal activity suggesting that function of RFRP-3 is not initiated until 1-week of age and thereafter it could participate in the regulation of gonadal development.

1. Introduction

Progressive development and growth of gonad occur in parallel with somatic growth until the attainment of puberty in some non-seasonal breeders. However, remarkable changes in the gonadal activity (spermatogenesis, testosterone level, etc.) occur in a short span of time just before and around the puberty in these species. Thereafter, sperm production and testosterone concentration remain at the maximum level during the reproductively active period of the species' life span. Regressive/degenerative changes start appearing in the seminiferous tubules with aging. Such changes have been reported in men, rats, oxen, mice and cats but their causes remain unclear (Lützen and Ueberberg, 1973; Humphrey and Ladds, 1975; Gosden et al., 1982; Elcock and Schoning, 1984; Hatakeyama et al., 2008). Similar degenerative changes are reported to be induced by irradiation, artificial cryptorchidism and experimental autoimmunization in laboratory animals (Nebel and Murphy, 1959; Sato et al., 1981; Jegou et al., 1983). Regressive testicular changes, such as those in aged men and animals, can also occur spontaneously in young men resulting in infertility, although the cause remains unknown (Wong et al., 1973).

Reproduction is under the control of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH), which is secreted into the hypophyseal portal system to stimulate synthesis and secretion of the gonadotropins. This releasing hormone originally isolated from mammals (Matsuo et al., 1971) and subsequently from birds (King and Millar, 1982; Miyamoto et al., 1982, 1984) and other vertebrates, is the primary factor responsible for hypothalamic control of gonadotropin secretions from the pituitary. Gonadal sex steroid and inhibin can also modulate gonadotropin secretion via feedbacks from the gonads. But, a neuropeptide inhibitor for gonadotropin secretion was unknown in vertebrates until the discovery of hypothalamic dodecapeptide (SIKPSAYLPLRF-NH_2) termed gonadotropin-inhibitory hormone (GnIH), which directly inhibits gonadotropin release from the cultured quail anterior pituitary (Tsutsui et al., 2000). Ubuka et al. (2003) also analyzed developmental changes in the expressions of GnIH precursor mRNA and the mature peptide GnIH during embryonic and posthatch ages in the quail diencephalon including the paraventricular nucleus and median eminence. The gene of the mammalian RFamide-related peptides (RFRPs) is orthologous to the avian GnIH gene. This RFRP gene gives rise to two biologically active peptides; RFRP-1 and RFRP-3 (Hinuma et al., 2000; Ukena et al., 2002; Kriegsfeld et al., 2006; Clarke et al., 2008). Until now, these mammalian GnIH orthologues have been identified in the bovine, rat, mouse and human brains (Fukusumi et al., 2001; Ukena et al., 2003; Yoshida et al., 2003; Ubuka et al., 2009). RFRPs are mainly expressed in the neurons of dorsomedial hypothalamic nucleus (DMH) and/or paraventricular nucleus (PVN) in mammals

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and are directly or indirectly known to have inhibiting influence on the gonadal function (Hinuma et al., 2000; Fukusumi et al., 2001; Ukena and Tsutsui, 2001; Yano et al., 2003; Kriegsfeld et al., 2006; Johnson et al., 2007; Ubuka et al., 2009; Smith and Clarke, 2010).

In spite of the identification, characterization and localization of this inhibitory peptide in mammalian system, relatively few reports are focused on the physiological aspects of this mammalian GnIH ortholog. Recently, Quennell et al. (2010) observed an increase in the hypothalamic RFRP gene expression of male rats between 2 and 4 weeks of age. Sethi et al. (2010) also reported the high levels of the expression of RFRP-3 neurons observed in sexually immature mice decreased in the sexually mature condition. Hence, in the present study, age-dependent variations were monitored in the expression of *ir*-RFRP-3 in mice brain. Testicular activity and immunohistochemistry of RFRP-3 neurons of mice of different age groups (ranging from day-old to prepubertal, postpubertal and 1.5-year-old mice) were studied to assess the possible correlation between the gonadal function (development, maturation and regression) and the expression of RFamide peptide in aging mice. The present findings indicate an inverse relation between two systems and suggest a functional role of RFRP-3 as a key neuropeptide involved in mammalian reproduction.

2. Materials and methods

2.1. Animals

Male laboratory mice (*Mus musculus*) of the Parkes (P) strain were obtained from a colony maintained in our laboratory. The mice were housed under hygienic conditions in a well-ventilated photoperiodically controlled room (light:dark 12:12), and were provided with commercial food (Pashu Aahar Kendra, Varanasi, India) and tap water *ad libitum*. All the experiments were conducted in accordance with institutional practices, and within the framework of the revised Animals (Scientific Procedures) Act of 2002 of the Government of India.

2.2. Source of tissues

Tissues for the aging experiment obtained from day-old, 1-, 3-, 5-, 7-, 9-, 11-, 13-week and 1.5-year-old mice ($n = 5$ per group) were weighed. Day of delivery/birth was designated as day-old. Small animals (day-old, 1-week and 3-week) were killed by cervical dislocation whereas large animals (5-, 7-, 9-, 11-, 13-week and 1.5-year-old) were killed under ether anesthesia prior to dissection. Blood was collected from the heart into a heparinized tube and centrifuged at 4000 rpm for 20 min at 4 °C to separate plasma and stored at -20 °C for the radioimmunoassay of testosterone. Mice were perfused transcardially with phosphate-buffered saline (PBS) followed by Zamboni's fixative [4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB); pH 7.4]. The length and width of the left testis was measured *in situ* with dial calipers, and the testicular volume was calculated using Bissonett's formula $4/3\pi ab^2$ ($a = 1/2$ of the long axis; $b = 1/2$ of the short axis) (Jaiwal and Chaturvedi, 1991).

The testes from both sides were excised and weighed, and the gonado-somatic index (GSI) was calculated as weight of the paired testes/100 g body weight. The brain was also dissected out and post-fixed to be processed for the immunohistochemistry of RFRP-3.

2.3. Histological preparations

The testes removed after perfusion from each age group mice were weighed and re-fixed overnight in the same fixative mentioned above for histological studies. Testes were then dehydrated in an ascending series of alcohol, treated with xylene and embedded in paraffin wax. 6-μm-thick sections were cut by a Weswox rotary microtome (Western Electric & Scientific Works, Ambala Cantt, India), and stained with haematoxylin–eosin. Histological sections of the testis were viewed under a microscope (Axioskop 2 *Plus*; Carl Zeiss AG, Oberkochen, Germany) and images were captured with a digital camera. Seminiferous tubule diameter was determined in 10 sections per mouse testis by using the image analyzer software Motic Images 2000 version 1.3 (Sethi and Chaturvedi, 2009).

2.4. Hormone assay

A radioimmunoassay (RIA) of plasma testosterone was performed using a commercial RIA kit (Immunotech, Marseille, France) according to the manufacturer's instructions. The antiserum used in the assay was specific for testosterone; cross-reactivity was less than 0.03% with estradiol, 0.03% with progesterone, 0.01% with dehydroepiandrosterone, and 0.6% with androstenedione. Sensitivity of the assay was 0.025 ng/ml. The intra- and inter-assay coefficients of variation were 14.8% and 15%, respectively.

2.5. Immunohistochemistry of RFRP-3

Brains, dissected out after whole body perfusion, and post-fixation were passed through a graded series of alcohol and embedded in paraffin wax. Six micrometers thick coronal sections of the brain passing through the region of the dorsomedial nucleus of hypothalamus (DMH) were deparaffinized in xylene, rehydrated and rinsed in PBS (0.02 M, pH 7.4). Immunohistochemistry of RFRP-3 was performed by the method of Sternberger and Sternberger (1986) with some modifications. In brief, endogenous peroxidase activity was eliminated from the sections by incubation with 0.3% H_2O_2 in absolute methanol for 20 min. After blocking nonspecific binding components with 5% normal goat serum in PBS for 1 h at room temperature, the sections were immersed in 1:1000 dilution of primary antiserum raised against quail GnIH (Tsutsui et al., 2000) for 16–20 h at 4 °C. Slides were rinsed in PBS and incubated with 1:1000 dilution of horseradish-peroxidase-conjugated secondary antibody, followed by another set of washes. Sections were then incubated for 1 h in avidin–biotin complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA). The resulting complex was visualized using 0.03% 3,3′-diaminobenzidine in 0.05 M Tris–HCl (pH 7.4) with 0.03% H_2O_2 for 10–20 min. After washing with Tris–HCl, the staining was stopped by washing in distilled water. Sections were dehydrated through an ethanol series, cleaned in xylene and mounted using DPX (a mixture of distyrene, a plasticizer and xylene). Slides were viewed under a microscope (Axioskop 2 *Plus*; Carl Zeiss AG, Oberkochen, Germany) and images were captured using a digital camera.

2.6. Image analysis

The number of immunoreactive RFRP-3 cell bodies was counted from the centre region of DMH using a X20 objective lens and MacBiophotonics ImageJ software. Around 25–35 sections were obtained from the dorsomedial nucleus and the RFRP-3 neuronal numbers were counted in the middle 5 sections only. Once images of these sections containing RFRP-3 neurons were captured, background threshold levels were adjusted to allow for automatic counting of *ir*-neurons (not fibres) in these sections by the software package. In addition, the ImageJ software placed a dot on each neuron counted, allowing the observer to verify accuracy during the counting process.