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Changes in Prodynorphin Gene Expression and Neuronal Morphology in the Hypothalamus of Postmenopausal Women

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Abstract

Human menopause is characterised by ovarian failure, gonadotrophin hypersecretion and hypertrophy of neurones expressing neurokinin B (NKB), KiSS-1 and oestrogen receptor α (ER α) gene transcripts within the hypothalamic infundibular (arcuate) nucleus. In the arcuate nucleus of experimental animals, dynorphin, an opioid peptide, is colocalised with NKB, kisspeptin, ER α and progesterone receptors. Moreover, ovariectomy decreases the expression of prodynorphin gene transcripts in the arcuate nucleus of the ewe. Therefore, we hypothesised that the hypertrophied neurones in the infundibular nucleus of postmenopausal women would express prodynorphin mRNA and that menopause would be accompanied by changes in prodynorphin gene transcripts. In the present study, *in situ* hybridisation was performed on hypothalamic sections from premenopausal and postmenopausal women using a radiolabelled cDNA probe targeted to prodynorphin mRNA. Autoradiography and computer-assisted microscopy were used to map and count labelled neurones, measure neurone size and compare prodynorphin gene expression between premenopausal and postmenopausal groups. Neurones expressing dynorphin mRNA in the infundibular nucleus of the postmenopausal women were larger and exhibited hypertrophied morphological features. Moreover, there were fewer neurones labelled with the prodynorphin probe in the infundibular nucleus of the postmenopausal group, compared to the premenopausal group. The number of dynorphin mRNA-expressing neurones was also reduced in the medial preoptic/anterior hypothalamic area of postmenopausal women without changes in cell size. No differences in cell number or size of dynorphin mRNA-expressing neurones were observed in any other hypothalamic region. Previous studies using animal models provide strong evidence that the changes in prodynorphin neuronal size and gene expression in postmenopausal women are secondary to the ovarian failure of menopause. Given the inhibitory effect of dynorphin on the reproductive axis, decreased dynorphin gene expression could play a role in the elevation in LH secretion that occurs in postmenopausal women.

Keywords

oestrogen; GnRH; human; menopause; kisspeptin; neurokinin B; dynorphin; oestrogen receptor

Introduction

Human menopause is characterised by ovarian follicle depletion (1,2), reduction of ovarian steroids to castrate levels, and a compensatory elevation of serum gonadotrophins. Increased GnRH secretion from the hypothalamus likely contributes to the rise in serum LH in

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postmenopausal women (3). Moreover, within the infundibular (arcuate) nucleus, there is hypertrophy of a subpopulation of neurones expressing KiSS-1, NKB, and ER α mRNA, accompanied by increased KiSS-1 and NKB gene expression (4–6). Ovariectomy of young cynomolgus monkeys produces nearly identical changes (6,7) providing compelling evidence that the neuronal hypertrophy and changes in neuropeptide gene expression observed in postmenopausal women is secondary to ovarian failure. Indeed, there are now numerous studies in animal models implicating arcuate kisspeptin and NKB neurones in the sex-steroid regulation of GnRH secretion (3,8–10). Taken together, these data suggest that hypertrophied infundibular neurones expressing KiSS-1, NKB and ER α mRNAs play an important role in the elevation of gonadotrophin secretion in postmenopausal women.

Endogenous opioid peptides are also important regulators of the reproductive axis, with inhibitory effects on hypothalamic GnRH neurones (11,12). Dynorphin, an endogenous opioid peptide derived from the prodynorphin gene, has been shown to mediate progesterone negative feedback on GnRH secretion via the kappa opioid receptor (13). Using electron microscopy, synapses between dynorphin terminals and GnRH somata have been identified in the hypothalamus of the ewe (13). Interestingly, dynorphin is coexpressed with KiSS-1, NKB, ER α or progesterone receptors in the arcuate nucleus of the ewe and rat (14–18) and the expression of prodynorphin mRNA is altered by ovariectomy (19). Therefore, we hypothesised that the hypertrophied neurones in the infundibular nucleus of postmenopausal women would express prodynorphin mRNA and that menopause would be accompanied by changes in prodynorphin gene transcripts. To address this hypothesis, we used *in situ* hybridisation and computer microscopy to compare the distribution, morphology and relative mRNA expression of prodynorphin neurones in the hypothalamus of premenopausal and postmenopausal women.

Materials and Methods

Hypothalami were collected from premenopausal (n = 3, ages 14, 23, 32 years of age) and postmenopausal (n = 3, 52, 66 and 67 years of age) women who died of sudden, unexpected causes. The specimens were collected in accordance with the guidelines set forth in the Federal Register and the Human Subjects Committee at the University of Arizona. The postmortem delay of the subjects was 14.2 ± 2.2 hours (mean $\pm$ SEM) and there was no significant difference in postmortem interval between the two groups. There was no history of oestrogen replacement, drug use, or chronic systemic illness other than atherosclerosis. At autopsy, each brain was bisected in the midsagittal plane, and hypothalamic blocks were dissected and frozen in isopentane at -30°C . The hypothalami were then sagittally sectioned (20 μm thickness) in a cryostat, thaw-mounted onto gelatinised slides and stored at -80°C until use.

Hybridisation histochemistry

Hybridisation histochemistry was performed on every tenth section throughout the medial hypothalamus of each subject. A synthetic [^{35}S]-labelled 48-base cDNA probe targeted to bases 862–909 of the rat prodynorphin gene was used (20). This sequence was 94% homologous to the human (21) and has been used extensively in our previous studies (5,22). Genebank searches showed no significant homology of this sequence to other mammalian central nervous system genes. Northern analysis using human tissues showed this probe to label the appropriate size transcript under the same conditions of stringency as the current study (5). The *in situ* hybridisation methodology has been previously described in detail (5,22). Following overnight hybridisation and stringent washes, the slides were dried, dipped into Kodak NTB-3 nuclear emulsion (diluted 1:1 with water) and stored in the dark at 4°C . Test slides were developed at different times to determine the optimum exposure length of

12 weeks. Sections were counterstained with toluidine blue. Control sections using radiolabelled sense probes yielded no labelling above background.

Morphological Analysis

Slides were coded to prevent experimenter bias. To analyse the distribution of neurones throughout the hypothalamus, the sections (6–10 per subject) were examined using brightfield and darkfield microscopy. For quantitative analysis of cell number, sections of the medial hypothalamus were matched to Fig. 4-4 from Nauta and Haymaker (23). Additional sections were selected for counting the number of neurones in the premammillary nucleus [see figure 5C from (22)]. These sections (2–3 per subject) were systematically scanned using an image-combining computer microscope equipped with a Lucivid miniature CRT, a motorised stage and Neurolucida Software (Microbrightfield, Colchester, VT). All labelled neurones (silver grains >5X background) were marked and counted. For comparisons of the numbers of neurones in specific regions, the boundaries of the infundibular nucleus, paraventricular nucleus, ventromedial nucleus, posterior hypothalamus and premammillary nucleus were digitised with the aid of the computer microscope. Because a definitive boundary could not be distinguished between the medial preoptic and anterior hypothalamic areas, this entire region was circumscribed to analyse cell numbers. This region will be referred to as the preoptic/anterior hypothalamic area.

Cell profile areas and grain numbers were quantified within the infundibular nucleus and preoptic/anterior hypothalamic area, the two regions that displayed changes in the number of labelled cells between groups. Within these areas, approximately 20 labelled neurones were randomly selected using Stereoinvestigator Software (Microbrightfield Inc.) and images were captured at 60X and imported into Simple PCI image-analysis software for quantification of grain number (Compix Inc., Cranberry Township, PA). The perimeter of each labelled neurone was manually drawn and this number was used to calculate cell profile areas. For statistical comparisons between groups, the average number of labelled cells/section, cell areas and number of autoradiographic grains/neurone were calculated for each subject. These values were used to compute group means and standard errors. Student's *t*-tests were used to compare groups.

Results

Numerous small prodynorphin neurones were identified within the rostral and middle infundibular nucleus in the premenopausal group (Figs. 1 and 2). Compared to the premenopausal women, the number of neurones expressing prodynorphin mRNA in the infundibular nucleus was significantly reduced by 73% in postmenopausal group ($t = 6.87$, $df = 4$, $p = 0.002$, Figs. 1 and 2). Moreover, prodynorphin-mRNA expressing neurones in the postmenopausal group exhibited morphological features of hypertrophy, which included increased Nissl substance and larger nuclei and nucleoli (Fig. 3). This finding was confirmed by quantitative analysis; the mean profile area of labelled neurones in the infundibular nucleus of postmenopausal group was more than twice the size of the premenopausal women ($t = -37.7$, $df = 4$, $p < 0.001$). There was no change between groups in the number of autoradiographic grains per neurone (Fig 2).

In the premenopausal women, scattered small to medium prodynorphin mRNA-expressing neurones were identified in the medial preoptic area, extending to the anterior hypothalamic area (Fig. 1). Only rare prodynorphin-mRNA expressing neurones were identified within this region in postmenopausal women (Fig. 1). Quantitative analysis revealed a 77% decline in the number of labelled prodynorphin neurones in the preoptic/anterior hypothalamic region in the postmenopausal group ($t = 3.026$, $df = 4$, $p < 0.04$, Fig. 2). There was no significant difference in the size of prodynorphin mRNA-expressing neurones or in the

number of autoradiographic grains per neurone between premenopausal and postmenopausal women in the preoptic/anterior hypothalamic region (Fig. 2).

In addition to the infundibular nucleus and preoptic/anterior hypothalamic areas described above, prodynorphin mRNA-expressing neurones were identified in many hypothalamic regions (Fig. 1). Medium-sized prodynorphin neurones were distributed in the ventromedial and dorsomedial nucleus and there was heavy labelling of neurones in the premammillary nucleus. Magnocellular prodynorphin mRNA-expressing neurones were identified in the paraventricular nucleus and numerous large neurones were labelled in the posterior hypothalamus. The distribution and morphology of these neurones were identical to our previous studies of prodynorphin gene expression in the human hypothalamus [for representative photomicrographs see (22)]. With the exception of the infundibular nucleus and the preoptic/anterior hypothalamic area, the distribution of labelled neurones was identical in the premenopausal and postmenopausal groups. In addition, no differences between groups were observed in the number of neurones in the paraventricular nucleus, ventromedial nucleus, posterior hypothalamus and premammillary nucleus (Table 1).

Discussion

Human menopause is associated with remarkable changes in hypothalamic neuronal morphology and neuropeptide gene expression. In the infundibular (arcuate) nucleus of postmenopausal women, there is hypertrophy of a subset of neurones expressing KiSS-1, NKB and ER α mRNA (4–6). Numerous studies provide evidence that these neurones play a role in the sex-steroid feedback on gonadotrophin secretion (3) and that the neuronal hypertrophy is secondary to the ovarian failure of menopause (4,6,7,24). More recently, dynorphin, an opioid peptide implicated in reproductive regulation, has been shown to be colocalised with kisspeptin, NKB, ER α or progesterone receptors in the arcuate nucleus of experimental animals (14–18). Based on these studies, we hypothesised that prodynorphin mRNA would be expressed in the hypertrophied neurones in postmenopausal women. The present study shows this prediction to be correct; prodynorphin-mRNA expressing neurones in the infundibular nucleus more than doubled in size in the postmenopausal women and exhibited the morphologic features of hypertrophied neurones described in our previous studies (4–6,25). Although definitive proof awaits multiple labelling experiments, these findings suggest that the colocalisation of dynorphin with kisspeptin, NKB or ER α that has been identified in other species (15–18) is conserved in the human.

In contrast to the increased number of neurones expressing KiSS-1 and NKB mRNA in the infundibular nucleus of postmenopausal women (5,6), the number of neurones labelled with the prodynorphin probe was reduced. Because there is no loss of cells in the infundibular nucleus of older women, the decrease in the number of neurones expressing dynorphin-mRNA is likely due to a reduction of gene expression below the level of detection rather than cell death (25). Thus, there appears to be differential effects of menopause on KiSS-1/ NKB and prodynorphin gene expression in the human infundibular nucleus.

Prodynorphin mRNA-expressing neurones were identified in multiple hypothalamic sites in agreement with prior *in situ* hybridisation and immunocytochemical studies (22,26,27). This diverse location is consistent with participation of dynorphin peptides in a variety of homeostatic functions including reproduction, cardiovascular regulation, water balance and thermoregulation (13,28–32). In postmenopausal women, the decrease in prodynorphin gene expression was only detected in two regions, the infundibular nucleus and the medial preoptic/anterior hypothalamic area. In the ewe, more than 90% of the dynorphin neurones in the infundibular nucleus, preoptic area and anterior hypothalamic area express progesterone receptors (14,15) which are highly coexpressed with oestrogen receptors (33).

Ovariectomy of ewes results in a remarkably similar pattern of decreased prodynorphin gene expression in the infundibular nucleus, preoptic area and anterior hypothalamus (19). These studies provide strong evidence that the reduction in the number of prodynorphin mRNA-expressing neurones in postmenopausal women described in the present study is secondary to ovarian failure.

The decrease in prodynorphin gene expression is consistent with clinical studies showing a reduction in the basal opioid inhibition of LH secretion after the menopause. Tonic opioid inhibition of the reproductive axis in normal cycling women can be demonstrated by measuring LH in peripheral plasma after administering naloxone, an opioid receptor antagonist. Administration of naloxone in the late follicular or luteal phase, but not the early follicular phase, results in increased plasma concentrations of LH (34–36). In postmenopausal or oophorectomised young women, naloxone has no effect on LH secretion (36–39). Oestrogen or progesterone replacement in postmenopausal women restores the ability of naloxone to induce LH secretion, indicating that the loss of opioid tone after the menopause is secondary to the removal of sex-steroids (37,38). However, experiments using peripheral administration of naloxone do not provide information on the specific opioid peptide or the site involved in the tonic opioid inhibition of LH and comparable studies using selective opioid antagonists are not available in the human. The present study suggests that the decrease in endogenous opioid tone in postmenopausal women may be a reflection of the decreased dynorphin gene expression in the infundibular nucleus and/or the preoptic/ anterior hypothalamus.

Numerous lines of evidence suggest that the hypertrophied neurones in the infundibular nucleus of postmenopausal women play a role in the regulation of sex-steroid feedback on the reproductive axis (3). Based on the evidence that dynorphin is an important inhibitory regulator of GnRH secretion (13), the presence of prodynorphin mRNA within the hypertrophied neurones further supports this hypothesis. Thus, a decrease in the inhibitory influence of dynorphin could contribute to the elevation of gonadotrophin secretion observed in postmenopausal women. The significance of the decrease in prodynorphin gene expression in the rostral hypothalamus of postmenopausal women is unknown. Classic studies in the rhesus monkey implicate the medial basal hypothalamus as the control centre for reproduction (40), but such studies do not exclude participation of other brain areas. Alternatively, dynorphin neurones in the preoptic area and anterior hypothalamus of postmenopausal women could participate in other homeostatic functions that are influenced by sex-steroids. For example, the rostral hypothalamus, including the preoptic area and anterior hypothalamus, is a major regulatory centre for thermoregulation. Dynorphin, or other kappa receptor agonists, alters the temperature sensitivity of warm sensitive neurones in preoptic slice preparations (41). Furthermore, central administration of dynorphin modifies body temperature (42) and behavioural thermoregulation (43). Interestingly, menopausal flushes have been hypothesised to be caused by a limited opioid withdrawal syndrome (38). Moreover, in morphine-addicted rodents, acute opioid withdrawal induces episodic elevations in tail skin temperature indicative of skin vasodilatation (44,45). These findings raise the intriguing possibility that the decrease in prodynorphin gene expression in the postmenopausal human hypothalamus is related to the mechanism of menopausal flushes.

In summary, the hypertrophied neurones in the infundibular nucleus of postmenopausal women express prodynorphin gene transcripts. Moreover, a selective decrease in the number of neurones expressing prodynorphin mRNA was observed in the infundibular nucleus and preoptic/anterior hypothalamic area in postmenopausal women. These data are consistent with previous demonstrations of a reduction in the inhibitory opioid tone on LH secretion in postmenopausal women. There is strong evidence that the neuronal hypertrophy and the

decrease in prodynorphin gene expression in postmenopausal women is secondary to the ovarian failure of menopause. Given the inhibitory role of dynorphin in reproductive processes, a reduction in prodynorphin gene expression could contribute to the elevation in gonadotrophin secretion after the menopause.

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Figure 1.

Computer-microscope maps showing the distribution of neurones expressing prodynorphin mRNA in representative sagittal sections of a premenopausal (A) and postmenopausal woman (B). Each symbol represents one labelled neurone. There were decreased numbers of neurones expressing prodynorphin mRNA in the infundibular nucleus and the medial preoptic/anterior hypothalamic area of postmenopausal women. Abbreviations: ac, anterior commissure; AHA, anterior hypothalamic area, fx, fornix; INF, infundibular nucleus; MB, mammillary body; oc, optic chiasm; POA, preoptic area; PH, posterior hypothalamus. Scale bar = 4 mm.

Figure 2.

Alterations in prodynorphin gene expression and neuronal morphology in the infundibular nucleus (A, B, C) or the preoptic/anterior hypothalamic area (D, E, F) of premenopausal (Pre) and postmenopausal (Post) women. The graphs show the number of prodynorphin mRNA-expressing neurones/section (top), profile area of prodynorphin mRNA-expressing neurones (middle) and the number of autoradiographic grains per labelled neurone (bottom). There were decreased numbers of prodynorphin mRNA-expressing neurones detected in the infundibular nucleus of postmenopausal women accompanied by a marked increase in cell size. A similar decline in cell number was seen in the preoptic/anterior hypothalamic area of postmenopausal women without differences in the size of labelled neurones. Values are expressed as mean ± SEM. * $p < 0.05$, ** $p \leq 0.002$.

Figure 3.

Photomicrographs of prodynorphin mRNA-expressing neurones in the infundibular nucleus (A, B) and medial preoptic nucleus (C, D) in premenopausal (A, C) and postmenopausal (B, D) women. The grains mark the location of prodynorphin mRNA and the sections are counterstained with toluidine blue. Prodynorphin neurones in the infundibular nucleus (but not the preoptic area) of the postmenopausal group had larger somata and displayed hypertrophied morphological features. Scale bar = 10 μm (applies to A–D).

Table 1

Number of neurones expressing dynorphin mRNA in various hypothalamic regions of premenopausal and postmenopausal women

Hypothalamic Region	Premenopausal	Postmenopausal
Infundibular Nucleus	101.7 ± 6.6	27.0 ± 8.7**
Preoptic/Anterior Hypothalamic Area	56.3 ± 13.3	12.7 ± 5.5*
Paraventricular Nucleus	66.7 ± 17.7	77.7 ± 14.5
Ventromedial Nucleus	80.3 ± 5.9	67.3 ± 5.8
Posterior Hypothalamus	308.7 ± 17.2	289.7 ± 13.4
Premammillary Nucleus	124.3 ± 16.6	121.3 ± 20.2

Values represent the mean (± SEM) number of labelled neurones/section, n = 3 per group.

* Significantly different from premenopausal, $p < 0.05$;

** Significantly different from premenopausal, $p = 0.002$