



NOTE

Theriogenology

Decreased Anti-Müllerian hormone and Anti-Müllerian hormone receptor type 2 in hypothalami of old Japanese Black cows

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ABSTRACT. Cow fertility decreases with age, but the hypothalamic pathomechanisms are not understood. Anti-Müllerian hormone (AMH) stimulates gonadotropin-releasing hormone (GnRH) neurons via AMH receptor type 2 (AMHR2), and most GnRH neurons in the preoptic area (POA), arcuate nucleus (ARC), and median eminence (ME) express AMH and AMHR2. Therefore, we hypothesized that both protein amounts would differ in the anterior hypothalamus (containing the POA) and posterior hypothalamus (containing the ARC and ME) between young post-pubertal heifers and old cows. Western blot analysis showed lower ($P < 0.05$) expressions of AMH and AMHR2 in the posterior hypothalamus, but not in the anterior hypothalamus, of old Japanese Black cows compared to young heifers. Therefore, AMH and AMHR2 were decreased in the posterior hypothalami of old cows.

KEY WORDS: female reproductive senescence, gonadotropin-releasing hormone neuron, Müllerian inhibiting substance, preoptic area, ruminant

J. Vet. Med. Sci.
82(8): 1113–1117, 2020
doi: 10.1292/jvms.20-0159

Received: 19 March 2020
Accepted: 3 June 2020
Advanced Epub:
17 June 2020

Fertility decreases during aging in human and bovine females [15, 16], but the exact pathophysiological mechanisms in the hypothalamus are not clarified yet. Anti-Müllerian hormone (AMH) is a glycoprotein that belongs to the transforming growth factor (TGF)- β superfamily. Plasma AMH concentrations can predict the fertility of adult female goats, ewes, cows, and women [12, 13]. We recently discovered the extragonadal functions of AMH mediated by its primary receptor, AMH receptor type 2 (AMHR2). Specifically, AMHR2 colocalizes with gonadotropin-releasing hormone (GnRH) receptors on the lipid rafts of gonadotrophs [7, 8], and AMH stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from bovine gonadotrophs. Moreover, recently, GnRH neurons in the areas of the brain relevant to neuroendocrine control of reproduction [the preoptic area (POA), arcuate nucleus (ARC), and median eminence (ME)] in humans and rodents were reported to express AMHR2 [2]. Additionally, we recently reported that 75–85% of cell bodies and fibers of bovine GnRH neurons are positive for both AMH and AMHR2 in the POA, ARC, and the internal and external zones of the ME [6]. Furthermore, AMH strongly activates GnRH neurons in adult female mice both *in vivo* and *in vitro* [2], and AMHR2-deficient mice showed abnormal development and function of GnRH neurons, which resulted in reduced fertility [11]. These data suggest that AMH and AMHR2 have important roles in the brain areas controlling reproductive functions. Therefore, in this study, we hypothesized that the levels of AMH and AMHR2 in the anterior hypothalamus (containing the POA; hereby referred to as POA tissue) as well as the posterior hypothalamus (containing the ARC and ME; hereby referred to as ARC&ME tissue) would differ between young post-pubertal heifers and old cows.

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and were approved by the Committee on Animal Experiments of Yamaguchi University.

We obtained brain samples from healthy post-pubertal Japanese Black heifers (25.9 ± 0.6 months of age; $n=5$; luteal phase; young group) and old Japanese Black cows (89.7 ± 20.3 months of age; 5.2 ± 0.5 parity; $n=5$; luteal phase, old group) managed by our contracted farmers in western Japan. They were slaughtered for harvesting beef according to the regulation of the Ministry of Agriculture, Forestry and Fisheries of Japan. All heifers and cows were non-lactating, non-pregnant, and with no follicular cysts, luteal cysts, or other ovarian disorders based on macroscopic examinations of the ovaries [5]. Old cows were slaughtered after completing parturition a sufficient number of times as planned by farmers to obtain beef, usually after 84 months of age.

We used a previously reported method of western blotting [7, 8] using the same anti-AMH rabbit polyclonal antibody (ARP54312_P050; Aviva Systems Biology, San Diego, CA, USA) and the same anti-bovine AMHR2 antibody. Both

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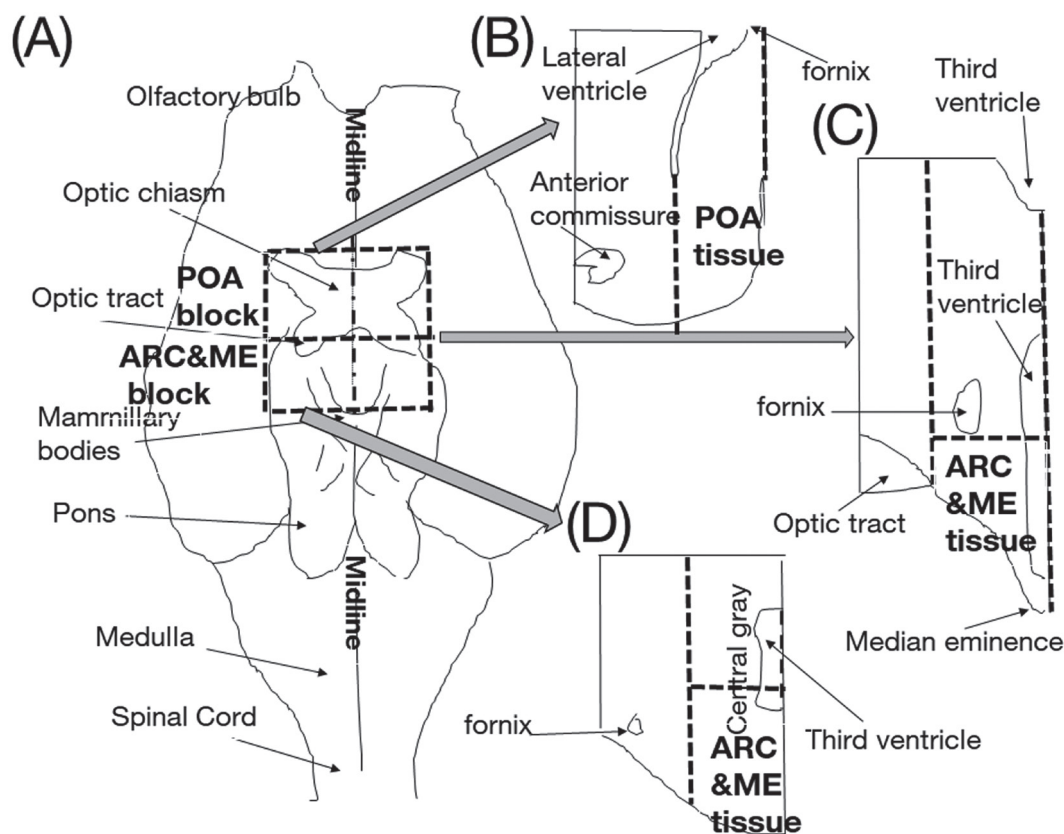


Fig. 1. Schematic illustration of brain tissue sampling according to a previous study [6]. Briefly, brain tissues were dissected as per the dotted line on the ventral side (A) with the following margins: rostrally-rostral border of the optic chiasm; caudally-rostral to the mammillary bodies; lateral to the optic chiasm; and 0.5 cm dorsal to the third ventricle. We then split the block into two parts by cutting rostral to the median eminence (ME), yielding an anterior part containing the preoptic area (POA block) and a posterior part containing the arcuate nucleus and median eminence (ARC&ME block) according to a method reported by Hassaneen *et al* [3]. The blocks were further cut using a previously reported method [6]. Both the POA and ARC&ME blocks were cut at the midlines to obtain left and right sides. Using the bovine brain atlas [10, 14] as a reference, the blocks were further cut using their exterior shapes and the third or lateral ventricles as landmarks as the dotted line (B, C, D). Finally, the size of each tissue sample containing the preoptic area (POA tissue) was less than 1 cm along its lateral axis; 2 cm along the rostrocaudal axis; and 3 cm along the vertical axis. The size of each tissue containing the arcuate nucleus and median eminence (ARC&ME tissue) was less than 1 cm along its lateral axis; 2 cm along the rostrocaudal axis; and 1 cm along the vertical axis.

antibodies had been verified using immunofluorescence analysis and western blotting in bovine small follicles [7, 8]. The anti-AMH antibody recognizes the mature C-terminal form of human AMH (corresponding to amino acids 468–517; SVDLRAERSVLIPETYQANNCQGVCGWPQSDRNPRYGNHVLLLLKMQARG). This sequence had 98% homology to amino acids 483–532 of the mature C-terminal form of bovine AMH but no homology to other bovine proteins. The anti-bovine AMHR2 chicken polyclonal antibody recognizes the extracellular region near the N-terminus of bovine AMHR2 (corresponding to amino acids 31 to 45; GVRGSTQNLGKLLDA).

We obtained POA and ARC&ME tissue samples based on the bovine brain atlas [10, 14] using methods detailed in previous studies [3, 6] and in Fig. 1. The collected POA and ARC&ME tissues were immediately frozen in liquid nitrogen and stored at -80°C until protein extraction.

We performed western blotting as previously described [6–8]. Briefly, proteins were extracted from frozen POA ($n=5$ for each group) and ARC&ME ($n=5$, for each group) tissues using a mortar, liquid nitrogen, and a tissue protein extraction reagent (T-PER; Thermo Fisher Scientific; Waltham, MA, USA) with protease inhibitors (Halt Protease Inhibitor Cocktail; Thermo Fisher Scientific). The total protein content of each tissue homogenate was estimated using a bicinchoninic acid kit (Thermo Fisher Scientific). The extracted protein sample ($33.4\ \mu\text{g}$ of total protein in $37.5\ \mu\text{l}$) was mixed in $12.5\ \mu\text{l}$ of $4\times$ Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 10% (v/v) β -mercaptoethanol, then boiled for 3 min at 100°C . The boiled protein samples were quickly cooled down in ice. The boiled protein samples, $15\ \mu\text{l}$ ($8\ \mu\text{g}$ of total protein) were loaded on a polyacrylamide gel (Any KD Criterion TGX precast gel; Bio-Rad) along with a molecular weight marker (Precision Plus Protein All Blue Standards; Bio-Rad) and four standard samples (2, 4, 8, and $16\ \mu\text{g}$ total protein) and were resolved by electrophoresis on sodium dodecyl sulfate polyacrylamide gels at 100 V for 90 min. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. Blocking was done with 0.1% Tween 20 and 5% non-fat dry milk for 1 hr at 25°C , and then immunoblotting

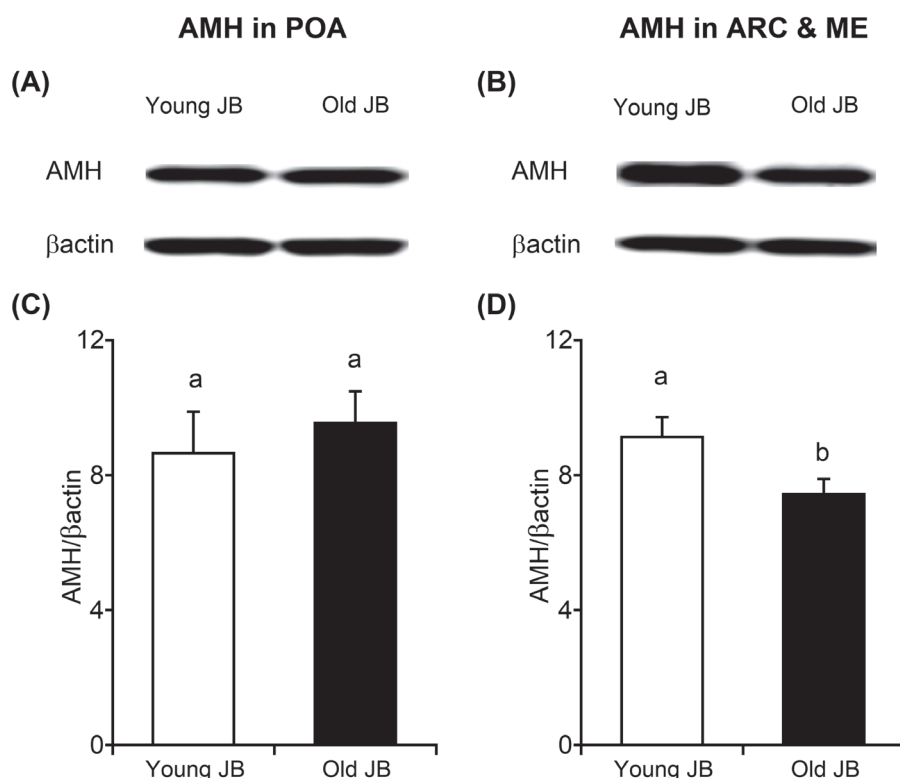


Fig. 2. Representative Anti-Müllerian hormone (AMH) (in mature C-terminal form) and β -actin immunoreactive protein bands in preoptic area (POA; A), and arcuate nucleus and median eminence (ARC&ME; B) tissues obtained from Japanese black young heifers and old cows. Comparison of AMH protein expression normalized to that of β -actin in young heifers ($n=5$) and old cows ($n=5$) in POA (C) and ARC&ME (D). Letters (a vs. b) indicate significant differences ($P<0.05$) between groups.

was performed with either anti-AMH rabbit antibody or anti-AMHR2 chicken antibody (1:25,000 dilution each) overnight at 4°C. After washing the membrane with 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20, the PVDF membrane was incubated with horseradish peroxidase (HRP)-conjugated goat antibody against rabbit IgG or anti-chicken IgG (Bethyl Laboratories, Montgomery, TX, USA; 1:50,000 dilution) at 25°C for 1 hr. Protein bands were visualized using an ECL-Prime chemiluminescence kit (GE Healthcare, Amersham, UK) and a CCD imaging system (Fujifilm, Tokyo, Japan).

MultiGauge v.3.0 software (Fujifilm) was used to quantify the signal intensity of the POA and ARC&ME protein bands. The band intensities for the 16-, 8-, 4-, and 2- μ g protein samples were set as 100%, 50%, 25%, and 12.5%, respectively, for both AMH and AMHR2, and the intensities of other samples were calculated as a percentage of these standards using MultiGauge software. After antibodies were removed from the PVDF membrane with stripping solution, the membrane was blotted with an anti- β -actin mouse monoclonal antibody. The intensities of the β -actin band for the 16-, 8-, 4-, and 2- μ g protein samples were set as 100%, 50%, 25%, and 12.5%, respectively, for both tissues, and the intensities of other samples were calculated as a percentage of these standards using MultiGauge software. AMH and AMHR2 expression levels were normalized to β -actin in each sample.

Statistical analysis was done by a non-paired *t*-test using StatView version 5.0 for Windows (SAS Institute, Cary, NC, USA). The level of significance was set at $P<0.05$. Data are expressed as means \pm standard errors of the mean (SEM).

Figures 2A, 2B, 3A, and 3B show representative immunoreactive protein bands for AMH (25 kDa), AMHR2 (70 kDa), and β -actin (41 kDa) in the POA and ARC&ME tissues of young and old groups. AMH and AMHR2 protein levels in POA tissue were not different (Figs. 2C and 3C) between young and old groups. In contrast, AMH and AMHR2 protein levels in ARC&ME tissue in the young group were higher than those in the old group ($P<0.05$; Figs. 2D and 3D).

The present study revealed a correlative relationship between aging and a decrease in AMH and AMHR2 protein in the ARC&ME tissues of the posterior hypothalamus. However, further studies are necessary to investigate a potential causal relationship between this decrease in AMH and AMHR2 protein and aging.

We also analyzed AMH and AMHR2 by immunofluorescence in the POA, ARC, and ME in young and old hypothalami (data not shown); however, no differences were detected since the immunofluorescence method was qualitative and not quantitative. While quantitative reverse transcription-polymerase chain reaction is a useful method for evaluating differences in AMH and AMHR2 mRNA expression, there are currently no appropriate steadily expressed housekeeping genes in the brain [1]. Possible mechanisms include decreased axonal transport from GnRH neurons in the POA, or decreased expression of AMH and AMHR2 in GnRH neurons in the POA.

Previous studies have demonstrated the importance of AMH and AMHR2 in GnRH-positive neurons in hypothalamic function.

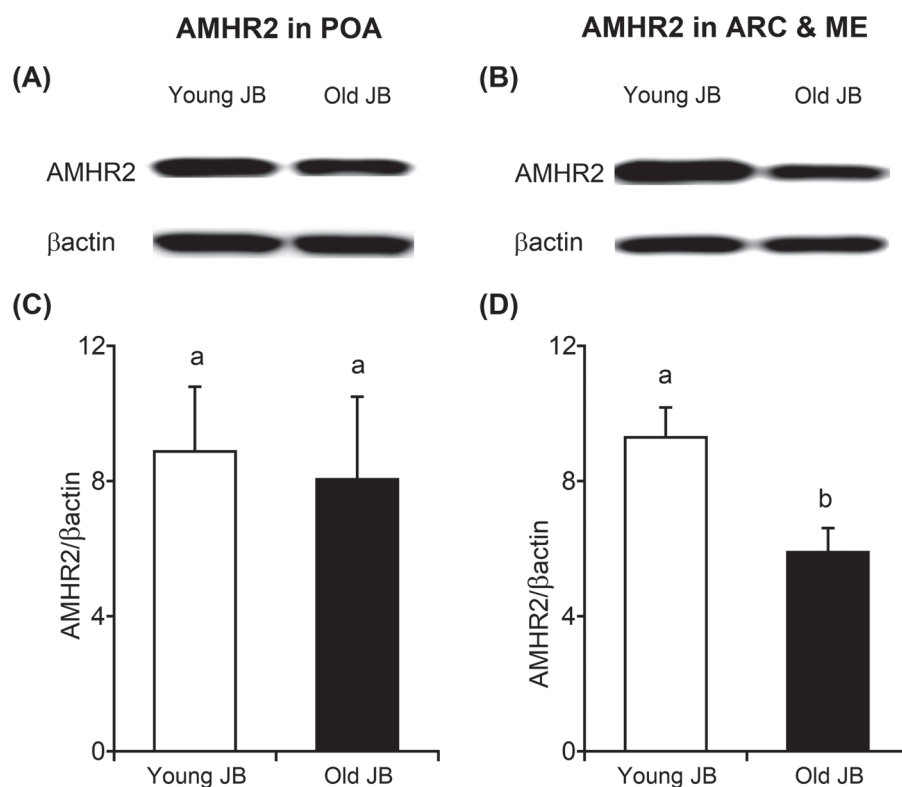


Fig. 3. Representative AMH receptor type 2 (AMHR2) and β -actin immunoreactive protein bands in preoptic area (POA; A), and arcuate nucleus and median eminence (ARC&ME; B) tissues obtained from young heifers and old cows. Comparison of AMHR2 protein expression level normalized to β -actin in young heifers ($n=5$) and old cows ($n=5$) in POA (C) and ARC&ME (D). Letters (a vs. b) indicate significant differences ($P<0.05$) between groups.

Approximately 85% of hypothalamic GnRH neuronal fibers are positive for both AMH and AMHR2, including those in the external zones of the ME [6]. The ME is the interface between the neural and peripheral endocrine systems through which GnRH is secreted into the pituitary portal blood vessels [4]. Using three-dimensional immunofluorescence, our recent study suggested that bovine GnRH neurons secrete AMH as well as GnRH into the pituitary portal blood [6], which may lead to AMH stimulation of LH and FSH secretion from gonadotrophs in the pituitary [7, 8]. However, it should be noted that less than 30% of AMH- and AMHR2-positive neurons are non-GnRH neurons in the POA, ARC, and ME according to our previous study [6]. To date, there is no published data on AMH or AMHR2 in non-GnRH neurons, or specifically on whether kisspeptin neurons express these proteins. Therefore, further studies are necessary to investigate a potential causal relationship between this decrease in AMH and AMHR2 protein and aging.

This study is not the first to demonstrate that aging specifically affects posterior hypothalamic areas such as the ARC and ME. Kermath *et al.* [9] compared neuroendocrine gene expressions in three hypothalamic regions—the ARC, ME, and anteroventral periventricular (AVPV) nucleus located close to the POA [14]. Interestingly, Kermath *et al.* [9] also reported that the majority of the aging-related changes occurred in the ARC and ME, whereas there were few in the AVPV nucleus, and the overall pattern was a decrease with aging. Their results suggest important roles of the ARC and ME during reproductive senescence. The majority of the GnRH neurons in the bovine hypothalamus express both AMH and AMHR2 [6]. AMH strongly activates GnRH neurons in adult female mice both *in vivo* and *in vitro* [2]. Combined with the results of Kermath *et al.*, our data suggests important roles of the ARC and ME in reproductive senescence.

In conclusion, both AMH and AMHR2 were decreased in the posterior hypothalamus containing ARC and ME in old cows, suggesting an important correlation between aging and both proteins. However, the present study did not demonstrate a causal relationship. Therefore, further studies are required to clarify the roles of the AMH-AMHR2 system in GnRH neurons during aging.

ACKNOWLEDGMENTS. O. Kereilwe was supported by MEXT (Ministry of Education, Culture, Sports, Science, and Technology) with the provision of a scholarship. This research was partly supported by a Grant-in Aid for Scientific Research (JSPS Kakenhi Grant Number 18H02329) from the Japan Society for the Promotion of Science (Tokyo, Japan) to H. Kadokawa.

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