BIOLOGY OF REPRODUCTION **81,** 388–395 (2009)
Published online before print 29 April 2009.
DOI 10.1095/biolreprod.109.076331

Porcine Hypothalamic Aromatase Cytochrome P450: Isoform Characterization, Sex-Dependent Activity, Regional Expression, and Regulation by Enzyme Inhibition in Neonatal Boars[1]

C.J. Corbin,[3] T. Berger,[4] J.J. Ford,[6] C.E. Roselli,[7] W. Sienkiewicz,[8] B.C. Trainor,[5] J.F. Roser,[4] J.D. Vidal,[3] N. Harada,[9] and A.J. Conley[2,3]

Departments of Population Health & Reproduction,[3] Animal Science,[4] and Psychology,[5] University of California Davis, Davis, California
USDA,[6] ARS, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska
Department of Physiology and Pharmacology,[7] Oregon Health and Science University, Portland, Oregon
Department of Functional Morphology,[8] University of Warmia and Mazury, Olsztyn, Poland
Fujita Health University,[9] Toyoake, Aichi, Japan

ABSTRACT

Domestic pigs have three *CYP19* genes encoding functional paralogues of the enzyme aromatase cytochrome P450 (P450arom) that are expressed in the gonads, placenta, and preimplantation blastocyst. All catalyze estrogen synthesis, but the gonadal-type enzyme is unique in also synthesizing a nonaromatizable biopotent testosterone metabolite, 1OH-testosterone (1OH-T). P450arom is expressed in the vertebrate brain, is higher in males than females, but has not been investigated in pigs, to our knowledge. Therefore, these studies defined which of the porcine *CYP19* genes was expressed, and at what level, in adult male and female hypothalamus. Regional expression was examined in mature boars, and regulation of P450arom expression in neonatal boars was investigated by inhibition of P450arom with letrozole, which is known to reprogram testicular expression. Pig hypothalami expressed the gonadal form of P450arom (redesignated the "gonadal/hypothalamic" porcine *CYP19* gene and paralogue) based on functional analysis confirmed by cloning and sequencing transcripts. Hypothalamic tissue synthesized 1OH-T and was sensitive to the selective P450arom inhibitor etomidate. Levels were 4-fold higher in male than female hypothalami, with expression in the medial preoptic area and lateral borders of the ventromedial hypothalamus of boars. In vivo, letrozole-treated neonates had increased aromatase activity in hypothalami but decreased activity in testes. Therefore, although the same *CYP19* gene is expressed in both tissues, expression is regulated differently in the hypothalamus than testis. These investigations, the first such studies in pig brain to our knowledge, demonstrate unusual aspects of P450arom expression and regulation in the hypothalamus, offering promise of gaining better insight into roles of P450arom in reproductive function.

aromatase, brain, brain sexual differentiation, hypothalamus, isoform, neuroendocrinology, porcine, regional expression, regulation, sexual dimorphism, steroid hormones, testis

INTRODUCTION

The functional differences in the hypothalamic-hypophyseal axis in male and female mammals was recognized more than 70 years ago [1] and has been an issue of considerable interest to reproductive biologists and behaviorists ever since. Many of the organizational events contributing to the development of the male behavioral and neuroendocrine phenotype are thought to be related to the development of sexually dimorphic nuclei [2] that have been found in the hypothalamus of many species [3, 4]. Fetal or neonatal exposure to testosterone [5] directs neural organization and activates male reproductive behaviors that emerge after puberty [6, 7]. This is due at least in part to effects of estradiol and not testosterone itself [8]. The conversion of androgens to estrogens by the enzyme aromatase cytochrome P450 (P450arom), which is expressed in the brain, has a central role in organizing neuroendocrine function [9] and a variety of reproductive and social behaviors [10]. For instance, P450arom expression in the preoptic area of the hypothalamus is higher in rams that prefer females to male sexual partners [11]. The sexually dimorphic expression of P450arom in the mammalian [12] and avian [13] brain provides additional support for the importance of the enzyme in these processes. Males express higher levels of P450arom in the brain than females [9], especially in functionally important regions of the hypothalamus and limbic system [14]. The aromatase hypothesis has focused attention on local conversion of testosterone to estradiol and activation of estrogen receptors as key events in sexual differentiation of the brain [12, 15–17]. However, recent investigations have reemphasized the role of testosterone or possibly other androgens that make additional important contributions more directly through androgen receptor activation [18]. Therefore, exactly how sex steroids interact to mold the development and function of the mammalian sexually dimorphic nuclei and associated neural substrates is still not entirely clear. The reproductive consequences resulting from changes in sexual differentiation of the hypothalamus also remain poorly understood, particularly the role of P450arom.

[1]Supported in part by National Research Initiative Competitive Grant No. 2008-35203-19082 from the USDA Cooperative State Research, Education, and Extension Service. Names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.
[2]Correspondence: A.J. Conley, Department of Population Health & Reproduction, University of California Davis, Davis, CA 95616. FAX: 530 752 4278; e-mail: ajconley@ucdavis.edu

Received: 20 January 2009.
First decision: 23 February 2009.
Accepted: 15 April 2009.

eISSN: 1259-7268 http://www.biolreprod.org
ISSN: 0006-3363

The P450arom protein is highly conserved in its function, if not its peptide sequence [19]. It is expressed in the brain of all vertebrates studied to date [20], including amphibians, birds, fish, and reptiles [21–23], suggesting that its role in neural function is ancient and fundamental. It is encoded by a single gene (*CYP19*) in mammals with one known taxonomic exception, the pig and related suiformes, which possess either two or three functional isoforms (paralogues) encoded by tandemly duplicated genes [24, 25]. Since the first report of distinct paralogous P450arom enzymes in pigs [26], our laboratory has focused on defining the physiologically relevant functional differences between the two more ancestral enzymes in the cluster [27]. The porcine *CYP19* genes are expressed in a tissue-specific fashion in three principal sites, the gonads (ovary [26] and testis [28]), the placenta [29], and the preimplantation blastocyst [30]. We have shown that the gonadal and placental types of the porcine P450arom enzymes have evolved distinct catalytic properties [29]. The placental-type enzyme is much more efficient in androgen metabolism [31], but the gonadal-type enzyme is unique in catalyzing 1β-hydroxylation of testosterone in addition to the 19-hydroxylation that leads to estradiol synthesis [32]. 1OH-testosterone is an unusual metabolite formed in a branching reaction and accumulates at a similar rate to estradiol because it is nonaromatizable and is capable of activating the androgen receptor [32]. A P450arom enzyme that synthesizes both estradiol and a bioactive nonaromatizable androgen might be expected to exert unusual influence over brain differentiation and neuroendocrine function. To our knowledge, which of the porcine paralogues might be expressed in the hypothalamus has not been explored, nor have any reports appeared to date investigating P450arom expression in the porcine brain. Studies of P450arom may provide unusual insight into sexual differentiation of the pig brain based on the functionally relevant evolution of the porcine P450arom enzyme paralogues [27].

Therefore, the following study was conducted to begin defining the expression of P450arom in the porcine brain by addressing several basic questions. Does the porcine hypothalamus express P450arom? If so, is it higher in males than females, and which of the three isoforms or gene(s) does it express? In situ hybridization was also undertaken to identify the sites of P450arom expression in the hypothalamus of adult boars. Finally, expression levels in the hypothalamus were evaluated in neonatal boars given the P450arom inhibitor letrozole, which we have shown reprograms lower levels of enzyme activity and P450arom expression in the adult testis [33].

MATERIALS AND METHODS

Tissues

The study utilized mature postpubertal male and female Meishan and commercial crossbred pigs. Boars were of two ages (mean ± SEM age, 242 ± 3 days [n = 8] and 302 ± 2 days [n = 9]). Females were generally older (mean ± SEM age, 431 ± 30 days), but the youngest overlapped in age with the older group of boars (age range, 292–486 days). Hypothalami, pituitaries, and (in the case of boars) testes were collected within 5 min of slaughter and frozen immediately on dry ice. Tissue was dissected using the optic chiasm rostrally, the mammillary bodies caudally, and the lateral sulci to a depth of the third ventricle. In addition, four adult boars received an intracardiac perfusion with 4% paraformaldehyde in PBS (0.1 M [pH 7.4]) immediately following slaughter. Brains from these animals were subsequently removed, postfixed in the same fixative, washed and stored in 18% sucrose solution in PBS until dissected using the same landmarks as already described, frozen, and cryosectioned. Hypothalamic tissues from 15 neonatal commercial crossbred males (from an unrelated study of accessory sex gland growth) were also harvested in the same manner. These animals were assigned at age 1 wk to a control group (corn oil [n = 4]) or to one of three treatment groups given a single dose of the aromatase inhibitor letrozole (0.1 mg/kg in corn oil) at age 1 wk only (n = 4), two doses (one at age 1 wk and another at age 5 wk [n = 4]), or three doses (one each at age 1, 3, and 5 wk [n = 3]) and were killed at age 45 days. Blood was collected by jugular venipuncture into heparinized tubes before each animal was euthanized with pentobarbitone administration. Hypothalamic, testicular, and adrenal tissues were then harvested, snap frozen, and stored at −80°C until analyzed for aromatase activity. Plasma was harvested from blood samples after centrifugation and stored frozen for radioimmunoassay. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee, University of California Davis.

Microsomal Isolation and Aromatase Activity Assay

Tissues were homogenized in 0.1 M K_3PO_4 (pH 7.4), 20% glycerol, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin and then briefly sonicated. Homogenates were centrifuged at 15 000 × *g* for 10 min. Supernatants were transferred to new tubes and spun for 1 h at 100 000 × *g*, and the resultant microsomal pellets were resuspended in buffer containing 1 mM 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPS). Protein concentrations were determined using the BCA Protein Reagent (Pierce, Rockford, IL). Aromatase activity was measured as validated previously [34] in microsomal protein (250 μg) by monitoring the incorporation of tritium from [1β-^{3}H]-androstenedione (Perkin-Elmer, Boston, MA) into ^3H_2O. All reactions were run at 37°C in the presence of 300 nM androstenedione (20% labeled, 80% cold; Steraloids, Wilton, NH) and a generating system consisting of 17 mM glucose-6-phosphate, 1 mM NADPH, 2 mM NADP, and 1 U of glucose-6-phosphate dehydrogenase (Sigma-Aldrich, St. Louis, MO). Preliminary experiments using pooled hypothalamic microsomal protein (3–100 μg) demonstrated that product accumulation was linear ($R^2 = 0.995$) in incubations conducted for 6 h. Therefore, experiments were conducted in 2-h incubations using 250 μg of microsomal protein for hypothalamus and pituitary and 50 μg for testis, limiting product accumulation to <5% of substrate to ensure linearity. Purified recombinant porcine placental and gonadal types of P450arom (1 pmole/reaction) were used as controls in reactions incubated for 30 min. The assay was stopped with 30% cold trichloroacetic acid, extracted with chloroform, and mixed with a suspension of 5% charcoal and 0.5% dextran. After centrifugation at 2000 × *g* for 30 min, the amount of ^3H_2O produced was quantified by scintillation counting. Because the isoform of porcine P450arom expressed in the gonads is selectively inhibited by the imidazole etomidate but the form expressed in placenta is not [26, 28, 35], etomidate (1 μM) was added to incubations to help define the form of the enzyme in hypothalamus and testis. In contrast, letrozole is a potent competitive inhibitor of P450arom but is not selective for the porcine P450arom enzymes and was added (1 μM) to parallel incubations as an additional control expected to block activities in all tissues and reconstituted assays. Recombinant enzymes were His-tagged, overexpressed, affinity purified over nickel columns from insect cells, and reconstituted as previously described [31].

Western Blot Analysis

Microsomal proteins (10–50 μg) were separated on 8% SDS-PAGE gels and electroblotted onto polyvinylidene fluoride membranes (Immobilon P; Millipore Corp., Bedford, MA). P450arom was detected with a polyclonal antibody against recombinant human protein (1:5000; N. Harada [36]). Proteins were visualized with horseradish peroxidase (HRP)-linked IgG (1:10 000) and luminol reagents according to the ECL protocol (Perkin-Elmer). Purified recombinant porcine (gonadal/hypothalamic) P450arom (0.1 pmole) was used as a positive control [31].

RT-PCR, Cloning, and Sequencing

The RT-PCR was conducted to amplify, clone, and sequence partial P450arom cDNAs from hypothalamic transcripts to verify which P450arom gene was brain specific. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) from the hypothalami of four female pigs and two male pigs, all having demonstrated P450arom activity. Equal amounts of individual RNA samples were combined to make separate female and male pools from which poly A+ mRNA was isolated according to the manufacturer's protocol (Oligotex mRNA Mini Kit; QIAGEN, Valencia, CA). The mRNA (1 μg) was transcribed with 200 U of M-MLV RT (Promega, Madison, WI) beginning in a region of exon 9 that is completely homologous between both the placental- and gonadal-type enzymes. The cDNA was then amplified using Taq polymerase (QIAGEN) for 35 cycles with an annealing temperature of 55°C between primers located in exons 3 and 5, therefore

TABLE 1. Aromatase activity (pmol/mg/h) in hypothalamic microsomal protein of postpubertal boars and sows (ages as shown).*

Parameter	Boars (1)	Boars (2)	Sows (1)	Sows (2)
Age (days)	242 ± 3[a]	304 ± 2[b]	292, 298	476 ± 3[c]
Number	8	9	2	6
Activity (pmol/mg/h)	2.14 ± 0.60[a]	1.87 ± 0.62[a]	0.56, 0.26	0.53 ± 0.08[b]

* Data are presented as means ± SEM.
[a,b,c] Means with different superscripts are significantly different ($P < 0.05$).

spanning a region of nucleotide diversity. The 380-base pair (bp) amplicon was gel purified (QIAquick Gel Extraction Kit; QIAGEN) and sequenced directly using the forward PCR primer.

Thin-Layer Chromatographic Metabolite Analysis

Microsomal proteins (10–100 μg) were incubated with the same generating system as the tritiated water assay using 1 μM [4-^{14}C]-testosterone (48 mCi/mmol; Perkin Elmer). The incubation at 37°C for 6 h was stopped by extraction with 10 volumes of methylene chloride; the organic phase was evaporated to dryness and resuspended in 25 μl of ethyl acetate. Samples were applied to silica gel plates (Whatman, Maidstone, Kent, England) along with cold standards (Steraloids) and developed once in 100% ethyl acetate. Plates were placed on storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) overnight, followed by scanning on a phosphor-imager (Typhoon 8600; Molecular Dynamics).

In Situ Hybridization

Paraformaldehyde-fixed brain tissues from three boars were sectioned coronally at 20 μm and processed for in situ hybridization histochemistry as described previously [37]. Briefly, an antisense aromatase cRNA was transcribed with ^{33}P-uridine triphosphate and SP6 from a 400-bp fragment spanning exons 2–4 of the porcine gonadal-type P450arom [26] in a plasmid that was linearized with Sal I and purified over a Nick column (New England Nuclear, Boston, MA). The labeled P450arom cRNA was diluted to 5×10^6 cpm/ml with hybridization buffer (50% formamide, 10% dextran sulfate, 1× Denhardt solution, 0.2 M NCL, 10 mM Tris, 1 mM EDT, 0.5 mg/ml yeast tRNA, and 10 mM dithiothreitol). Sections were thawed, incubated with 0.001% proteinase K, acetylated, dehydrated, and desiccated under vacuum. Hybridization solution (80 μl) containing probe was applied, and each slide was coverslipped to prevent evaporation during hybridization (16–20 h at 58°C). Following hybridization, the coverslips were removed, and sections were rinsed in 4× saline-sodium citrate (SSC), treated with 2% RNase A for 30 min at 37°C, and washed to a final stringency of 0.1× SSC at 65°C. The slides were exposed to x-ray film (Hyperfilm Betamax; Amersham Biosciences, Piscataway, NJ) after they had been dehydrated through graded ethanol solutions and dried under vacuum. Control tissue sections that were pretreated with RNase (20 μg/ml for 30 min at 37°C) before incubation with antisense probe showed no specific hybridization signal.

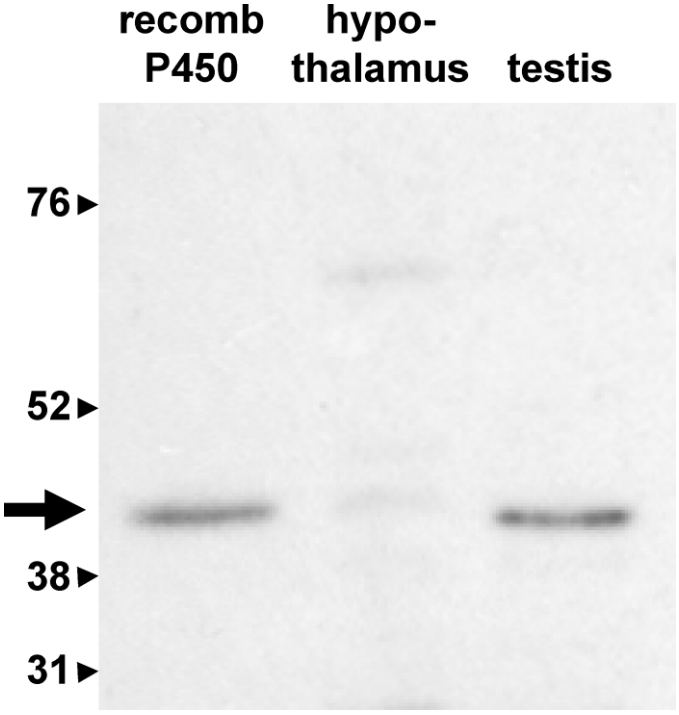

FIG. 1. Western immunoblot analysis for P450arom in porcine hypothalamus (50 μg/lane) and testes (10 μg/lane) by Western immunoblot analysis. Purified recombinant porcine (recomb P450 [0.1 pmole]) is included as a positive control (arrow). A single band detected in testicular protein was the same size as the recombinant enzyme, but expression of the enzyme in hypothalamus was not readily detectable even at 5-fold higher protein loading. Molecular size markers (in kilodaltons) are shown at left.

TABLE 2. Effects of aromatase inhibitors etomidate and letrozole (each at 1 μM) on aromatase activity expressed as a percentage of control activity (100%) in hypothalamic and testicular microsomal protein, and in reconstituted reactions containing recombinant porcine gonadal- and placental-type enzymes.*

Inhibitor	Hypothalamus	Testis	Recombinant gonadal	Recombinant placental
Etomidate	11.9 ± 2.7	2.6 ± 0.4	13.3 ± 1.7	96.3 ± 1.9
Letrozole	0 ± 0	0.53 ± 0.06	0.62 ± 0.28	0.30 ± 0.03

* Data are presented as means ± SEM.

Radioimmunoassay

Testosterone and estradiol levels were determined in plasma samples by radioimmunoassays validated previously for the pig, as described and reported elsewhere [38]. Briefly, the estradiol assay used a sheep antiestradiol 17β-6-bovine serum albumin antibody (Niswender No. 224; G. Niswender, Colorado State University, Fort Collins, CO), ^{3}H-estradiol (NET-317; Perkin Elmer), and estradiol standards (E950; Steraloids). Samples (400 μl) were extracted twice with ethyl ether (Fisher Scientific, Fair Lawn, NJ) to remove conjugated estrogens. The testosterone assay used a sheep antitestosterone antibody (Niswender No. S250), ^{3}H-testosterone (NET370; Perkin Elmer), and testosterone standards (A6940; Steraloids). All extracts were analyzed in the same assay. Intraassay coefficients of variation were <10% for both assays.

Statistical Analysis

Aromatase activity and steroid concentrations were analyzed parametrically by one-way ANOVA (STATA-IC 10.1 for Windows; StataCorp LP, College Station, TX) and nonparametrically (StatXact version 8.0; Cytel Software Corporation, Cambridge, MA) by a Kruskal-Wallis test because sample size precluded evaluations for normality of the data. Comparisons among treatment means (activities within tissues, as well as steroid concentrations) were made using Bonferroni procedures. Because groups were stratified representing zero (control), one (at age 1 wk only), two (at ages 1 and 5 wk), or three (at ages 1, 3, and 5 wk) treatments, a Jonckheere-Terpstra test was also performed to evaluate the presence or absence of a monotonic response trend associated with the number of times that treatments were administered. Equivalent results were obtained by both parametric and nonparametric analyses. Statistical probabilities reported reflect the results of parametric analyses. In addition, correlations were examined between activities measured in hypothalamic, pituitary, and testicular samples.

RESULTS

Hypothalamic aromatase activity was detected in all hypothalamic tissue samples but was 4-fold higher in postpubertal males than females ($P < 0.05$) (Table 1). Aromatase activities were similar in the younger sows to those older and still substantially below those of the older 304-day-old boars (Table 1). Levels of testicular aromatase activity were almost 100-fold higher than those in hypothalamus (mean ± SEM, 170 ± 11 vs. 1.97 ± 0.42 pmol/mg per hour), which were higher than those in male pituitary (0.43 ± 0.12 pmol/mg

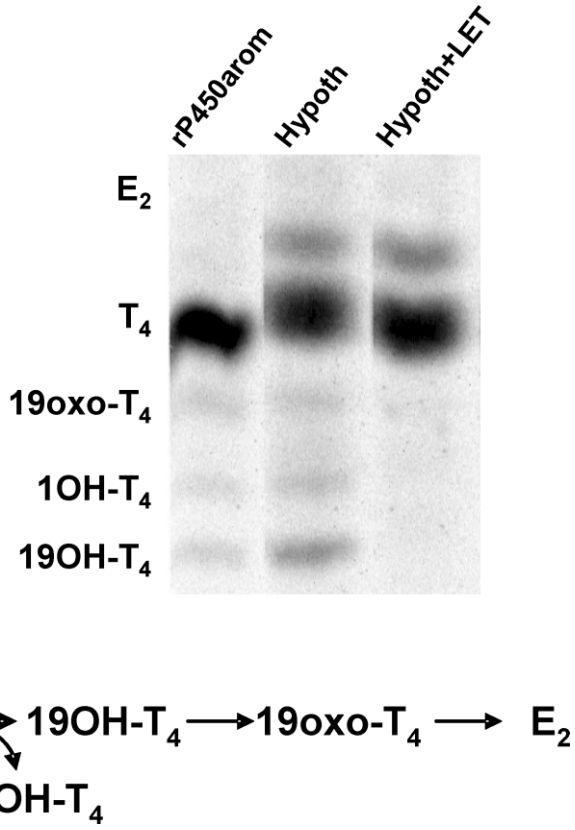

FIG. 2. [4-^{14}C]-testosterone (T_4) metabolism by recombinant porcine gonadal/brain-type P450arom (rP450arom), porcine hypothalamus microsomes (Hypoth [100 μg]), and hypothalamus plus letrozole (Hypoth+LET). Proteins were incubated for 6 h and extracted, and the products were separated by thin-layer chromatography. The migration of standards is shown at left, including 1OH-T_4, 19OH-T_4, and 19oxo-T_4, previously verified by gas chromatography-mass spectroscopy analysis. The reaction was stopped before estradiol (E_2) was detectable to maximize accumulation of these intermediates. The complete reaction scheme is shown at the bottom.

per hour). Immunoblot analysis confirmed that the differences in levels of activity corresponded with P450arom expression levels in testis and hypothalamus. A single immunoreactive band that comigrated closely with purified recombinant P450arom was clearly detectable in 10 μg of testicular microsomal protein but not in as much as 50 μg of hypothalamic microsomal protein (Fig. 1). There was no detectable activity in female pituitary microsomal protein, and no correlations were detectable among hypothalamic, pituitary, or testicular aromatase activities (data not shown). Etomidate (1 μM) inhibited enzyme activities in hypothalamus (male and female), testis, pituitary (data not shown), and recombinant gonadal-type P450arom by >85% but did not inhibit activity of the recombinant placental enzyme (Table 2). In contrast, letrozole inhibited activity in all tissues and controls, including the placental-type enzyme by >99%.

The products of testosterone metabolism by porcine hypothalamic microsomal protein were also investigated using thin-layer chromatography. Based on comigration with authentic standard, as well as RF values (distance migrated relative to the solvent front) previously determined and validated by gas chromatography-mass spectroscopy, hypothalamic microsomal protein metabolized testosterone to 1OH-testosterone (1OH-T) and to the estradiol intermediates 19OH-testosterone and 19oxo-testosterone (Fig. 2). The product profile generated with hypothalamic microsomal protein mirrored the metabolism of testosterone by purified recombinant porcine gonadal enzyme with one exception. Hypothalamic microsomes metabolized testosterone to an additional unidentified product that was less polar than the substrate. Letrozole inhibited products that comigrated with the known intermediates in aromatization, 19OH-testosterone, and 19oxo-testosterone, as well as the novel metabolite previously identified as 1OH-T. Accumulation of the unidentified product was not affected by letrozole treatment (Fig. 2).

Further proof of the identity of which of the porcine *CYP19* genes was expressed in porcine hypothalamus was obtained by sequence analysis of an amplicon generated from hypothalamic RNA. The RT-PCR of transcripts from male and female hypothalamus resulted in the amplification of a single amplicon that was cloned and analyzed subsequently. Sequence analysis of the 320-bp fragment revealed that, of 24 nucleotides in the amplicon known to differ between the gonadal- and placental-type transcripts based on previously cloned sequences [26], all but one matched the gonadal-type sequence (Fig. 3). The analysis of regional expression of P450arom in hypothalamus of boars using a porcine gonadal-type P450arom probe showed abundant expression of signal in the medial preoptic area, the bed nucleus of the stria terminalis (data not shown), the periventricular preoptic nucleus, and the lateral margins of the ventromedial hypothalamic nucleus (Fig. 4). Only background

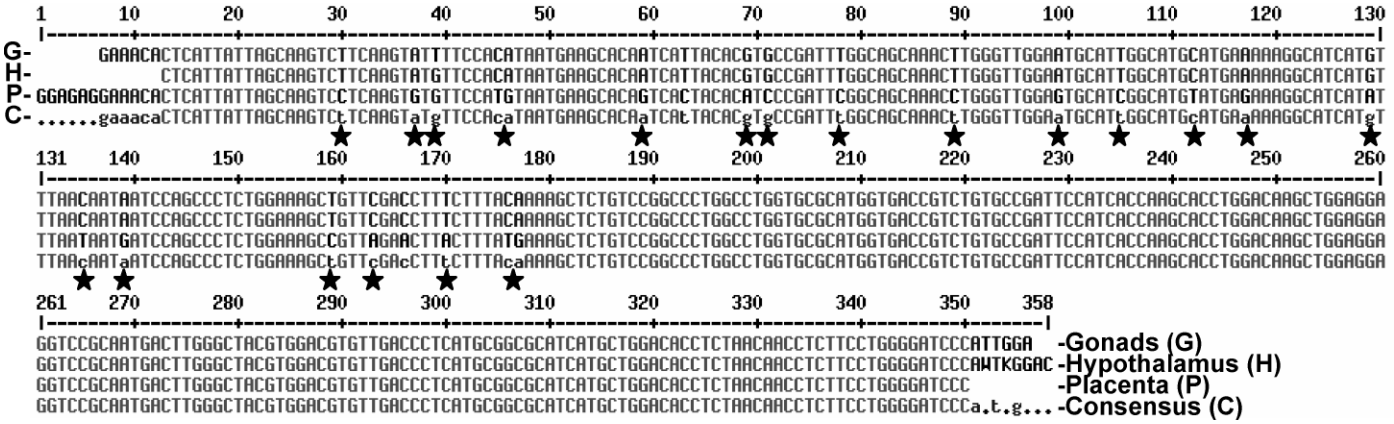

FIG. 3. Nucleotide sequence alignment of P450arom amplicon cloned from mRNA isolated from boar hypothalamus with sequences for porcine P450arom paralogues expressed in the gonads (G [AAB51388]) and placenta (P [AAB51387]). Nucleotide differences are highlighted below the consensus sequence (C) by a star.

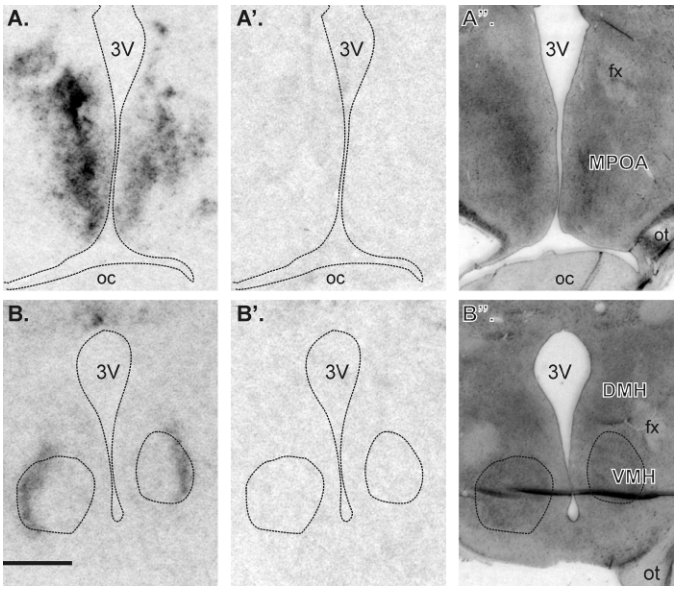

FIG. 4. Hypothalamic expression of P450arom as detected by in situ hybridization. Shown are digitized autoradiographs from representative sections of the medial preoptic area (**A**) and ventromedial hypothalamus (**B**) from a boar using ^{32}P-antisense P450arom cRNA, control autoradiographs from brain sections preincubated with RNase (**A**' and **B**'), and thionin histology (**A**" and **B**"). The digital images were all scanned at the same magnification; bar = 2 mm. Borders have been drawn to delineate the ventricles in the autoradiographs. The ventromedial hypothalamus has been outlined to illustrate that P450arom mRNA expression is confined to the lateral region of this nucleus. 3V, third ventricle; DMH, dorsomedial hypothalamic nucleus; fx, fornix; MPOA, medial preoptic nucleus; oc, optic chiasm; ot, optic tract; VMH, ventromedial hypothalamic nucleus.

levels of signal were present in tissue exposed to RNase before antisense hybridization (Fig. 4).

Letrozole treatment increased hypothalamic aromatase activity in boars at age 45 days over controls by 151%, 194%, and 233% for groups treated at ages 1 wk, 1 and 5 wk, and 1, 3, and 5 wk, respectively (Table 3) ($P < 0.05$). Levels in the groups treated at ages 1, 3, and 5 wk were higher than those in controls ($P < 0.05$). In contrast, testicular aromatase activity was decreased by letrozole treatment to 58%, 28%, and 9% of control values in the groups treated at ages 1 wk, 1 and 5 wk, and 1, 3, and 5 wk, respectively (Table 3) ($P < 0.01$). Activities were reduced in all treatment groups compared with controls ($P < 0.05$). Testicular aromatase activity was negatively correlated with activity in the hypothalamus ($R^2 = 0.59$, $P < 0.02$). Aromatase activity in the adrenal gland (data not shown) was not affected by treatment ($P > 0.5$), nor was it correlated with activities in testis or hypothalamus. There was no significant effect of letrozole treatment on plasma steroid hormone concentrations, although on average testosterone level was numerically higher and estradiol level lower in the treated boars than in the control boars (Table 3) ($P > 0.05$). Neither plasma steroid level was correlated with hypothalamic aromatase activity.

DISCUSSION

The pig and its suiforme relatives are the only mammals known to have multiple *CYP19* genes [39], having survived millions of years after duplication and reduplication occurred in an ancestor [40], but which of these genes is expressed in brain has not been investigated. Using multiple approaches, we show that the form of the enzyme previously shown to be expressed in the gonads (testis and ovaries) is the form expressed in hypothalamus of male and female pigs. This was established functionally by demonstrating 1OH-T synthesis and its inhibition by letrozole, evidence demonstrating that it was a product of P450arom, in contrast to at least one unidentified metabolite that was not. Additional functional evidence was provided by showing the sensitivity of hypothalamic activity to inhibition by the imidazole etomidate. Both 1OH-T synthesis and sensitivity of activity to etomidate are unusual but characteristic properties of the form of the porcine P450arom expressed in testis and ovaries [35]. It was further established by analysis of sequence of partial cDNA clones amplified from hypothalamic transcripts that closely (23 of 24 bp that differ between the gonadal- and placental-type sequences) matched the form previously considered gonad specific. The 1-bp mismatch is likely a variant, many of which are described for other steroidogenic enzymes [41], or perhaps even an amplification artifact. Regardless, these data firmly establish that what has to this point been referred to as the gonadal-type P450arom must now be recognized more correctly as the gonadal/hypothalamic form or paralogue. In contrast to pigs, several species of fish have two *CYP19* genes [21, 42, 43], one of which is expressed in the ovaries, and the other is brain specific. Unfortunately, there are few data on the fish P450arom enzymes [44], particularly with respect to their adaptive or functional significance. However, biochemical analysis of purified recombinant porcine aromatases supports the hypothesis that the P450arom paralogues expressed in the gonads (testes and ovaries) and placenta evolved catalytically to increase ovulation rate and to promote litter-bearing as a reproductive strategy [27]. The results of the present study suggest that evolutionary selection of the gonadal/hypothalamic form of porcine P450arom must necessarily include neuroendocrine and gonadal function.

Brain aromatase expression is a sexually divergent trait in vertebrates, being higher in males than females. The present data are consistent with this general principle, with boars averaging 4-fold higher levels of activity than those in sows. The pigs used to establish male and female levels of P450arom in the hypothalamus were all postpubertal, although females

TABLE 3. Aromatase activity (pmol/mg/h) in tissues, and testosterone (ng/ml) and estradiol (pg/ml) concentrations in systemic plasma, from 45-day-old neonatal boars treated orally with letrozole or given vehicle only (control).

	Letrozole treatment (0.1 mg/kg)			
Parameter	Control (n = 4)	1 Week (n = 4)	1+5 Weeks (n = 4)	1+3+5 Weeks (n = 3)
Hypothalamus	1.56 ± 0.44[a]	2.67 ± 0.48[a,b]	3.20 ± 0.39[b]	3.64 ± 0.17[b]
Testis	192 ± 30[a]	94 ± 20[b]	48 ± 9[b,c]	18 ± 8[c]
Testosterone	0.7 ± 0.2[a]	1.4 ± 0.2[a]	1.7 ± 0.2[a]	1.9 ± 0.2[a]
Estradiol	10.9 ± 2.0[a]	12.3 ± 2.0[a]	8.4 ± 2.0[a]	7.4 ± 2.0[a]

* Data are presented as means ± SEM.
[a,b,c] Means with different superscripts are significantly different ($P < 0.05$).

were significantly older than males. However, it is unlikely that this confounded the present data for several reasons. The youngest females (ages 292 and 296 days) had similar enzyme activities to the oldest sows and were similar in age to the older boars, but still their aromatase activities were substantially less (Table 1). Furthermore, there is no decrease in hypothalamic aromatase activity with age in rats [45]. The levels of aromatase activity measured in the hypothalamus of the boar, exceeding 6 pmol/mg per hour in one sample, appear to be considerably higher than hypothalamic values of other mammals measured in comparable samples [9]. This is true even when comparing the present data in boars with those of rams in investigations in which specific hypothalamic sites have been subsampled. The highest expression of P450arom is in the preoptic area of rams [46], but even there it still barely exceeds 0.5 pmol/mg per hour [47], a quarter of the average levels seen herein in boars without subsampling. More precise microdissection of the porcine hypothalamus is necessary to determine just how much higher P450arom activity is in pigs than in sheep and other species.

The boar is also unusual among male mammals in having high circulating levels of estrogens [48] that peak neonatally and again peripuberally [38, 49, 50], remaining higher than preovulatory peaks in estrous females [51]. This is reflected by high levels of testicular aromatase activity that increase in the first few weeks of life [28, 52], declining steadily thereafter [38] as testicular weight increases [53]. Testicular aromatase activity of boars was almost 100-fold higher than activity in the hypothalamus, but there was no correlation between these two sites of expression. The reverse is true in rams (with respect to levels), in which the preoptic area has 10-fold higher enzyme activity than that in testes [47]. In neonatal mice, aromatase activity is around 0.5 pmol/mg per hour in hypothalamic microsomes (Conley and Vidal, unpublished results) but is below limits of detection in testes [54]. Thus, while exhibiting the same sexual divergence in levels of brain P450arom favoring males over females, it appears also that both hypothalamic and testicular levels increased markedly over those of ancestors as pigs evolved.

Despite seemingly sizable differences in levels of basal expression of P450arom in porcine hypothalamus compared with those in other mammals, regional expression was found to be broadly similar to that described in other species [12]. This suggests that neurons in the medial preoptic area and ventromedial hypothalamus are sites of P450arom expression in the hypothalamus of boars, but studies are required to more comprehensively map the distribution and dimorphisms between male and female hypothalami. These brain regions are involved in the physiological and behavioral aspects of male reproduction [4, 17] and correspond to regions expressing estrogen receptors in pigs [55] and several other species [12, 37]. They form part of an interconnected sexually dimorphic brain circuit that develops under the influence of androgens and/or estrogens depending on the species examined [56]. The observation that these regions contain high P450arom expression supports other data suggesting that aromatization is involved in morphological differentiation of the brain, as well as behavior [57, 58].

Neuronal P450arom expression is stimulated by estrogens in birds [17] and fish [21] and by androgens in the mammals studied thus far [12]. Given the high levels of P450arom expression in the porcine hypothalamus and testis, the regulation of brain expression might be expected to differ in this species from that in others. The results of the present study show that treatment of neonatal boars with the selective aromatase inhibitor letrozole increased hypothalamic aromatase activity in boars killed 10 days or longer after the last dose without significantly altering circulating testosterone levels. Nor were any correlations found between either circulating testosterone or estradiol levels and aromatase activity in the hypothalamus. These data are consistent with results of a similar study in male guinea pigs. Letrozole treatment increased brain aromatase activity of adult intact and castrated males without changing androgen or estrogen levels [59]. Choate and Resko [59] speculated that this paradoxical increase in brain P450arom expression induced by an inhibitor might arise by ligand-induced stabilization of the protein, changing its effective half-life. Our data argue strongly against this hypothesis based on the opposite response observed in testes, which our data demonstrate express the same *CYP19* gene. It is possible that P450arom expression in the porcine hypothalamus may actually be inhibited by estradiol. Even if due to androgen accumulating after inhibition of aromatization, it is more likely to involve changes in local synthesis of steroids in the hypothalamus itself [60]. Clearly, brain P450arom and testicular P450arom are regulated differently in the pig, and more extensive studies are required to understand exactly how.

Previous investigations have shown that, in addition to aromatization, the form of P450arom expressed in the gonads metabolizes testosterone to the nonaromatizable potent androgen 1OH-T. The present data indicate that the same form of the enzyme is expressed in the porcine hypothalamus and that it also synthesizes 1OH-T, the accumulation of which was inhibited by letrozole treatment, just as 19OH-testosterone and 19oxo-testosterone were. Earlier investigations demonstrated that 1OH-T is nonaromatizable and activates the androgen receptor [32]. This has potentially important implications for sexual differentiation of the brain, which is increasingly recognized to be influenced by androgenic and estrogenic organization [18]. Thus, inhibition of P450arom in pigs would potentially decrease the influence of both androgens and estrogens on this process, with possibly greater effect on reproductive function or potential. In addition, aromatase activity was measurable in the male pituitary, although at $<25\%$ of male hypothalamic levels, but was undetectable in female pituitary tissues. Thus, the porcine pituitary also appears to exhibit sexually dimorphic expression of P450arom, as does the rat [61, 62] and human [63]. Human investigations indicate that the inhibitory effect of testosterone on luteinizing hormone secretion is due at least in part to aromatization in the pituitary [64], but nothing is known of it role in pigs. It is likely that the novel activity of the porcine gonadal/hypothalamus P450arom enzyme, as well as its levels and sites of expression in the porcine hypothalamus and pituitary, will provide insight into the function of P450arom in regulating the neuroendocrine axis.

ACKNOWLEDGMENTS

The authors thank Dr. Bob Kraeling for advice and guidance, Kent Parker and staff at the University of California Davis Swine Center for help with animal care and tissue collection, Lil Sibley for steroid determinations, and Dr. Phil Kass for assistance with statistical analysis.

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