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# Molecular Profiling of Postnatal Development of the Hypothalamus in Female and Male Rats[1]

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## ABSTRACT

**Reproductive function is highly dynamic during postnatal developmental. Here, we performed molecular profiling of gene expression patterns in the hypothalamus of developing male and female rats to identify which genes are sexually dimorphic, to gain insight into a more complex network of hypothalamic genes, and to ascertain dynamic changes in their relationships with one another and with sex steroid hormones during development. Using a low-density PCR platform, we quantified mRNA levels in the preoptic area (POA) and medial basal hypothalamus (MBH), and assayed circulating estradiol, testosterone, and progesterone at six ages from birth through adulthood. Numerous genes underwent developmental change, particularly postnatal increases, decreases, or peaks/plateaus at puberty. Surprisingly, there were few sex differences; only *Esr1, Kiss1,* and *Tac2* were dimorphic (higher in females). Cluster analysis of gene expression revealed sexually dimorphic correlations in the POA but not the MBH from P30 (Postnatal Day 30) to P60. Hormone measurements showed few sex differences in developmental profiles of estradiol; higher levels of progesterone in females only after P30; and a developmental pattern of testosterone with a nadir at P30 followed by a dramatic increase through P60 (males). Furthermore, bionetwork analysis revealed that hypothalamic gene expression profiles and their relationships to hormones undergo dynamic developmental changes that differ considerably from adults. These data underscore the importance of developmental stage in considering the effects of hormones on the regulation of neuroendocrine genes in the hypothalamus. Moreover, the finding that few neuroendocrine genes are sexually dimorphic highlights the need to consider postnatal development from a network approach that allows assessment of interactions and patterns of expression.**

*gene expression, hypothalamus, puberty, sexual differentiation, steroid hormones*

## INTRODUCTION

For an individual to be able to reproduce, its reproductive system must be in synchrony with favorable external stimuli and internal factors that permit and even optimize reproductive success. For example, serum hormone levels and patterns of release, adequate nutritional status, and physiological and psychological maturity affect reproductive capacity, as do light cycles, seasonal variations, and immune function [1]. Of all of the body's systems, the reproductive system is one of the most highly dynamic in the context of the life cycle, with reproductive competence not being attained until after a complex and often protracted postnatal developmental and pubertal process has occurred. Much of this coordination is orchestrated through a hypothalamic neural and glial network that converges upon the gonadotropin-releasing hormone (GnRH) neurons that provide the final output from the brain to the pituitary gland. For example, the onset of puberty is initiated by an increase in the pulsatile release of GnRH from these neurons, but GnRH neurons are already mature at birth. This has led to the hypothesis that neural and glial inputs to GnRH neurons are responsible for initially clamping GnRH release at low levels prepubertally, then enabling pulsatile GnRH release to increase postnatally through the progression of puberty [2, 3].

The hypothalamus is structurally and functionally sexually dimorphic [4], something that plays out as sex differences in GnRH release, hypothalamic neural functioning, and reproductive behavior. Sexual differentiation of the hypothalamus is strongly influenced by differential exposure of male and female brains to sex steroid hormones in early life [5]. However, GnRH neurons themselves do not express most sex steroid hormone receptors [6, 7]. Therefore, the effects of developmental steroids on this neural network are mediated by afferent inputs to GnRH neurons. Excitatory inputs thought to regulate GnRH include kisspeptin (*Kiss1*), neurokinin B (*Tac2*), and glutamate, whereas inhibitory inputs include the opioid, dynorphin [8, 9]. Gamma-aminobutyric acid exerts both excitatory and inhibitory influences, and this balance may contribute to developmental regulation of GnRH [10]. Transcription factors that activate or repress gene expression also play a key role [9]. Nevertheless, it remains unclear how these and other factors interact to result in the attainment of reproductive competence.

There is considerable literature on how peripheral sex hormones, especially estradiol ($E_2$) and testosterone (T), regulate gene expression in the hypothalamus. Most of this work was conducted in adults using models of castration and/or hormone treatments [11–22]. A separate body of work has addressed how circulating serum hormones are involved during sexual differentiation of the brain during early life [5]. However, there is surprisingly little research on the relationship between hormones and gene expression throughout postnatal development in an intact model [23]. Considering that the brain remains sensitive to steroid hormones postnatally [24], during which time there are large changes in both hormone and neurotransmitter release, this is an important gap in research.

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While previous studies have measured gene expression in the hypothalamus and characterized circulating hormones, much of this work has been cross-sectional, used few ages, did not compare the sexes, or studied one or a few genes. The literature for individual genes and hormones varies widely because of these experimental differences. Thus, our goal was to provide a more unified perspective on hormones, gene expression, and developmental sex differences through quantification of 48 hypothalamic genes and assaying serum concentrations of sex steroid hormones in male and female animals across postnatal development.

## MATERIALS AND METHODS

### Animals

All the animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin Institutional Animal Care and Use Committee. Sex differences in neuroendocrine gene expression and serum hormone concentrations were investigated throughout postnatal development on Postnatal Days (P) 1, 5, 15, 30, 45, and 60. Sprague-Dawley rats were purchased from Harlan Laboratories (Houston, TX) and impregnated in house. Dams were allowed to give birth, and on the day after birth (P1), the litter composition and birth weights were recorded. The study was designed to distribute male and female littermates across as many different ages as possible. For earlier time points (P1 and P5), two same-sex siblings were pooled to ensure enough RNA and serum for all the assays. For later time points (P15, P30, P45, and P60), individual rats were used. At P1 and P5, samples from two littermates were pooled to get a targeted $n = 8$ (see the figures for the final numbers). A total of 10 litters were used to achieve this number. Pups were weaned on P21 and housed in same-sex groups (two to three per cage) with food and water provided ad libitum, at a constant temperature (21°C–22°C), and with a partially reversed 12:12 light cycle (lights-on at 2300 h; lights-off at 1100 h). A secondary sex characteristic of the onset of puberty was monitored daily (preputial separation in males and vaginal opening in females). After vaginal opening, the females were subjected to daily vaginal smears.

### Tissue Collection and Storage

Rats were euthanized by rapid decapitation between 1030 and 1130 h. Postpubertal females were euthanized on the day of proestrus as indicated by vaginal cell cytology. Brains were removed and the preoptic area (POA) and medial basal hypothalamus (MBH) were dissected using standard brain landmarks as described [25, 26] and snap frozen on dry ice. Trunk bloods were collected and allowed to clot, and the serum was separated via centrifugation ($1500 \times g$ for 5 min). Tissues and serum were stored at −80°C until use.

### RNA Extraction

RNA was extracted from frozen POA and MBH tissues using an in-house double detergent lysis buffer system [26]. Samples were homogenized using a 22-gauge needle and 1-ml syringe; cytoplasmic RNA was treated with proteinase K, extracted with phenol chloroform, and precipitated in ethanol. Resuspended RNA was treated with 1 unit of TURBO DNase (Applied Biosystems Inc., Foster City, CA) to rid samples of genomic DNA. All the samples were run on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) to assess RNA purity, integrity, and concentration.

### Taqman Microfluidic Real-time PCR Cards

Samples were run on custom-designed microfluidic 48-gene PCR cards (Applied Biosystems) with specific gene assays chosen based on a priori hypotheses and published reports on their importance in neuroendocrine function, timing of puberty, steroid hormone responsiveness, and sex differences (46 genes of interest and 2 housekeeping genes; a complete list is provided in Supplemental Table S1; all the Supplemental Data are available online at www.biolreprod.org). Because it is a Taqman PCR-based card, it does not require further PCR validation (which would be redundant). Nevertheless, we have previously validated this assay by comparing it with conventional gene-by-gene PCR assays and had excellent replication of results from identical samples run by the two methods [23]. Inter- and intrasample variability on the cards is low [23], and we have since been using single samples because of the high cost of the assay together with its very low variability in our hands. Cytoplasmic RNA (2 μg) was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to manufacturer's protocol. The product was stored at −20°C until use at which time samples were diluted 1:10 before PCR reactions were conducted.

Real-time RT-PCR was carried out on an ABI 7900 using Taqman Universal Mastermix (Applied Biosystems) and the following run parameters: 50°C for 2 min, 95°C for 10 min, 45 cycles of 94.5°C for 30 sec, and 59.7°C for 1 min. Relative expression was determined for each sample using the comparative Ct method [27–29]. Samples were normalized to *Gapdh* as described previously [23, 30] and calibrated to the median delta-Ct of the group with the lowest expression to determine fold change in the expression for each gene.

### Serum Hormone Assays

Serum hormone concentrations were measured using radioimmunoassay for $E_2$, progesterone ($P_4$), and T (Beckman Coulter, Webster, TX) according to the manufacturer's recommended protocols. Samples were run in duplicate if possible. For the youngest animals (P1 and P5), serum was pooled when necessary to ensure there was sufficient serum for all the assays. Samples having a coefficient of variation of 10% or greater were either rerun when possible or dropped from the analysis ($E_2 = 5$ samples dropped, $T = 1$ sample dropped, $P_4 = 2$ samples dropped). In a few cases, there was not enough serum to run all the serum hormones for the P1 animals, and so serum from other P1 animals was used for the T and $E_2$ assays to maintain the sample size as close to $n = 8$ as possible.

For the $E_2$ assay, two assays were run with assay sensitivity of 2.2 pg/ml and intraassay variability of 4.22% (14 samples) and 4.06% (90 samples). Interassay variability was 11.49%. For the T assay, two assays were run with assay sensitivity of 0.08 ng/ml and intraassay variability of 2.47% (88 sample) and 3.2% (27 samples). Interassay variability was 36.86%. While the interassay variability is high, each assay had representatives from each age and sex; there was not a significant difference between the two runs in serum concentrations obtained; and group data show small standard error bars suggesting that the variability did not affect the validity of serum results. These T levels were also in line with those reported previously in our laboratory [23] and others [31]. For the $P_4$ assay, one assay was run with assay sensitivity of 0.12 ng/ml and intraassay variability of 2.96%.

### Statistics

For gene expression data, statistics were performed using relative expression for each sample. Multiple regression analysis was conducted using PASW software (IBM, Armonk NY) to compare each endpoint (genes and hormones) using age and sex as independent variables. For those endpoints where a significant main effect of age or a sex-by-age interaction was observed, a Tukey-Kramer post hoc analysis was performed to determine specific differences between each group. If the data did not meet the assumptions for multiple regression, the data were transformed (natural log or square root) and reanalyzed. In a few cases, transformed data did not meet the assumptions for statistical analysis by multiple regression. In those cases, data were analyzed using a Kruskal-Wallis test followed by Mann-Whitney test between each group. For hormone concentrations, an effect was considered significant at $P < 0.05$. For gene expression data, a Benjamini and Hochberg false-discovery rate correction [32, 33] was used to correct our $P$ values in order to account for the large number of variables measured. Gene expression data were tested for outliers using the z-score of the residuals from the initial regression. A data point was considered an outlier if the residual was greater than 2.5 standard deviations from initial line of best fit. For hormone concentrations, data were tested for outliers using the Grubbs outlier test. Confirmed outliers were excluded from the final analysis.

To examine possible relationships between gene expression and serum hormones throughout development, the data were analyzed using a bootstrap technique [34]. Briefly, original paired data was resampled with replacement for 1000 repetitions, and the Pearson correlation coefficient was computed on each of these new bootstrapped data sets to build a distribution of coefficients. The significance of the Pearson correlation coefficient for each gene/gene or gene/hormone interaction was determined from the bootstrapped distributions. Only those correlation coefficients that survived a Benjamini and Hochberg false discovery rate [32, 33] at a $P$ value $< 0.05$ were considered significant. The bootstrap and subsequent analyses were performed using Matlab (The Mathworks, Natick, MA) software.

Hierarchical cluster analysis and heat maps were performed using Multiple Experiment Viewer V4.8.1 (MeV 4.8.1; TM4.org), and clusters were validated using R statistical packages.

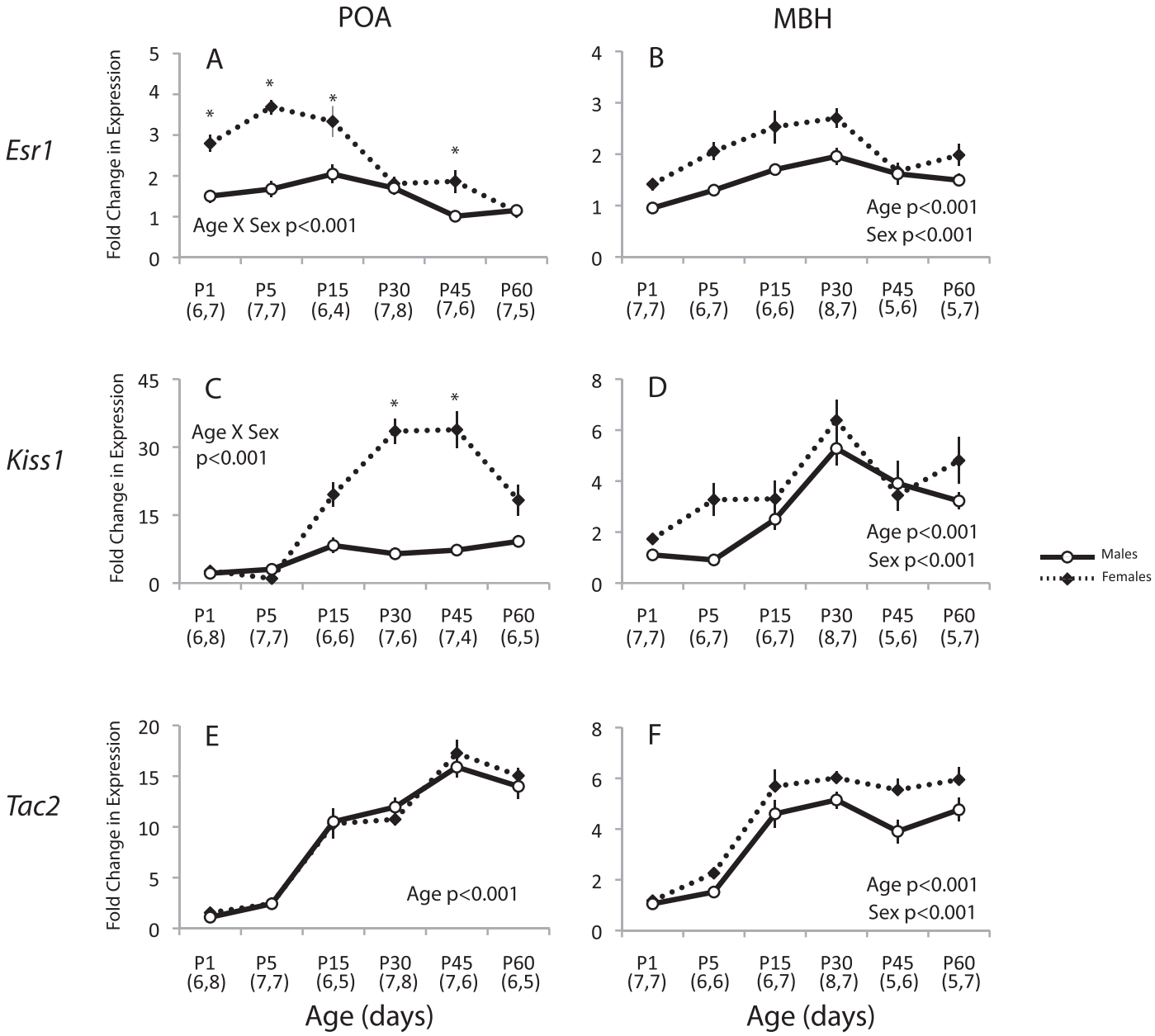


FIG. 1. Expression of the three sexually dimorphic genes is shown in the POA (left) and the MBH (right) on Postnatal Days (P) 1, 5, 15, 30, 45, and 60 in males (solid lines) and females (dotted lines). Data are expressed as fold change in relative expression, with the age/sex having the lowest expression level set at 1.0. *Esr1* (**A**, **B**) and *Kiss1* (**C**, **D**) had a significant interaction of age and sex in the POA ($P < 0.001$), and there were significant main effects of age ($P < 0.001$) and sex ($P < 0.001$) in MBH. *Tac2* (**E**, **F**) was sexually dimorphic in the MBH only ($P < 0.001$) and underwent a significant developmental increase in both regions ($P < 0.001$). Significant sex differences for each age are indicated by asterisk. Too many significant age-related changes were observed in both males and females to depict on the figure, and are summarized here. In the POA, *Esr1* decreases significantly from P15 to P30 ($P < 0.009$) in females and from P30 to P45 in males ($P = 0.019$). *Kiss1* increases from P5 to P15 in both males ($P = 0.002$) and females ($P < 0.001$). *Tac2* significantly increases from P1 to P5 ($P < 0.001$), P5 to P15 ($P < 0.001$), and P30 to P45 ($P = 0.002$). In the MBH, *Esr1* increases from P1 to P15 ($P = 0.009$). *Kiss1* significantly increases from P1 to P15 ($P < 0.001$) and P5 to P30 ($P < 0.001$). *Tac2* increases from P1 to P5 ($P < 0.001$) and P5 to P15 ($P < 0.001$). The numbers of rats here and in other figures are shown parenthetically for males (left) and females (right).

## RESULTS

### *Sex Differences in Developmental Profiles of Neuroendocrine Genes in the POA and MBH*

Expression of 48 genes was measured by real-time RT-PCR throughout development in the POA and MBH of females and males using the Taqman low-density array. Of these, only three genes exhibited any sexual dimorphism, with a significant age-by-sex effect for *Esr1* and *Kiss1* in the POA (Fig. 1, A and C), and main effects of sex for *Esr1*, *Kiss1*, and *Tac2* in the MBH.

In the POA, *Esr1* expression was significantly greater (~3-fold) in females than males early in development (P1–P15; $P < 0.05$), a difference that disappeared after adolescence (P30–P60; Fig. 1A). Additionally, a significant main effect of age was found for *Esr1* expression, with developmental decreases in both female and male POA, albeit a much smaller magnitude of change in males. *Kiss1* mRNA (Fig. 1C) expression was also

sexually dimorphic in the POA, with significantly greater expression (~3-fold) in females when compared to males from P30 to P45 ($P < 0.01$). Additionally, age related increases were observed for *Kiss1* in both males and females, (~30-fold increase in females from P5 to 30, and ~10-fold increase in males from P5 to P15). *Tac2* mRNA expression in the POA (Fig. 1E) demonstrated a significant age-related increase, but no sex difference. Post hoc analysis revealed a significant increase (~5-fold) in expression from P1 to P15 followed by another significant 2-fold increase from P30 to P45 ($P < 0.01$) in both sexes.

In the MBH, there were significant main effects of sex ($P < 0.001$) and age ($P < 0.001$) for *Esr1, Kiss1,* and *Tac2* (Fig. 1, B, D, and F) but no significant interactions. *Esr1* expression was greater in developing females than males, with significant increases from P1 to P15 (~2-fold). *Kiss1* (Fig. 1D) expression increased significantly from P1 to P15 (~2-fold) and P5 to P30 (~3-fold) in males and females. Females had significantly greater *Kiss1* expression than males, especially early in postnatal life. There was a trend for an interaction of sex and age ($P = 0.02$); however, this effect did not survive a false-discovery rate correction. Finally, *Tac2* (Fig. 1F) expression was greater in females than males ($P < 0.001$), and the expression increased ~ 3-fold from P5 to P15 in both sexes.

### Developmental Changes in Neuroendocrine Gene Expression in the POA and MBH

The real-time PCR array contains two internal controls for normalization: *Gapdh*, and *18s*. Both controls displayed small (~20%) but significant age-related changes. Therefore, we took a conservative approach: only genes that survived a false discovery rate correction and displayed greater than a 2-fold change in expression were included for further analysis. (This was also taken into consideration in the gene analysis described above for *Esr1, Kiss1,* and *Tac2*.) In the POA and MBH, 31 and 29 genes, respectively, met these criteria. In order to maintain consistency with past publications that used the same technology, all the gene expression was normalized to *Gapdh* expression [23]. For graphic purposes, genes were categorized into related functional groups (Fig. 2). Because no sex differences were observed, the data were collapsed across the sexes for graphing and analysis. Of those genes undergoing substantial developmental change, the most common patterns were a developmental decrease or developmental increase through puberty, with regional differences for a subset of genes.

In order to determine the relationships among specific genes across postnatal development, hierarchical cluster analysis was conducted using MeV 4.8.1 and validated using R statistical packages. Clustergrams were constructed using MeV 4.8.1 software (Fig. 3) for each brain region and sex using the correlation coefficients to express similarity and the average linkage method. Validated clusters are indicated in red. In general, the genes clustered into two broad groups identified from inspection of individual developmental profiles (Figs. 1 and 2): those that decreased from P1 and those that increased from P1. Clusters by sex and age reveal sex differences in the POA (Fig. 3A) from P30 to P60 that were not observed in the MBH (Fig. 3B). In the POA (Fig. 3A), males and females clustered together from P1 to P15, but from P30 to P60, the sexes segregated into two separate clusters. In the MBH (Fig. 3B), females and males clustered together by age from P1 through P60 indicating that while there were few sex differences in the expression patterns of gene expression, there were sex differences in the correlation of genes in the POA but not the MBH.

### Sex Differences in the Developmental Profiles of Serum Hormone Concentrations

Serum concentrations of $E_2$ (Fig. 4A), T (Fig. 4B), and $P_4$ (Fig. 4C) were measured in the same developing rats as those used for gene expression. In both sexes, $E_2$ concentrations were significantly greater on P15 when compared to any other time point ($P < 0.05$), but there were no sex differences. In males, T exhibited a nadir at P30, followed by a dramatic increase to P60. Mann-Whitney post hoc analysis revealed that concentrations were significantly greater in males when compared to females on all days except P30. In females, serum T was significantly greater on P15 when compared to all the other days. Finally, serum T was significantly greater on P60 in males than all the other ages regardless of sex ($P < 0.05$). For $P_4$, significant age-by-sex interactions as well as significant main effects of age and sex ($P < 0.001$), were detected. Progesterone concentrations were identical in males and females from P1 to P30. From P45 to P60, $P_4$ levels were significantly greater in females than males, as well as all the other ages in both males and females ($P < 0.01$).

### Integration of Genes and Hormones as Developmental Networks

To examine the relationship between gonadal hormones and hypothalamic gene expression throughout development, we used the network analysis platform Cytoscape [35–37] to generate networks based on significant Pearson correlation coefficients between hormones and relative gene expression in each brain region. Data were first collapsed across the sexes for each age in each region to identify relationships that change throughout postnatal development regardless of sex (Figs. 5 and 6). In order to display the networks in a legible format, the networks for P1 and P5 in both the POA and MBH are not shown in Figures 5 and 6 but are included in Supplemental Figures S1 and S2.

In the POA, the highest number of significant correlations was detected on P1 (Supplemental Fig. S1A) and P30 (Fig. 5B). On P15 (Fig. 5A), there were a number of negative correlations, many of them with sex steroid hormones and their receptors, an effect not observed for the other ages. From P30 to P60 (Fig. 5, B–D) in the POA, the majority of the correlations were positive. Finally on P60, *Gnrh1* was negatively correlated with seven genes; four are growth factors or their receptors, two are associated with neurotransmitters (*Gabbr2* and *Grin2c*), and one is the steroidogenic enzyme, aromatase p450 (*Cyp19a1*). In the MBH (Fig. 6, A–D), there were more correlations on P5 (Supplemental Fig. S2B) than on any other day. Additionally on P15, *Pdyn* was negatively correlated with a number of genes, and on P45 *Hsd17b2* was negatively correlated with seven genes, six of which were neurotransmitters.

## DISCUSSION

In the current study, we profiled a network of neuroendocrine-related hypothalamic genes and serum hormones in the context of postnatal development of the two sexes. Our results revealed novel relationships and surprisingly few sex differences, leading us to believe that the developing hypothalamus has very different hormone-dependent and -independent properties from those in adults. A number of published studies have identified sex differences in gene expression [15, 38–43],

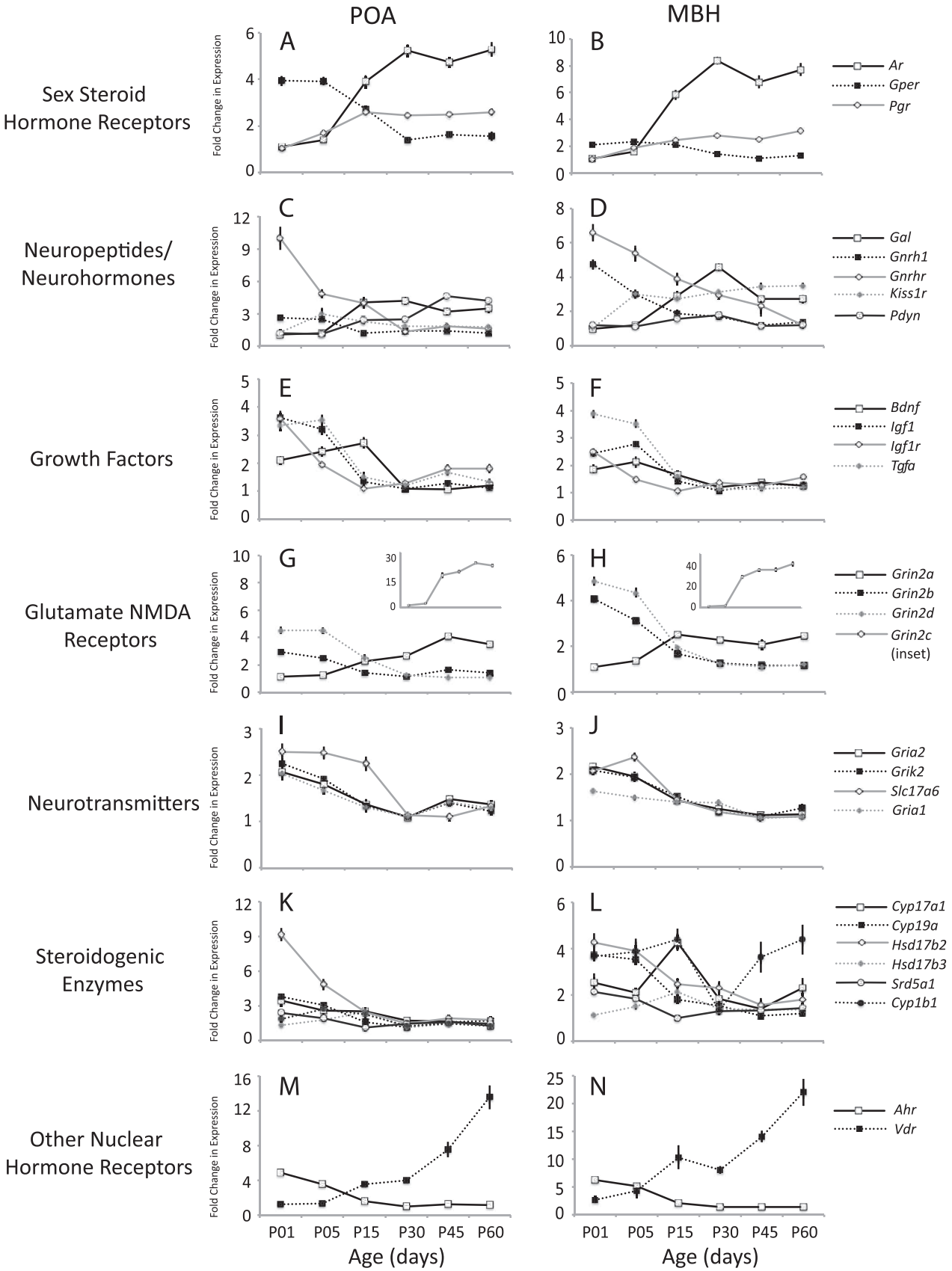


FIG. 2. Nonsexually dimorphic genes that underwent developmental change are shown in the POA (left; **A**, **C**, **E**, **G**, **I**, **K**, **M**) and MBH (right; **B**, **D**, **F**, **H**, **J**, **L**, **N**) of developing rats. Data are shown combined for males and females because there were no sex differences, and the results are displayed as relative fold change. Genes were graphed as functional groups. In **G** and **H**, because *Grin2c* underwent substantially higher fold change than other N-methyl-D-aspartate (NMDA) receptor subunits, its presentation is broken out into an inset.

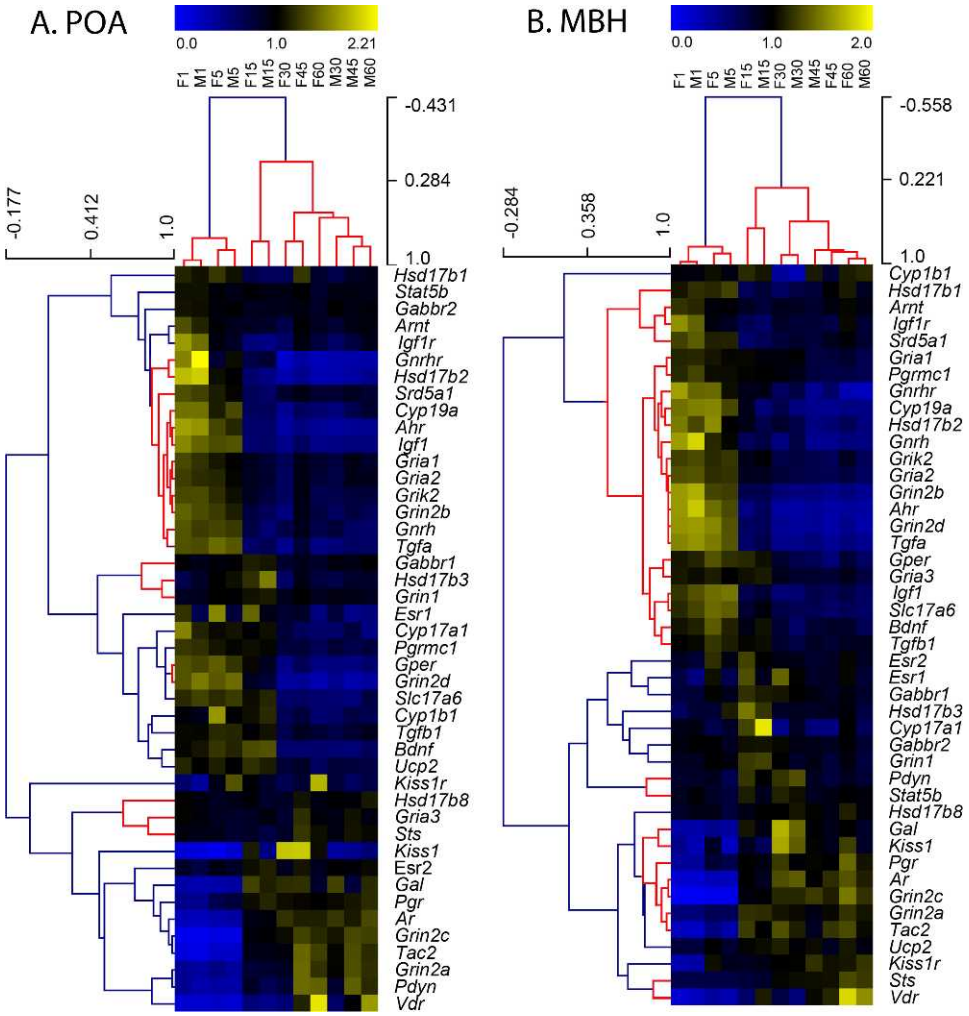


FIG. 3. Hierarchical cluster analysis using correlation coefficients to express similarity and the average linkage method was conducted using the Multiple Experiment Viewer V4.8.1 (TM4.org), and clusters were validated using R statistical packages to reveal genes that grouped by expression patterns in the POA (**A**) and MBH (**B**). Data are displayed as highest expression in yellow and lowest expression in blue, and validated clusters are indicated in red brackets for genes (to the left of the clustergrams) and for sex and age (above the clustergrams). For both POA and MBH, genes clustered into two general expression patterns; those increasing from P1 (bottom) or decreasing from P1 (top). In the POA, males and females clustered together by age from P1 to P15. However, from P30 to P60 the females and males clustered within the sexes. In the MBH, males and females clustered by age throughout development.

protein expression [44–47], and epigenetic changes [40, 48, 49] in the brain during postnatal development. However, our study differs from all the other published work in several fundamental ways. First, using a real-time PCR panel, we were able to study a network of 48 neuroendocrine genes implicated in the hypothalamic control of pubertal development, allowing us to do specific hypothesis testing about important genes implicated in brain sexual differentiation (e.g., *Esr1*, *Kiss1*, *Tac2*) and at the same time, identify potentially novel pathways not systematically studied in the hypothalamus. Second, we utilized developing male and female littermates distributed across six ages chosen to span perinatal, prepubertal, peripubertal, and postpubertal periods. This type of systematic approach is important because it eliminates confounds of overreliance on littermates within a single age group. Third, we compared rostral (POA) and caudal (MBH) hypothalamic regions to enhance understanding of tissue specificity in expression. Fourth, we measured the three major gonadal steroids in these same animals, something rarely done in a single study containing both sexes [31, 50] and not previously done in the context of hypothalamic gene expression. Therefore, we believe that we are the first to determine not only how numerous neuroendocrine genes change over time but also whether and how their relationships change in a sex-specific manner, together with their associations with serum hormones.

### Esr1, Kiss1, *and* Tac2 *mRNAs Are Sexually Dimorphic in the Hypothalamus*

One of the most surprising results of this study was that only three of the 48 genes analyzed showed significant sexual dimorphisms: *Esr1*, *Kiss1*, and *Tac2*, all necessary for reproductive function and the pubertal transition [2, 8, 51]. Furthermore, these genes are coexpressed in the same kisspeptinergic neurons in parts of the hypothalamus. More specifically, in the POA, nearly all of the Kiss1 neurons coexpress *Esr1*, and in the MBH, there is a population of Kiss1-expressing neurons that also expresses *Tac2* and *Pdyn*, often referred to as KNDy neurons. The KNDy neurons also express *Esr1* and are sensitive to fluctuations in $E_2$ (reviewed in [52]). Our findings of robust developmental changes in a

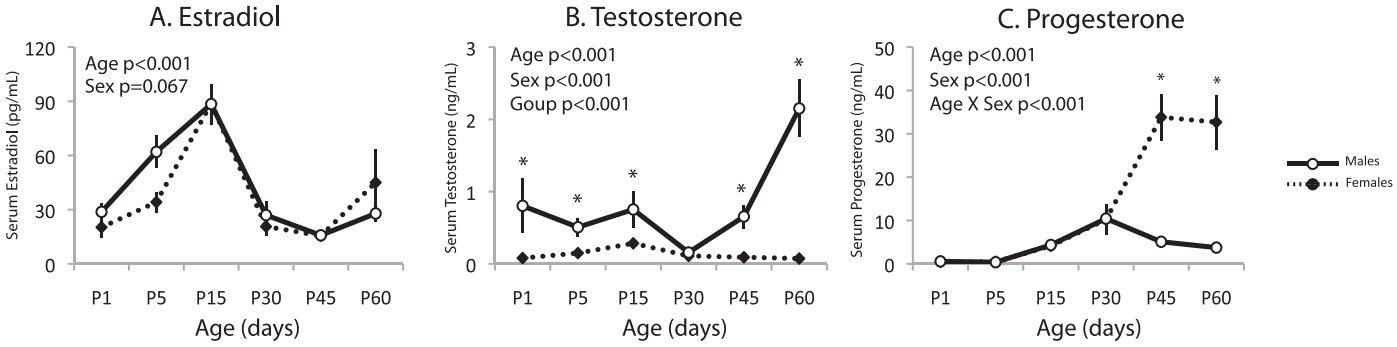


FIG. 4. Serum $E_2$ (**A**), T (**B**), and $P_4$ (**C**) concentrations are shown in male (solid line) and female (dotted line) rats throughout postnatal development. Serum $E_2$ (**A**) changed significantly in males and females throughout postnatal development ($P < 0.001$) and was highest on P15 in both sexes. Serum T (**B**) changed significantly with age ($P < 0.001$). In males, serum T dropped to a nadir on P30 and then increased into adulthood, whereas in females, serum T peaked at P15 ($P < 0.05$). For $E_2$ and T, a significant group effect was also determined by nonparametric analysis. Serum $P_4$ had significant changes by age ($P < 0.001$), sex ($P < 0.001$), and a significant interaction of sex and age ($P < 0.001$). In both sexes, serum $P_4$ increased until P30 but diverged thereafter, with levels falling in males and increasing in females. Asterisk denotes significant sex differences at each age. Age-related changes were also observed that were too numerous to depict on the figure. Serum $E_2$ increases from P1 to P5 ($P = 0.004$) and decreases from P15 to P30 ($P < 0.001$). In males, serum T is greatest on P60 ($P < 0.05$) and lowest on P30 ($P < 0.05$) when compared to all the other days. In females, T is significantly greater on P15 when compared to all the other days ($P < 0.05$). In both males and females serum $P_4$ significantly increases from P5 to P15 in males and females ($P < 0.001$) and from P30 to P45 ($P < 0.001$) in females only.

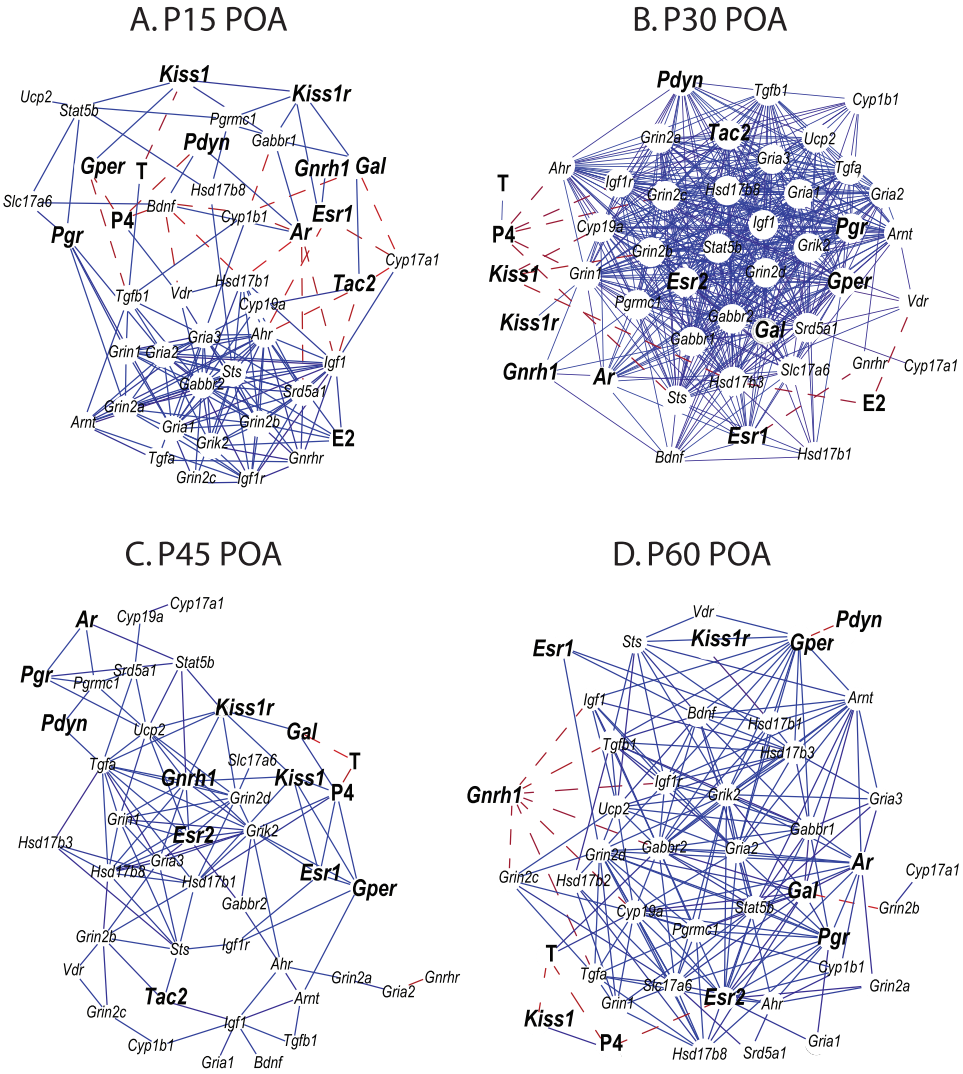


FIG. 5. Networks of gene and hormone correlations in the POA are shown on those developmental days with the greatest change (P15, P30, P45, and P60; **A**–**D**, respectively). Positive correlations are indicated as blue solid lines, and negative correlations are indicated as red dotted lines. Thirteen specific genes and hormones are indicated by the larger font and bolded text as a selected subset (see the text for a description).

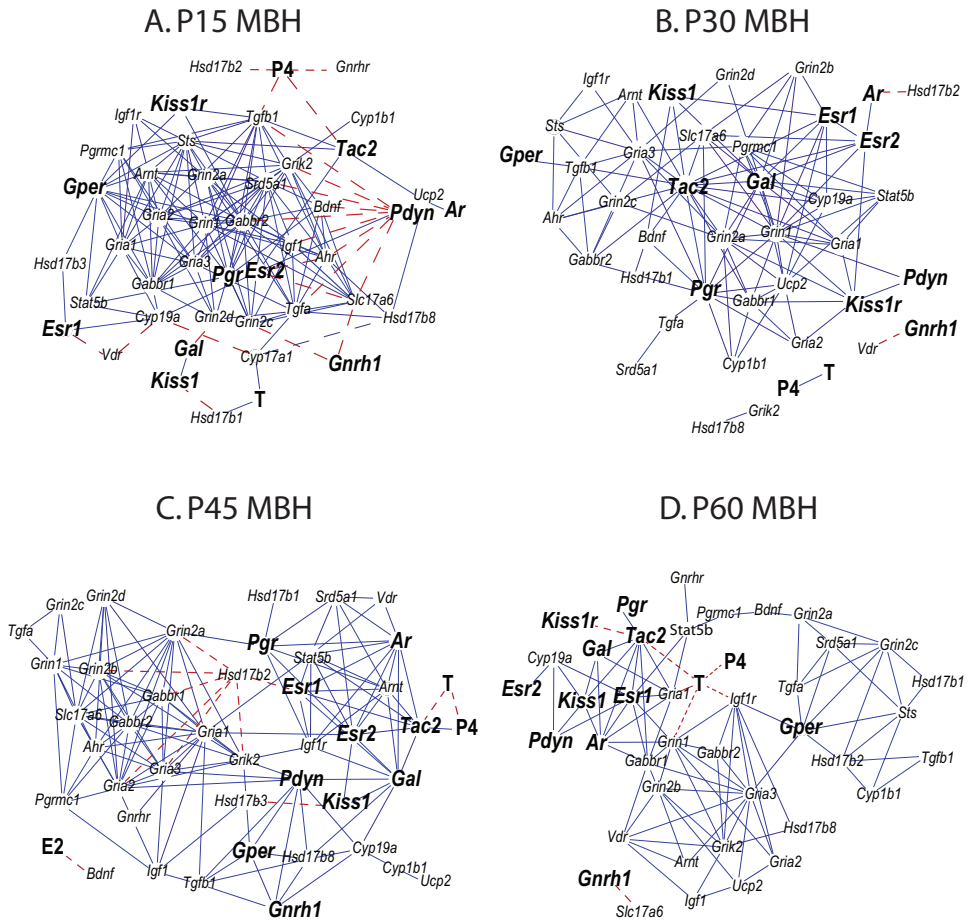


FIG. 6. Networks of gene and hormone correlations in the MBH are shown on those developmental days with the greatest change (P15, P30, P45, and P60; **A**–**D**, respectively). Positive correlations are indicated as blue solid lines, and negative correlations are indicated as red dotted lines. Thirteen specific genes and hormones are indicated by the larger font and bolded text as a selected subset (see the text for a description).

sex- and region-specific manner support the importance of these genes in the maturation of the hypothalamus.

In both hypothalamic regions studied, *Kiss1* and *Tac2* increased developmentally. By contrast, *Esr1* decreased in the POA, and in the MBH, it increased to P30, then decreased from P30 to P60. While the decrease in *Esr1* expression was surprising, a recent study observed a similar expression pattern [41] in several hypothalamic nuclei (anteroventral periventricular nucleus [AVPV], medial POA, arcuate nucleus [ARC], and ventromedial nucleus). Interestingly, knocking out *Esr1* specifically in kisspeptin neurons advanced the timing of puberty in females [53], suggesting that there may be a functional significance to the developmental decrease seen in the current study. The sexual dimorphism of *Kiss1* and *Esr1* found herein replicated previous studies in young animals [38, 41–43]. However, one novel finding of our study is that *Kiss1* and *Esr1* are not sexually dimorphic in the adult (P60) POA. Numerous studies have shown that *Kiss1* is greater in the female AVPV (reviewed in [54]) of adult animals. However, in the present study, *Kiss1* expression is only significantly greater in the female POA on P30 and P45 (with a trend on P15; $P = 0.073$). Additionally, it should be noted that we do not observe sex differences in expression of numerous genes that been reported to be sexually dimorphic in the rostral hypothalamus (reviewed in [55]) including, but not limited to, *Esr1* [56] and androgen receptor [15, 57]. We hypothesize that these differences are likely the result of differences in experimental design, including the time of day the animals were euthanized (our animals are euthanized prior to lights out), cycle stage of the females (euthanized here on proestrus), and gonadal status (our rats were intact). Furthermore, the POA and MBH dissections utilized here include several smaller subnuclei that may be differentially regulated by age, sex, and/or hormones, something that we are currently addressing in ongoing work using hypothalamic microdissections. Even within more discrete hypothalamic regions, it is also necessary to recognize the fundamental heterogeneity of nervous system tissue—with a mixture of neuronal phenotypes, glial cells, blood vessels, etc.—requiring interpretation of all the data in light of this mix of cells. Therefore, a limitation of gene expression work on homogenized tissue is the ability to determine specific cellular changes, something that needs to be accomplished by other methodologies such as immunohistochemistry, in situ hybridization, or single cell profiling. Despite these potential limitations, our results in POA and MBH highlight the complex and differential interplay between sex steroid hormones, neuroendocrine genes, and hypothalamic region in response to circadian cycles and gonadal status. Indeed, recent studies in females suggest that *Kiss1* expression is under circadian control in the AVPV of female rodents (males were not investigated) [58, 59]. Future studies should include intact animals euthanized at different circadian intervals to gain an understanding of these complex interactions.

There is little published research on dimorphisms of *Tac2*, found here in the MBH but not the POA of our developing rats. While previous work reported that *Tac2* is not sexually dimorphic in the KNDy neurons in the ARC [17, 60, 61], there are the same caveats as mentioned above for *Kiss1*. Furthermore, *Tac2* in the MBH and potentially in the KNDy neurons themselves may also be sensitive to circadian/estrous regulation. In support of this hypothesis, Tac2 expression in the ARC is greatest on proestrus in females [62] (again, males were not investigated). While the functional role of the KNDy neurons in the ARC is only beginning to be understood, Navarro et al. [17, 60] have hypothesized that *Tac2* may function as a pacemaking signal for *Kiss1* release from these neurons, a hypothesis that is supported by our current study.

### *Developmental Nonsexually Dimorphic Changes in Gene Expression*

The PCR platform identified an additional 28 or 26 genes that changed significantly and displayed a greater than 2-fold change throughout postnatal development in either the POA or MBH, respectively (Fig. 2). While there were some differences in the extent of fold changes observed between the POA and MBH, many of the genes shared similar expression patterns throughout postnatal development. However, there were notable differences between the two regions. In the POA but not MBH, two genes changed significantly with developmental age: *Pdyn* increased 4-fold (Fig. 2, C and D) and *Gria1* (Fig. 2, I and J) decreased 2-fold from P1 to P60. Additionally, two steroidogenic enzymes, *Cyp1b* and *Cyp17a1,* had different expression patterns in the MBH and POA. *Cyp1b1* metabolizes 17β-estradiol and *Cyp17a1* catalyzes the conversion of cholesterol to progestins [63]. Differences in these enzymes might indicate a drop in local available steroid neurohormones. There is an intriguing body of work suggesting that polymorphisms in Cyp17 alleles may be associated with the advancement of puberty in young women [64, 65], implicating this gene in the tempo of reproductive maturation.

Interestingly, the few genes on our array implicated in the inhibition of GnRH secretion, namely, *Pdyn* (MBH only), *Gabbr1, Gabbr2, Tgfb1,* and *Pgrmc1,* showed little change in expression throughout development (data not shown with the exception of *Pdyn*). However, genes associated with excitatory inputs to the GnRH system changed substantially in the current study. Previously, it has been hypothesized that the onset of puberty is regulated by an increase in excitatory and a decrease in inhibitory inputs onto the GnRH system [2, 3]. The current findings provide evidence that the mechanisms regulating these inputs may differ in the hypothalamus during development. Importantly, in our study, the developmental expression patterns seen for stimulatory inputs undergo dynamic change, suggesting that there is some reorganization of the excitatory inputs and not just a simple increase in stimulation to the GnRH neurons. We hypothesize that during postnatal development, stimulatory signals may switch to regulation by neuropeptides that are more responsive to peripheral changes in sex steroid hormones, including *Kiss1, Gal,* and *Tac2*, all of which increase dramatically during this period. While this hypothesis needs further evaluation, our data provide intriguing preliminary evidence of this possibility. Our data also do not negate the potential importance of inhibitory neurotransmitters and their relationship to postnatal development, as posttranscriptional changes may occur to these factors that are not necessarily paralleled by mRNA changes.

### *Cluster Analysis Reveals Sex Differences in Expression Patterns*

We used cluster analysis to evaluate sex and age differences in correlations of genes as a way to predict common regulators of expression (Fig. 3). In general, genes in the POA and MBH clustered according to two expression profiles: those that increased from P1 (bottom cluster) or decreased from P1 (top cluster). Interestingly, in the POA, males and females cluster together by age on P1, P5, and P15, but the two sexes cluster separately from P30 to P60, suggesting that sex differences in correlations in the POA emerge after P30. This effect was not observed in the MBH where the males and females of each age cluster together throughout development. Taken together these data provide evidence that sexual dimorphisms in expression in the POA are age dependent and that the developmental stage of the animals should be taken into account in conducting sex differences studies.

### *Sex Differences in Serum Hormone Concentrations*

Developmental concentrations of sex hormones have previously been described in rodents [23, 31, 42, 66–70], but to our knowledge only one previous publication has characterized sex differences in the three major gonadal hormones across development within a single study [31]. Concentrations of $E_2$, $P_4$, and T in our rats were quite similar to those from the literature, and with only one exception (discussed below), postnatal patterns and sex differences were also consistent with the published literature.

Other reports of postnatal developmental changes in circulating $E_2$ demonstrate that levels are at their highest early in life and then experience a decrease, but the exact age at which the peak occurs varies among studies. Two groups [31, 71] reported that $E_2$ levels were highest on P1, declined over the next few days, then had a smaller peak at about Day 9, falling again thereafter. In a previous study, when we measured serum $E_2$ levels at P1, P5, P15, P30, P45, and P60 in males, our results matched these other reports, with an overall decrease from P1 through P30 [23]. By contrast, in the current study, $E_2$ concentrations were highest in both sexes on P15. The developmental changes in serum $E_2$ observed here were also similar to those reported for hamsters [70] and male rats [69]. The overall conclusion that we draw from current and past work is that serum $E_2$ concentrations are similar in developing female and male rats and that they undergo a decrease as the animals mature. While we do not know the explanation for differences among the studies, they may be attributable to intrauterine position, prenatal litter size and sex ratio; postnatal litter composition, including pup numbers and sex ratio after culling of litters; time of day that animals were euthanized; or differences in hormone-binding proteins such as alpha-fetoprotein, among others.

Serum T in the current study was greater in males than females for most of postnatal development with the exception of P30 when male T concentrations dropped to undetectable levels. This finding in males replicated our and others' previous reports [23, 31]. Additionally, we noted a significant peak of T in females on P15, similar to an earlier study [31].

Likewise, our results on serum $P_4$ were consistent with the literature [31, 66]. We observed significant sex differences in serum $P_4$ after puberty, with much higher $P_4$ concentrations observed in females on P45 and P60. Again similar to previous findings, serum $P_4$ peaked in male rats on P30, the same day when serum T is undetectable in males [23, 31, 66]. Taken together, these data provide important developmental time

points that may be important for the sex differences in the timing of puberty.

### *Network Analysis of Genes and Hormones Reveals Novel Interactions During Postnatal Development*

As in our previous study [23], we noticed that a number of genes reported to be regulated by steroid hormones displayed developmental patterns that diverged from predicted relationships to specific serum hormone concentrations. For example, *Pgr* (progesterone receptor) is regulated by *Esr1* and its ligand $E_2$ in numerous areas of the female brain but not the male brain [11, 72, 73]. However, in our study serum $E_2$ decreased from P15 to P45, a time when *Pgr* is unchanged in both the male and female POA and MBH. Therefore, we used Cytoscape to assemble networks of genes and hormones with significant correlations according to developmental age in the hopes of generating new hypotheses about how genes and hormones interact in an intact rat model throughout development. Furthermore, because expression of a gene is not necessarily paralleled by expression of its protein product, results on genes that undergo developmental change and/or sex differences should be followed up by Western blot analysis or immunohistochemistry. In fact, we hope that our gene bionetwork results will serve as a resource to guide future experimentation in this arena. Here, to simplify our model, we combined data from the two sexes and highlighted thirteen selected endpoints (indicated in larger font and bold text in Figs. 5 and 6 and Supplemental Figs. S1 and S2) to facilitate the identification of regulatory networks. However, we present the entire network so that readers can search for relationships among other endpoints of interest. This network analysis showed surprising interactions among hormones and sex steroid hormone receptors that challenge the traditional dogma of how sex steroid hormone receptors are regulated by their ligands [23]. For example, at no point in development is *Pgr* positively correlated with $E_2$ (in fact, on P5 in the POA, *Pgr* is negatively correlated with $E_2$). With the exception of the P15 POA, it is rare that any of the serum hormones serves as a regulatory hub in either the POA or the MBH. As a whole, the network analysis shows the complexity of sex steroid hormone interactions with gene expression in the brain of developing rats.

Because sex steroid hormone receptors are necessary for sexual differentiation of the brain (reviewed in [55]) and reproductive function (reviewed in [52]), we expected that they would be major hubs in our networks throughout postnatal development. However, this was rarely the case. One exception was *Esr2* (estrogen receptor beta), which was a hub at every age in the POA except P15. Another consistent relationship that was observed throughout development in both the POA and MBH was between *Pgr* with *Ar* (androgen receptor) and $P_4$ with T. On P1, P45, and P60 in the POA and on P1, P5, and P45 in the MBH, *Ar* and *Pgr* are positively correlated, but at P45 and P60, serum T and $P_4$ are negatively correlated. Together with similar results from a previous study [23], our data indicate that there is a strong relationship between *Ar* and *Pgr* that deserves further investigation.

### *Implications of This Work*

Previous work investigating relationships among hormones and gene expression has relied almost entirely upon models of gonadectomy, with or without hormone treatment, with most work done on adults. Our studies of intact developing male and female rats represent a different perspective and revealed several surprising results. First, we found that there are relatively few sex differences in the expression patterns of genes throughout postnatal development. Second, the subset of genes that was sexually dimorphic (*Esr1, Kiss1, Tac2*) represents a defined category of molecules implicated in the control of postnatal reproductive development [8]. Third, we identified novel relationships of sex steroid hormones, their receptors, and neuropeptides in the POA and MBH indicating a complex mechanism of regulation that is dependent on the developmental time point and sex. The correlation and network analysis approaches can be used to generate hypotheses for future testing in studies of postnatal development of the hypothalamus.

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