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Androgenic Regulation of Sexually Dimorphic Expression of RNA Binding Motif Protein 48 in the Developing Mouse Cortex and Hippocampus

Houng-Wei Tsai[*], **Michael Franklin**, **Chris Armoskus**, **Saori Taniguchi**, **Courtney Moder**, **Kathy Trang**, **Marilisa Santacruz**, **Allyson Milla**
Department of Biological Sciences, California State University Long Beach, Long Beach, CA 90840, USA

Abstract

To further reveal the molecular mechanism underlying sexual differentiation of the mouse cerebral cortex and hippocampus, we reanalyzed our previous microarray study with Gene Ontology (GO) term enrichment and found that the GO term "RNA binding" was over-represented among the 89 sexually dimorphic candidate genes. Thus, we selected 16 autosomal genes annotated to the term RNA binding and profiled their mRNA expression in the developing male and female mouse cortex/hippocampus. During the first three weeks after birth, sex differences in mRNA levels of *Khdrbs2, Nanos2, Rbm48,* and *Tdrd3* were observed in the mouse cortex/hippocampus. Of these genes, only the female-biased expression of *Rbm48* in neonates was abolished by prenatal exposure to testosterone propionate (TP), while postnatal treatment of TP three weeks after birth increased *Rbm48* and *Tdrd3* mRNA levels in both sexes. Regardless of sex, the postnatal cortex/hippocampus also showed a marked increase in the content of androgen receptor (*Ar*) and estrogen receptor β (*Esr2*), but a decrease in estrogen receptor α *(Esr1)* and aromatase *(Cyp19a1),* which might confer the different responses of *Rbm48* to prenatal and postnatal TP. Our results suggest that androgen-regulated, sexually dimorphic *Rbm48* expression might present a novel molecular mechanism by which perinatal androgens control development of sexual dimorphism in cortical and hippocampal structure and function.

Keywords

Sex difference; Cerebral cortex; Hippocampus; Sex-biased gene; RNA binding protein; Androgen

1. Introduction

In humans and other mammals, sexual differentiation is essential for sex-specific development and function of both reproductive and non-reproductive organs, including the

[*]Correspondence to: Department of Biological Sciences, California State University Long Beach, 1250 Bellflower Boulevard, Long Beach, CA 90840-3702, United States. Fax: +1 562-985-8878. Houng-Wei.Tsai@csulb.edu (H.-W. Tsai).

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brain (Cahill 2006). This critical physiological process is dependent on not only the actions of sex hormones and other gonadal secretions following gonadal determination, but also the genetic mechanisms mediated by the genes located on the X and Y chromosomes (Arnold 2017). Besides normal neural functions, sex or gender differences are also noted in the prevalence and symptomatology of many neurological diseases and mental illnesses, such as Alzheimer's disease, autism, depression, and schizophrenia (Bao and Swaab 2010; Pinares-Garcia et al., 2018). As sexual differentiation may confer inherent sex-specific disease vulnerability (Bao and Swaab 2011; McCarthy 2016), elucidation of the mechanisms underlying sexual dimorphism in brain structure and function will provide important insights into the etiologies of gender-biased neuropsychiatric disorders and promote the development of new therapies.

During early development, the mouse testes secrete testosterone with two perinatal peaks, the first occurring during late gestation and the second peaking on the day of birth (Motelica-Heino et al., 1988; Vom Saal and Bronson 1980). In mice and other rodent models, the developmental increase in circulating testosterone has been proven to program the expression of sex-specific reproductive and aggressive behaviors and the underlying dimorphic structural correlates in the male and female hypothalamus and amygdala (organizational effect) (Arnold 2009; Ngun et al., 2011; Quadagno et al., 1975; vom Saal 1979). To shape sexual dimorphism in the brain circuits and behavioral phenotypes, testosterone could act directly on androgen receptor (AR) and/or indirectly on estrogen receptors (ERs) via local conversion of testosterone to estradiol by aromatase (Bodo and Rissman 2007; Honda et al., 1998; Ogawa et al., 2000; Sato et al., 2004).

In addition to reproduction and aggression, many complex cognitive functions and social behaviors controlled by the cerebral cortex and hippocampus also differ between the sexes in rodent models (Gresack and Frick 2003; Isgor and Sengelaub 1998; Karlsson et al., 2015; Rizk et al., 2005). Neonatal manipulations of gonadal hormones have similarly been found to lead to sex differences in learning and cognition (Isgor and Sengelaub 1998). Associated with behavioral differences, a number of structures in these two brain regions of mice and rats have been found to be sexually dimorphic. For example, male rats show greater volumes and soma sizes in their hippocampal CA1 and CA3 pyramidal cell layer than females, and prenatal exposure of females to androgens or estrogens masculinizes the CA1 and CA3 pyramidal cell morphology (Isgor and Sengelaub 1998). These sex differences in the hippocampus may result from a greater rate of neurogenesis in males than females during the first week after birth via the activation of AR and ERs (Bowers et al., 2010; Zhang et al., 2008). Besides the hippocampus, the cerebral cortex is thicker in mice with testes but not in those with ovaries, irrespective of their sex chromosome complement (XX vs. XY), suggesting an effect of gonadal secretions on sexual differentiation of the cortex (Markham et al., 2003). AR and ERs are abundantly expressed in the developing mouse cortex and hippocampus (Kerr et al., 1995; Mogi et al., 2015; Tsai et al., 2015), and once activated, these receptors may act as transcription factors to modulate gene expression. However, the AR- or ER-specific downstream targets that are relevant to sex differences in these brain regions remain unclear.

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To address this question, we previously used gene expression microarrays to identify 90 genes differentially expressed in the cortex/hippocampus of neonatal male and female mice (Armoskus et al., 2014b). By searching the Gene Ontology (GO) terms annotated to these genes, a variety of molecular functions and biological processes, including histone modifications, cell proliferation/death, androgen/estrogen signaling pathways, and synaptic organization, have been implicated in brain sexual differentiation. To further uncover meaningful associations of these genes, we performed GO enrichment analysis of our microarray data and found that the GO term, “RNA binding”, was overrepresented, suggesting that those molecular events exclusively governed by a network of sex-dimorphically expressed RNA-binding proteins might constitute a novel mechanism for controlling sexual differentiation of the developing cortex and hippocampus. In support of this possibility, an early study observed that the hippocampal mRNA levels of two splicing factors, polypyrimidine tract-binding protein-associated splicing factor *(Sfpq/Psf)* and splicing factor arginine/serine-rich 3 *(Sfrs3/Srp20),* were higher in male mice than in females, and hippocampus-dependent learning tasks upregulated expression of both splicing factors differently between the sexes (Antunes-Martins et al., 2007). Based on the growing list of neurological diseases and psychiatric disorders, in which the cortex and hippocampus are implicated, and which are linked to defects of splicing and other RNA processing mechanisms (Brinegar and Cooper 2016; Jung and Goldman 2018; Licatalosi and Darnell 2006; Manning and Cooper 2017), it has become increasingly probable that RNA binding proteins may play important roles in the regulation of neural functions served by these two brain regions. Thus, we hypothesized that during early development, sexually dimorphic expression of the genes encoding RNA binding proteins in the developing mouse cortex and hippocampus might activate a regulatory gene network to mediate the masculinizing effects of gonadal steroids on differential neural function and behavior between the sexes. To test our hypothesis, we first determined mRNA levels of 16 candidate genes annotated to RNA binding in the developing male and female mouse cortex/hippocampus. Next, we characterized expression changes of the selected genes having sexually dimorphic expression as a function of prenatal or postnatal testosterone treatment to assess their potential as downstream androgen targets that direct brain sexual differentiation.

2. Experimental Procedures

2.1. Animals

Male and female C57BL/6J mice were housed in the CSULB Animal Care Facility with a controlled 12:12h light:dark cycle (lights on at 0600 h). Food (Teklad Mouse/Rat Diet #7012; Harlan Laboratories, Inc., Indianapolis, IN) and water were provided *ad libitum.* Adult female mice were individually paired with a fertile male and checked daily for the presence of mating plugs. The day when a mating plug is found was designated as embryonic day 0 (E0). All experimental procedures have been approved by CSULB Institutional Animal Care and Use Committee and performed according to Association for Assessment and Accreditation of Laboratory Animal Care International guidelines.

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2.2. Experimental Design

Experiment 1.—Sixteen adult female mice were individually paired with nine fertile male to produce the pups used for this experiment. Male and female pups from a total of 29 litters were sacrificed by rapid decapitation at postnatal day (PN) 0, 7, 14, and 21 (n = 8–20 per group). Their brains were immediately removed from the skulls after decapitation and coronally blocked as described previously (Armoskus et al., 2014b; Tsai et al., 2009). The cerebral cortex/hippocampus was dissected out, frozen on dry ice, and then stored at −80°C until processed for RNA extraction. The sexes of the pups were determined by examining the pigmentation in their anogenital regions as well as their gonads and reproductive tracts.

Experiment 2.—To examine if sexually dimorphic expression of selected genes was regulated by prenatal androgens, pregnant mice received a subcutaneous injection of either vehicle (0.05 ml sesame oil) or testosterone propionate (TP, 100 μg in 0.05 ml; Sigma-Aldrich) daily beginning on E16 (16 days after a mating plug was seen) until PN0 as described previously (Armoskus et al., 2014a). This treatment was to mimic the testosterone surge observed in male embryos during late gestation. The dosage and duration of TP administered were modified from previous studies showing that prenatal TP injections in pregnant dams masculinized reproductive morphology and behavior in their female offspring (Hotchkiss et al., 2007; vom Saal 1979; Wolf et al., 2002; Yucel et al., 2003). In addition, the effectiveness of our TP treatment on brain development was confirmed as it caused an increase in the density of dendritic spines in the frontal cortex of prenatally testosterone-exposed mice (Hatanaka et al., 2015). Vehicle- and TP-treated, male and female pups from nine litters (n = 10 per group) were sacrificed at PN0, and their cortex/hippocampus was similarly collected and stored as described above. The pups used in this experiment were produced by five male and six female breeders. Besides the pigmentation in the anogenital regions and the gonads, anogenital distance (AGD) was also measured using a digital caliper (Fisher) to indicate the effects of prenatal TP on genital masculinization.

Experiment 3.—To investigate the effect of testosterone on mRNA levels of sexually dimorphic genes after the critical period of brain sexual differentiation, male and female pups were treated with a subcutaneous injection of either vehicle (0.05 ml sesame oil) or TP (100 μg in 0.05 ml; Sigma-Aldrich) daily at 1000 h beginning at PN21. While our TP treatment paradigm has not been previously reported in the literature, the same dose of TP administered to newborn females was effective at masculinizing their brains and behaviors (Hisasue et al., 2010; Seney et al., 2012). The cortex/hippocampus of vehicle-and TP-treated, male and female pups from 11 litters (n = 8–11 per group) was collected 3–6 h after the last injection at PN23. AGD of individual pups was similarly measured at sacrifice, and their sexes were confirmed by examining the gonads and reproductive tracts as described above. The pups used in Experiment 3 were produced by six male and six female breeders.

2.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the cortex/hippocampus sample using an RNeasy® Lipid Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration of each RNA sample was determined by measuring the absorbance at 260 nm

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(A_{260}) and 280 nm (A_{280}) using the BioRad SmartSpec™Plus spectrophotometer (Hercules, CA), and its purity was determined by calculating the A_{260}:A_{280} ratio.

Using the Bio-Rad iScript™ cDNA Synthesis kit and Bio-Rad MyCycler™ thermocycler, cDNA was synthesized from 1 μg total RNA in a 20-μl reaction at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. The cDNA was then stored at −20°C later used in PCR and qPCR to measure relative mRNA levels of selected genes.

2.4. Primer Tests

The PCR primers used in the current study were purchased from Eurofins MWG Operon LLC (Louisville, KY). The sequences of all PCR primers were first found from the Harvard Primer Bank (https://pga.mgh.harvard.edu/primerbank) and checked for specificity using NCBI's Primer-BLAST program against RefSeq mRNA database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers (10 mM) were tested in PCR assays with 40 cycles using GoTaq® Green Master Mix (Promega Corporation, Madison, WI). All PCR primer tests were conducted with the annealing temperatures listed in Table 2. PCR products were separated on a 1.5% agarose gel containing 10 mg/ml ethidium bromide and then visualized using the FluorChem E™ System (ProteinSimple, San Jose, CA). All PCR primer sets showed a single PCR product of the expected size and no product in the negative control (without cDNA).

2.5. RT-qPCR

Serially diluted, male and female mouse cortical/hippocampal cDNA samples, ranging from 1:4 to 1: 512, were used to test for primer efficiency. Total volume of each qPCR reaction comprised of 4 μl cDNA, 1 μl forward primer (840 nM), 1 μl reverse primer (840 nM), and 6 μl ABsolute QPCR SYBR® Green Mix with ROX and MgCh (Thermo Scientific, Waltham, MA). All qPCR reactions were amplified on the StepOnePlus™ Real-Time PCR System (Life Technologies, Grand Island, NY) with an initial 15 min denaturation/polymerase-activation at 95°C, 40 cycles of denaturation at 95°C for 15 s, annealing at the optimal temperature (Table 2) for 1 min, and extension at 72° for 30 s, followed by a dissociation step at 95°C for 1 min, at 55°C for 30 s, and at 95°C for 30 s to produce a melt curve. Negative controls were also included in each assay. Ct values were analyzed by linear regression with log base 2 of the dilution as the predictor and Ct of the reaction as the response. Primers were deemed unsuitable for qPCR if the R^2 value of the regression was below 0.95, or Ct-values of 1:16 dilution were above 32. The sequences, PCR product sizes, efficiencies, and annealing temperatures of all the primer used are listed in Table 2.

Using RT-qPCR with the optimal conditions as described in the dilution tests, relative mRNA levels of target genes were measured in duplicate for each cDNA sample (in 1:16 dilution) to reduce intersample variation. For each sample, relative expression of target and housekeeping genes were calculated using Pfaffl's method against the average Ct values of the PN0 females in Experiment 1 and against the average Ct values of the vehicle-treated females in Experiments 2 and 3 (Pfaffl 2001).

Normalization and quantifications of the genes of interest was performed with the comparative Cts method relative to the housekeeping genes, *Actb* and *Rpl113a* (Armoskus et

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al., 2014b). For Experiments 2 and 3, target gene expression was calculated as normalized to the average Ct values of vehicle-treated females.

2.6. Statistical Analyses

The data from individual groups were first tested for equality of variances using Levene's test. When significant deviations were detected ($p<0.05$), the data were log transformed and then retested for equal variance. After ensuring equal variance, the data were analyzed by two-way ANOVA to evaluate: (1) the effects of sex, age, and their interaction in Experiment 1, and (2) the effects of sex, TP treatment, and their interaction in Experiments 2 and 3. When two-way ANOVA showed significant effect or interactions, Tukey's post-hoc test was further used for pair-wise comparison to determine which two groups were significantly different from each other. A p value <0.05 was considered statistically different.

3. Results

3.1 GO Enrichment Analysis of Microarray Data

We previously identified 90 candidate genes differentially expressed in the neonatal mouse cortex/hippocampus between male and female mice using gene expression microarrays (Armoskus et al., 2014b). In the current study, we reanalyzed those genes with GO term enrichment analysis except loc101056177 (not annotated) and found some of them to be significantly associated with the molecular function GO term, "RNA binding" (GO: 0003723) ($p = 0.009$). Among the 89 genes, 13 were annotated with "RNA binding", including *Ddx3y, Dhx8, Eif2s3x, Eif2s3y, Elavl4, Nanos2, Ncbp2, Ppargc1b, Rbm12, Rbm48, Srsf10, Tdrd3,* and *Thoc3* (Table 1). *Ddx3y* and *Eif2s3y* are located on the Y chromosome and exclusively expressed in males, while *Eif2s3x* is an X escape gene expressed from both active and inactive X chromosomes with higher levels in females than males (Armoskus et al., 2014b). Using GO annotation search, these genes were further explored with regard to their connections to the mechanisms of post-transcriptional control regulated by RNA binding proteins. *Ncbp2* was annotated with "RNA 3'-end processing" (GO:0031123) while *Dhx8, Ncbp2, Srsf10,* and *Thoc3* were linked to "RNA splicing" (GO: 0008380). *Ncbp2* and *Thoc3* were involved in "RNA transport" (GO:0050658). *Eif2s3x, Eif2s3y, Elovl4, Nanos2,* and *Ncbp2* were annotated with "translation" (GO:0006412), and *Elavl4, Nanos2*, and *Ncbp2* were linked with "RNA catabolic process" (GO:0006401). This suggests that RNA binding proteins may regulate brain sexual differentiation post-transcriptionally via mechanisms including mRNA processing (polyadenylation and splicing), export and localization, decay, and translation. Three genes, *Ddx3y, Rbm12,* and *Rbm48,* lack the GO biological process annotations.

Using RT-qPCR, we confirmed the sex differences in mRNA levels of *Ddx3y, Eif2s3x,* and *Eif2s3y* in the neonatal cortex/hippocampus (Armoskus et al., 2014b), and found that their expression is independent of prenatal testosterone levels (Armoskus et al., 2014a). In this study, we continued to profile the expression of the other 10 candidates in the developing mouse cortex/hippocampus using RT-qPCR. In addition, six non-dimorphic genes, *Dusp11, Khdrbs2, Sfpq, Srsf3, Strbp,* and *Tut1,* were also included in the analysis; *Dusp11, Khdrbs2, Strbp,* and *Tut1* displayed a 1.2 or greater fold change between the sexes in neonates with a

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trend towards statistical significance by microarrays while male-biased expression of *Sfpq* and *Srsf3* was observed in the juvenile and/or adult mouse hippocampus (Antunes-Martins et al., 2007).

3.2 Effect of Age and Sex on mRNA Levels of Selected Sexually Dimorphic Candidate Genes in the Developing Mouse Cortex/Hippocampus

Body weight of each mouse used in this experiment was obtained at sacrifice (Table 3). Two-way ANOVA revealed a significant increase in body weight as age advanced ($p < 0.001$), but no effect of sex ($p = 0.799$) or interaction between age and sex ($p = 0.457$) on body weight.

We then used RT-qPCR to determine mRNA expression of the 16 selected genes in the cortex/hippocampus of male and female mice at PN0, PN7, PN14, and PN21. Two-way ANOVA revealed a significant effect of sex on relative mRNA levels of *Khdrbs2* ($p = 0.015$), *Nanos2* ($p = 0.019$), *Rbm48* ($p < 0.001$), and *Tdrd3* ($p = 0.003$) (Figure 1). *Rbm48* ($p = 0.003$) and *Tdrd3* ($p < 0.001$) displayed a significant effect of interaction between age and sex, and Tukey's post hoc analysis further revealed a femalebiased expression of both genes at PN0 and PN7, but not PN14 or PN21. Additionally, all four genes displayed a significant effect of age on their expression ($p < 0.001$). *Khdrbs2* and *Tdrd3* mRNA levels steadily declined with age after birth, but increased again at PN21. *Nanos2* expression rapidly declined after birth and remained low during early development. *Rbm48* mRNA levels increased at PN7, dropped to low levels at PN14, but then increased again at PN21.

Eleven of the 12 other genes, with the exception of *Srsf10,* exhibited no sex-, but age-dependent changes in their mRNA levels in the developing cortex/hippocampus. A two-way ANOVA revealed a significant effect of age on relative mRNA levels of *Dhx8* ($p < 0.001$), *Dusp11* ($p < 0.001$), *Elov14* ($p < 0.001$), *Ncbp2* ($p < 0.001$), *Pporgc1b* ($p < 0.001$), *Rbm12* ($p = 0.001$), *Sfpq* ($p = 0.033$), *Srsf3* ($p < 0.001$), *Strbp* ($p < 0.001$), *Thoc3* ($p < 0.001$), and *Tut1* ($p < 0.001$) (Figure 2). Post-hoc tests further showed that expression of *Dhx8, Elov14, Ncbp2, Pporgc1b,* and *Strbp* increased with age within the first three weeks after birth whereas *Srsf3* displayed a decline in expression by PN7 ($p < 0.05$). *Dusp11, Rbm12,* and *Tut1* mRNA levels transiently decreased between PN7 and PN14, but *Sfpq* and *Thoc3* displayed a brief rise in expression at PN7.

3.3 Effects of Prenatal TP Treatment on Expression of Khdrbs2, Nanos2, Rbm48, and Tdrd3 in the Cortex/Hippocampus of Neonatal Male and Female Mice

To determine if sexually dimorphic expression of *Khdrbs2, Nanos2, Rbm48, and Tdrd3* in the developing cortex/hippocampus was due to prenatal testosterone secreted by the developing testes, we determined relative mRNA levels of these genes in the cortex/hippocampus of male and female neonatal mice prenatally treated with either vehicle or TP. Body weight and AGD of each mouse in this experiment were measured at sacrifice. A two-way ANOVA revealed no effect of sex, treatment, or their interaction on body weight (Table 3). In contrast, two-way ANOVA of AGD data showed significant effects of sex ($p < 0.001$), treatment ($p = 0.004$), and their interaction ($p = 0.012$) (Figure 3, upper panel). Tukey's post hoc analysis further indicated that prenatal TP treatment significantly increased AGD in

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females, not males, but the AGD of TP-treated females was shorter than TP-treated males ($p < 0.05$). These observations confirmed the masculinization by fetal androgen exposure in female animals.

Next, we determined relative mRNA levels of *Khdrbs2, Nanos2, Rbm48, and Tdrd3* in the cortex/hippocampus of male and female neonatal mice treated with either vehicle or TP during late gestation (Figure 4). A two-way ANOVA revealed a significant effect of sex ($p = 0.009$), treatment ($p = 0.017$), and their interaction ($p = 0.008$) on *Rbm48* mRNA levels, and Tukey's post hoc analysis further indicated a female-biased expression in vehicle-treated mice, but not in mice prenatally treated with testosterone (Figure 4, bottom left panel). Additionally, the analysis indicated no effect of sex ($p = 0.274$), or treatment ($p = 0.196$), but an interaction between sex and treatment ($p = 0.048$) on *Nanos2* mRNA levels. However, post hoc analysis indicated no significant differences between individual groups. There was no effect of sex ($p = 0.259$ and $p = 0.113$), treatment ($p = 0.104$ and p = 0.970), or interaction between sex and treatment ($p = 0.418$ and $p = 0.055$) on *Khdrbs2* or *Tdrd3* mRNA levels in the neonatal mouse cortex/hippocampus.

To verify the presence of sex steroid receptors and aromatase (*Cyp19o1*) in the cortex/hippocampus of prenatally vehicle- and TP-treated, neonatal mice, we determined relative mRNA levels of *Ar, Esr1, Esr2,* and *Cyp19a1.* There was no effect of sex, treatment, or their interaction on mRNA expression of *Ar, Esr1, Esr2,* and *Cyp19a1* in the neonatal mouse cortex/hippocampus receiving prenatal treatments (Table 4). However, there was a trend towards a sex difference in *Esr2* mRNA ($p = 0.059$) and a trend of interaction between sex and treatment in *Esr1* mRNA ($p = 0.058$). Although *Ar* was detected in the neonatal mouse cortex/hippocampus, high cycles of threshold (Ct) values (greater than 30) observed in qPCR assays indicated low quantities of *Ar* mRNA.

3.4 Effects of Postnatal TP Treatment on Expression of Khdrbs2, Nanos2, Rbm48, and Tdrd3 in the Cortex/Hippocampus of Juvenile Male and Female Mice

To determine if *Khdrbs2, Nanos2, Rbm48 and Tdrd3* remained responsive to androgens after the critical period, we measured their relative mRNA levels in the cortex/hippocampus of male and female mice treated with vehicle or TP during PN21–23. Body weights of the mice used in this experiment are shown in Table 3. A two-way ANOVA revealed no effect of sex ($p = 0.065$), treatment ($p = 0.058$), or their interaction ($p = 0.675$) on body weight. AGD was also measured on each mouse, and a two-way ANOVA showed significant effects of sex ($p < 0.001$) on mean AGD, with a longer AGD in males than females (Figure 3, lower panel). On the other hand, there was no effect of treatment ($p = 0.662$) or interaction between sex and treatment ($p = 0.252$) on AGD.

A two-way ANOVA revealed that postnatal TP treatment significantly increased *Rbm48* and *Tdrd3* mRNA levels ($p = 0.013$ and p = 0.047), but there was no effect of sex ($p = 0.538$ and $p = 0.643$) or interaction between sex and treatment ($p = 0.810$ and p = 0.689) (Figure 5). In contrast, there was no effect of sex ($p = 0.828$ and $p = 0.371$), treatment ($p = 0.575$ and $p = 0.596$), or interaction ($p = 0.980$ and $p = 0.427$) on expression of *Khdrbs2* and *Nanos2.*

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We then determined relative mRNA levels of *Ar, Esr1, Esr2,* and *Cyp19a1* in the cortex/hippocampus of vehicle- and TP-treated mice to confirm their presence in the postnatal cortex/hippocampus (Figure 6). There was no effect of TP treatment, sex, or their interaction on *Esrl, Esr2,* or *Cyp19a1* mRNA levels in the mouse cortex/hippocampus at PN23. Interestingly, postnatal TP significantly increased *Ar* expression in the cortex/hippocampus ($p = 0.017$), but no effect of sex ($p = 0.646$) or interaction between treatment and sex ($p = 0.358$) on its mRNA levels was observed.

3.5 Effect of Age and Sex on mRNA Levels of Ar, Cyp19a1, Esr1, and Esr2 in the Developing Mouse Cortex/Hippocampus

To determine if differential expression of steroid receptors and aromatase with age might play a role in conferring age-related changes in the androgenic responsiveness of the *Rbm48* gene, we determined mRNA levels of *Ar, Cyp19a1, Esr1,* and *Esr2* in the mouse cortex/hippocampus at PN0, 7, 14, and 21. Two-way ANOVA revealed a significant effect of age, but not sex with no interaction, on relative mRNA levels of *Ar, Cyp19a1, Esr1,* and *Esr2* in the male and female mouse cortex/hippocampus during early development (Figure 7). Within the first three weeks after birth, expression of *Ar* ($p < 0.001$) and *Esr2* ($p = 0.033$) increased with age. Post hoc analyses further demonstrated that *Ar* mRNA levels in the cortex/hippocampus were significantly higher on PN7 than PN0 and continued to increase at PN14 and PN21. However, the age-dependent rise in *Esr2* expression didn't occur until PN21. In contrast to *Ar* and *Esr2, Esrl* and *Cyp19a1* both showed a transient rise in mRNA levels at PN7, followed by a gradual decrease with levels below those at PN0 by PN21 ($p < 0.05$).

4. Discussion

During late gestation and on the day of birth, significant elevations of circulating testosterone are secreted by the developing testes, and after these surges, testosterone levels decrease and remain low in male mice until the onset of puberty (Clarkson and Herbison 2016). The perinatal rise in testosterone establishes brain masculinization that in turn underpins sexually dimorphic behavior and physiology in male mice (Ngun et al., 2011; Quadagno et al., 1975; vom Saal 1979). Synchronously with these increases in testosterone levels during early development, *Rbm48* expression is suppressed in the male compared to the female mouse cortex/hippocampus at both PN0 and PN7 specifically (Figure 1). Our finding that prenatal TP treatment in female neonates reduces *Rbm48* mRNA levels to male-like levels further indicates that the sex difference in neonatal cortical and hippocampal *Rbm48* expression might be due to the androgen-dependent downregulation of the *Rbm48* gene (Figure 5). Testosterone has been demonstrated to exert its effects on brain masculinization through AR directly and/or ERs indirectly after local aromatization of testosterone to estradiol (Bodo et al., 2006; Bodo and Rissman 2007; Ogawa et al., 1997; Simerly et al., 1997; vom Saal 1979). In the current study, we have confirmed the presence of *Ar, Cyp19a1, Esr1,* and *Esr2* mRNA in the neonatal mouse cortex/hippocampus (Figure 5), suggesting that both AR and ERs may be involved in the transcriptional regulation of *Rbm48.* While no effect of sex or TP on *Ar, Cyp19a1, Esr1,* and *Esr2* mRNA levels in neonates was detected, the differences in circulating testosterone between the sexes during

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the perinatal period can still activate AR and/or ERs in a sex-dependent fashion to regulate *Rbm48* transcription. Together, the differences in sex steroid milieu between male and female newborn pups appears to be the determinant of the sex difference in *Rbm48* mRNA levels in the developing mouse cortex and hippocampus although the protein levels of *Ar, Cyp19o1, Esr1,* and *Esr2* remain to be verified.

Acting as ligand-inducible transcription factors, liganded AR and ERs bind to the androgen response element (ARE) and estrogen response element (ERE), respectively, on the promoters of the genes they regulate (Denayer et al., 2010; Sanchez et al., 2002). Using a genome-wide analysis of promoter elements for estrogen receptor binding sites, Kamalakaran *et al.* (2005) not only confirmed the presence of ERE in the genes known to be regulated by estrogen, but also identified several new estrogen-responsive genes whose putative promoters contained EREs (Kamalakaran et al., 2005). To explore if the same molecular mechanisms may be involved in androgenic regulation of *Rbm48* expression, we searched the consensus sequences of ARE and ERE in the *Rbm48* promoter (−1000 to 100 bp) using the mmEPDnew, the Mus musculus (mouse) curated promoter database from Eukaryotic Promoter Database (https://epd.epfl.ch/mouse/mousedatabase.php?db=mouse). We found that its putative promoter contains one potential ARE (at position −50) and several potential EREs (at positions −802, −712, −606, −538, , −195, and −24 for ESR1, and −479, −117, −25/−24, and 44 for ESR2), which supports the *Rbm48* gene as a direct transcriptional target for both liganded AR and ERs. However, our prediction cannot rule out the possibility that ligand-activated ERs may regulate *Rbm48* expression via functional protein-protein interactions with other transcription factors bound to their cognate regulatory elements, such as AP-1 (at position −501), on the *Rbm48* promoter in an ERE-independent manner (Kushner et al., 2000; Safe 2001). To further evaluate if the predicted ARE and EREs as well as other motifs are the functional binding sits to AR and ERs, we can perform luciferase promoter constructs containing each element and DNA-binding assays *in vitro* and chromatin immunoprecipitation assays *in vivo.*

In contrast to the neonates, male and female juvenile mice both showed increased *Rbm48* mRNA levels in response to TP treatment at the same dose three weeks after birth (Figure 6), indicating that the *Rbm48* gene in the developing cortex and hippocampus remains androgen-responsive during early development, but seems to be regulated by distinct mechanisms depending on age. The opposite responses of *Rbm48* transcription to prenatal and postnatal testosterone might be inferred from the developmental changes in abundance of *Cyp19a1* and *Ar. Cyp19a*1 expression in the mouse cortex/hippocampus is lower in both males and females at PN21 as compared to neonates (Figure 8). Similar to our data, *Cyp19a1* mRNA expression in the mouse hippocampus remains elevated during the first two weeks after birth and then declines as adults (Ivanova and Beyer 2000). Decreased expression of *Cyp19a1* might prohibit local estrogen biosynthesis in the juvenile mouse cortex/hippocampus and then dampen estrogen responsiveness by reducing transactivation of ERα and/or ERβ by ligands. On the other hand, in agreement with our previous findings (Tsai et al., 2015), *Ar* mRNA levels drastically increase with age during the first three weeks after birth (Figure 8), which could enhance the responsiveness of the juvenile cortex/hippocampus to androgens through augmented *Ar* expression. In addition, it has been reported that AR overexpression could also inhibit estradiol-stimulated ER target gene

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transcription by competing for transcriptional coregulators (Lanzino et al., 2005) or binding to EREs in breast cancer cells (Peters et al., 2009). Accordingly, we speculate that AR might play the major role in the androgen-dependent upregulation of *Rbm48* expression three weeks after birth while the androgenic downregulation of *Rbm48* transcription in neonates might be largely mediated by the activation of ERs by locally synthesized estradiol.

The *Rbm48* gene encodes a 413-amino acid protein, RNA binding motif protein 48 (RBM48; 48 kDa), with a single copy of the RNA recognition motif (RRM) domain at the amino terminus (residues 78–177; RefSeq: NP_766579.3 and Ensembl: ENSMUST00000042753.13). RRM is a conserved, single-stranded RNA binding domain of 80–90 amino acids, and many RRM-containing proteins have been found to be the key regulators required for RNA biogenesis, stability, function, transport and cellular localization (Maris et al., 2005). According to the Ensembl (http://uswest.ensembl.org/Musmusculus/Info/Index), RBM48 and 270 other mouse gene products belong to the RNA-binding domain (RBD) superfamily with the presence of RRM and/or other canonical RBDs. Currently, neither RNA targets nor molecular function of RBM48 in mice have yet been identified. As proteins with structurally conserved motifs often share similar functions, we performed GO enrichment analysis of the 271 mouse RBD superfamily genes to explore the possible involvement of RBM48 in certain biological processes and signaling pathways. The results showed significant enrichment for terms related exclusively to the chemical reactions and cellular pathways that involve RNA, including RNA processing (GO:0006396), RNA splicing (GO:0008380), gene expression (GO:0010467), RNA metabolic process (GO:0016070), RNA localization (GO:0006403), RNA transport (GO:0050658), RNA stabilization (GO:0043489), and translation (GO:0006412), unsurprisingly suggesting that like many members of the RBD superfamily, RBM48 may control the fate of its bound RNAs by governing a single or multiple aspects of RNA biology, from transcription, splicing and polyadenylation to RNA modification, transport, localization, translation and turnover. Bai and colleagues (2019) have recently discovered RBM48 to function as a minor spliceosome factor in U12 splicing in plants (corn), and *Rbm48* mutations cause aberrant splicing of U12-type introns and the defects that repress endosperm cell differentiation during development (Bai et al., 2019). Since RBM48 is a highly conserved protein, mouse RBM48 is predicted to also function in U12 splicing. In addition, an *in vitro* study has found that RBM48 may contain anti-apoptotic activity as CRISPR knockout of *RBM48* in HCT116 and HeLa cells results in cleavage of both caspase 7 and PARP, the signs of apoptosis (Hart et al., 2015), which implies that RBM48 may be involved in apoptosis. In support of this, a list of RBM proteins, including RBM3, RBM4, RBM5, RBM6, RBM7, RBM8, RBM 10, RBM11, RBM12, RBM15, and RBM25, have been identified as apoptosis modulators in humans and mice (Cui and Placzek 2018; Jackson et al., 2015; Martinez-Arribas et al., 2006; Sutherland et al., 2005). Hormonally-mediated differences in cell death between the sexes are central to the sexual differentiation of many brain areas (Forger and de Vries 2010). Nunez *et al.* (2001) found that at the age of 6 and 7 days, male rats had a higher density of apoptotic cells in their primary visual cortex than females as the result of androgen exposure during perinatal period(Nunez et al., 2001). Based on these findings, we speculate that androgen-downregulation of *Rbm48* expression might be critical for the

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control of apoptotic processes, resulting in sexually dimorphic neural circuits in the developing mouse cortex/hippocampus.

RT-qPCR has been commonly used as a validation tool for confirming gene expression results obtained from microarray analysis. However, of the 13 selected sexually dimorphic candidate genes identified by microarrays, only five *(Ddx3y, Eif2s3x, Eif2×3y, Nanos2,* and *Rbm48;* 38%) were confirmed with the same sex differences in expression in the developing mouse cortex/hippocampus in previous (Armoskus et al., 2014b) and current studies (Figure 1). Interestingly, *Tdrd3,* one of the eight false positives, had been identified as a male-biased gene by microarrays, but expressed at higher levels in females than males as measured by RT-qPCR (Figure 1). In addition, *Khdrbs2* exhibited an insignificant sex bias trend in expression by microarrays, but a female-biased increase in mRNA levels by RT-qPCR (Figure 1). The majority of the selected candidates displayed a small fold change in expression, ranging from 1.16 to 1.46, and a p value of more than 0.001 between the sexes by microarrays except *Ddx3y* (2.8 fold, $p < 0.0001$), *Eif2×3x* (1.4 fold, $p < 0.0001$) and *Eif2×3y* (285.9 fold, $p < 0.0001$) (Armoskus et al., 2014b). Low correlations between microarrays and RT-qPCR have been previously reported for the measurement of mRNA expression of the genes showing less than a 1.4-fold change or a p-value greater than 0.0001 (Etienne et al., 2004; Morey et al., 2006; Wurmbach et al., 2003). The discrepancy between the results obtained from microarrays and RT-qPCR could be due to several fundamental technical differences between the two methodologies. First, microarray and RT-qPCR differ on calculation of expression data. The former requires global normalization while the latter simply utilizes the expression of one or two reference genes, such as *Rpl13a* and *Actb,* against which target gene expression is calibrated. Second, although the microarray probes and PCR primers are designed to be gene-specific, the specificity and sensitivity of array probes might be constrained by the length and sequence from allowing simultaneous hybridization of all probes and labeled cRNAs to occur at the same temperature. In contrast, the temperature at which PCR primers anneal to the cDNA template varies with the length and base composition of each primer set with optimal hybridization specificity and efficiency. Lastly, different locations on the target gene and lengths of microarray probes and PCR primers could also influence the correlation between the two methods. Even with some inconsistencies between the microarray and RT-qPCR data, microarray is still a powerful and effective tool for large-scale gene expression profiling, which helps scientists narrow the list of potential targets for further investigation of the complex molecular mechanisms underlying brain sexual differentiation.

In summary, our study has identified the *Rbm48* gene as a potential downstream effector to mediate the action of perinatal testosterone in driving sexual differentiation of the cerebral cortex and hippocampus during early development. With more abundant ERs and aromatase, but low AR, expressed in the neonatal cortex/hippocampus, the perinatal rises of testosterone in male mice seem to activate ERs to suppress *Rbm48* expression. As age advances, the *Rbm48* gene switches from being androgen-suppressed via ERs to being androgen-stimulated through AR as AR levels rise robustly after birth. The hormonally regulated *Rbm48* gene expression during early development may provide a starting point for future investigations to better understand the process of brain sexual differentiation. This will also provide new insights into the molecular mechanisms that generate gender bias in a variety of

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neurological diseases linked to dysfunction in these brain regions, such as Alzheimer's disease and autism.

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Highlights

- *Rbm48* mRNA expression in the developing mouse cortex/hippocampus is female-biased
- Prenatal testosterone reduces *Rbm48* levels in the neonatal cortex/hippocampus
- Postnatal testosterone increases *Rbm48* levels in the juvenile cortex/hippocampus
- Levels of steroid receptors and aromatase confer androgenic responsiveness of *Rbm48*

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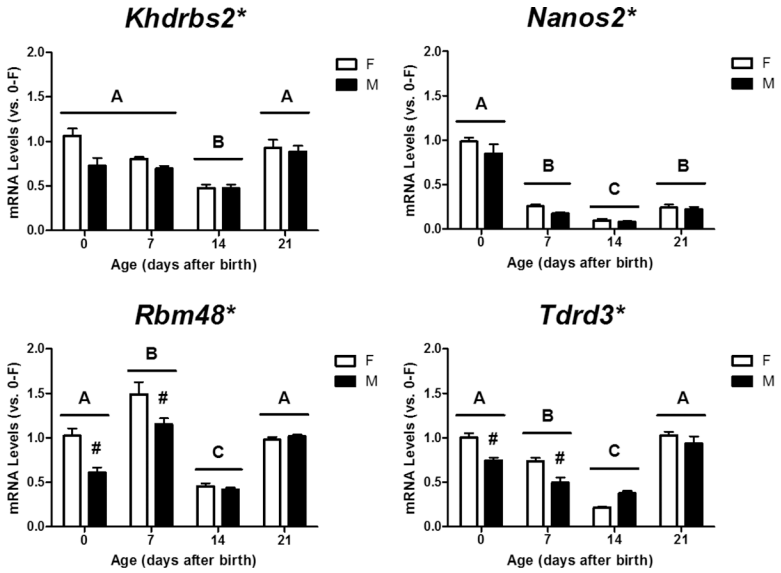

Figure 1.

Sex- and age-dependent changes in relative mRNA levels of *Khdrbs2, Nanos2, Rbm48,* and *Tdrd3* in the developing female (F) and male (M) mouse cortex/hippocampus. Expression of each gene was calculated and expressed relative to the average of neonatal females (0-F as 1-fold). * Indicates a significant difference of gene expression between the sexes ($p < 0.05$). # indicates a significant difference vs. females at the same age ($p < 0.05$). A, B, C indicate significant differences between ages ($p < 0.05$).

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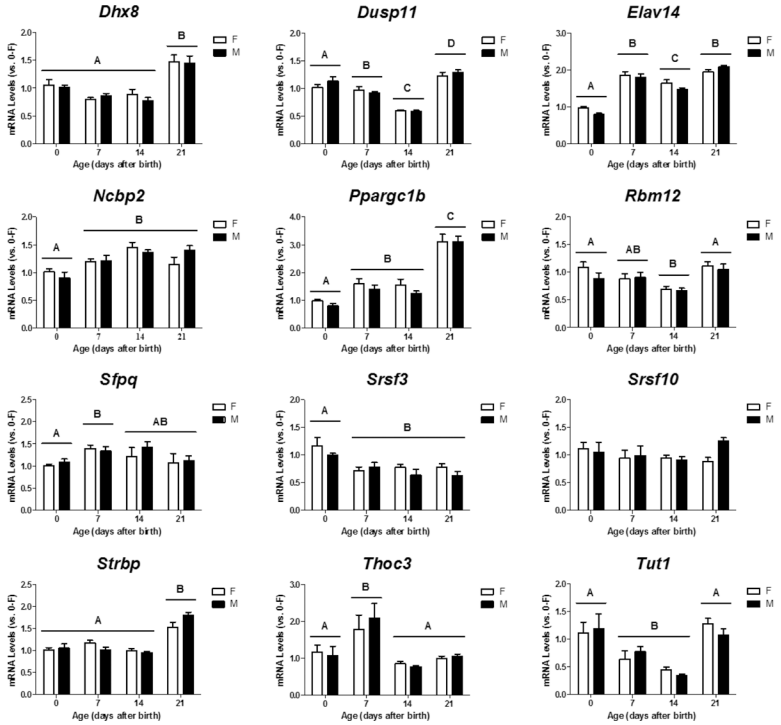

Figure 2.

Age-dependent changes in mRNA expression of *Dhx8, Dusp11, Elav14, Ncbp2, Ppargc1b, Rbm12, Sfpq, Srsf3, Srsf10, Strbp*, *Thoc3,* and *Tut1* in the developing female (F) and male (M) mouse cortex/hippocampus. Relative mRNA levels of individual genes were calculated and expressed relative to the average of neonatal females (0-F as 1-fold). A, B, C, D indicate significant differences between ages ($p < 0.05$).

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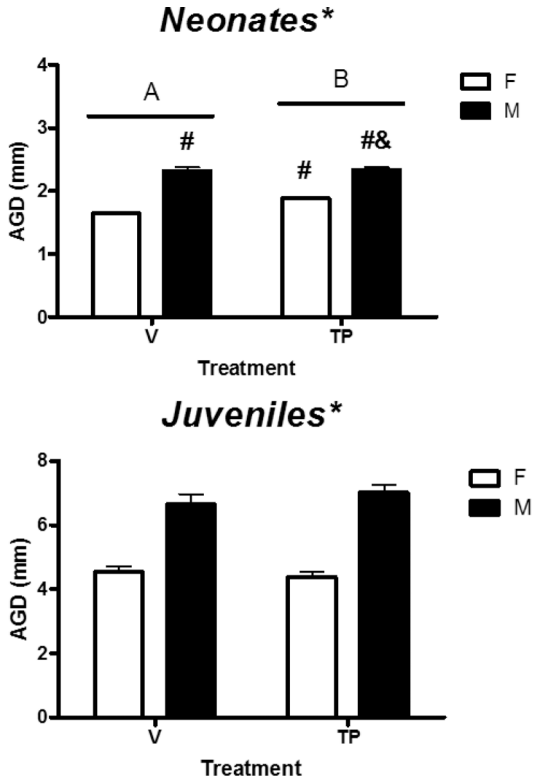

Figure 3.

Effects of prenatal (upper panel) and postnatal (lower panel) exposure to testosterone propionate (TP) on anogenital distance (AGD) in female (F) and male (M) neonatal and juvenile mice. * indicates a significant difference between the sexes ($p < 0.05$). # indicates significant difference vs. vehicle (V)-treated females ($p < 0.05$). & indicates significant difference vs. TP-F ($p < 0.05$). A, B indicate significant differences between the treatments ($p < 0.05$).

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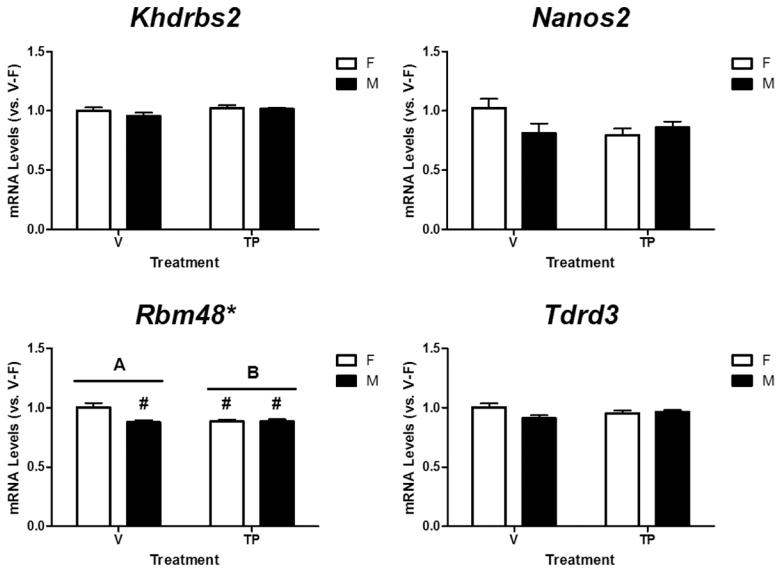

Figure 4.

Effects of prenatal exposure to testosterone propionate (TP, 100 μg/0.05ml) on mRNA expression of *Khdrbs2, Nanos2, Rbm48* and *Tdrd3* in the female (F) and male (M) neonatal mouse cortex/hippocampus. Relative mRNA levels of each gene were calculated and expressed relative to the average of vehicle (V)-treated females (V-F as 1-fold). * indicates significant difference between the sexes ($p < 0.05$). # indicates significant difference versus V-F ($p < 0.05$). A, B indicate significant differences between the treatments ($p < 0.05$).

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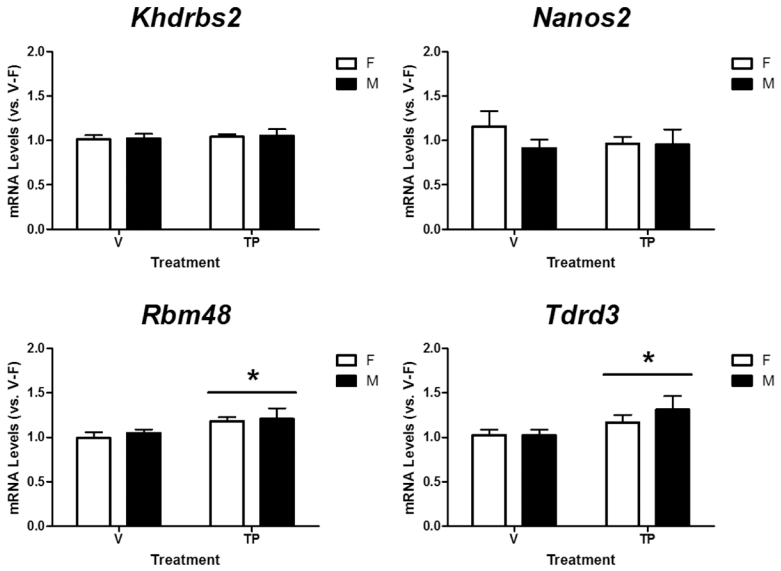

Figure 5.

Effects of postnatal exposure to testosterone propionate (TP, 100 μg/0.05 ml) on expression of *Khdrbs2, Nanos2, Rbm48,* and *Tdrd3* in the female (F) and male (M) mouse cortex/hippocampus at postnatal day 23. Relative mRNA levels were calculated relative to the average of vehicle (V)-treated females (V-F as 1-fold). * Indicates significant difference vs. vehicle-treated mice ($p < 0.05$).

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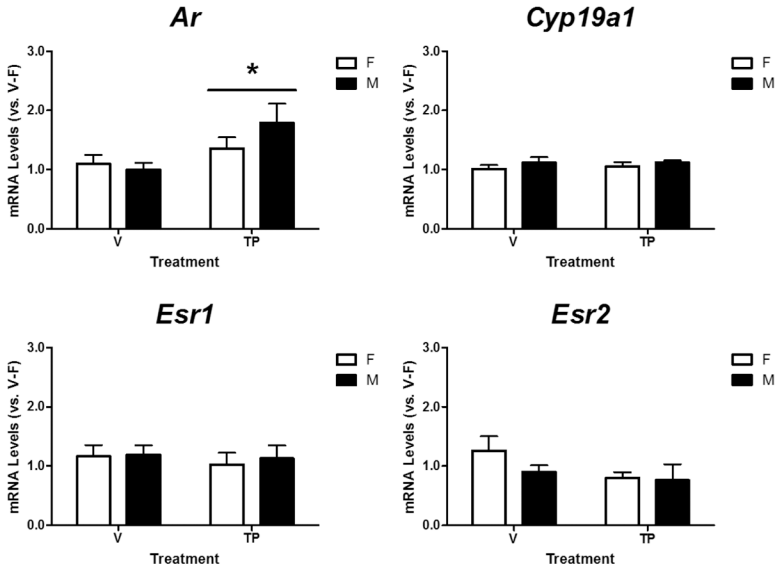

Figure 6.

Effects of postnatal exposure to testosterone propionate (TP, 100 μg/0.05 ml) on expression of *Ar, Cyp19a1, Esr1*, and *Esr2* in the female (F) and male (M) mouse cortex/hippocampus at postnatal day 23. Relative mRNA levels were calculated relative to the average of vehicle (V)-treated females (V-F as 1 fold). * Indicates significant difference vs. vehicle-treated mice ($p < 0.05$).

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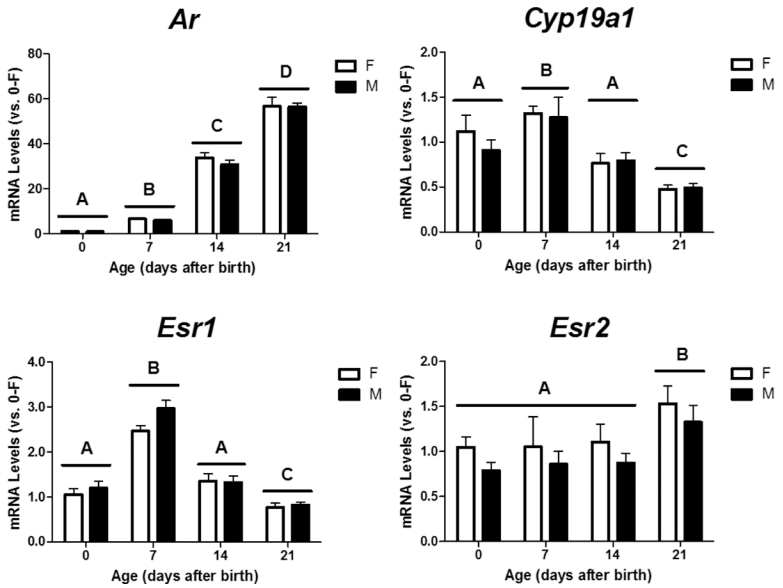

Figure 7.

Developmental changes in *Ar*, *Esr1,* and *Esr2* expression in the female (F) and male (M) mouse cortex/hippocampus at postnatal days 0, 7, 14, and 21. Gene expression was calculated relative to the average of neonatal females (0-F as 1 fold). A, B, C, D indicate significant differences between ages ($p < 0.05$).

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TABLE 1.

List of the selected genes annotated to the Gene Ontology term, RNA binding and expressed in the neonatal mouse cortex/hippocampus.

Gene Symbol	Name	RefSeq Accession	Chr[a]	Gene Ontology Annotations
Sexually dimorphic genes identified by microarrays				
Ddx3y[b]	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	NM_012008.2	Y	ATP binding, helicase activity, hydrolase activity, RNA binding
Dhx8	DEAH (Asp-Glu-Ala-His) box polypeptide 8	NM_144831.2	11	ATP binding, ATP-dependent helicase activity, hydrolase activity, RNA binding,
Eif2s3x[b]	Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	NM_012010.3	X	GTP binding, GTPase activity, translation initiation factor activity
Eif2s3y[b]	Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	NM_012011.2	Y	GTP binding, GTPase activity, translation initiation factor activity
Elavl4	ELAV like RNA binding protein 4	NM_001163399.1	4	AU-rich element binding, mRNA 3'-UTR binding, translation regulator activity
Nanos2[b]	Nanos C2HC-type zinc finger 2	NM_194064.2	7	mRNA binding, zinc ion binding
Ncbp2	Nuclear cap binding protein subunit 2	NM_026554.4	16	RNA 7-methylguanosine cap binding, snRNA binding
Ppargc1b	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	NM_133249.2	18	AF-2 domain binding, estrogen receptor binding, nuclear receptor transcription coactivator activity, RNA binding
Rbm12	RNA binding motif protein 12	NM_170598.2	2	RNA binding
Rbm48[b]	RNA binding motif protein 48	NM_172991.4	5	RNA binding
Srsf10	Serine/arginine-rich splicing factor 10	NM_010178.3	4	RNA binding, unfolded protein binding
Tdrd3[b]	Tudor domain containing 3	NM_001253755.1	14	Methylated histone binding, RNA binding, transcription coactivator activity
Thoc3	THO complex 3	NM_028597.3	13	RNA binding
Non-dimorphic genes identified by microarrays				
Dusp11[c]	Dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	NM_028099.4	6	Hydrolase activity, polynucleotide 5'- phosphatase activity, protein tyrosine/serine/threonine phosphatase activity, RNA binding
Khdrbs2[bc]	KH domain containing, RNA binding, signal transduction associated 2	NM_133235.3	1	ploy(A)/poly(U) RNA binding, protein heterodimerization activity, SH3/SH2 adaptor activity
sfpq[d]	Splicing factor proline/glutamine rich	NM_023603.3	4	E-box binding, histone deacetylase binding, protein homodimerization activity, RNA binding, RNA polymerase II distal enhancer sequence-specific DNA binding, transcription regulatory region sequence-specific DNA binding
Srsf3[d]	Serine/arginine-rich splicing factor 3	NM_013663.5	17	Phospholipase binding, sequence-specific mRNA binding
Strbp[c]	Spermatid perinuclear RNA binding protein	NM_009261.4	2	double/single-stranded RNA binding, microtubule binding, tubulin binding
Tut1[c]	Terminal uridylyl transferase 1, U6 snRNA-specific	NM_197993.3	19	ATP binding, mRNA 3'-UTR binding, polynucleotide adenylyltransferase activity, RNA uridylyltransferase activity, zinc ion binding

[a]Chromosome.

[b] indicates the genes showing sex difference in expression using microarrays and RT-qPCR Armoskus *et al.*, 2014].

[c] indicates the genes with a 1.2 or greater fold change in expression between the sexes by microarrays, but not reaching a statistically significant difference.

[d] indicates the genes reported by Antunes-Martins *et al.*(2007)

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TABLE 2.

List of the oligonucleotide primers used for RT-PCR and RT-qPCR.

Gene Symbol	Primer Sequence	RefSeq Accession	Annealing Temp (°C)	PCR Product Size (bp)
Actb	Forward: 5' CCAGATCATGTTTGAGACCTTCAA 3' Reverse: 5' CCAGAGGCATACAGGGACAGC 3'	NM_007393.5	61	78
Ar	Forward: 5' AGAATCCCACATCCTGCTCAA 3' Reverse: 5' AAGTCCACGCTCACCATATGG 3'	NM_013476.4	58	133
Cyp19a1	Forward: 5' ACCTCGGGCTACGTGGATGTG 3' Reverse: 5' GAT GTTT GGTTT GAT GAGGAGAGC 3'	NM_007810.4	60	159
Dhx8	Forward: 5' AATCCAGAGAACCAACCTGGC 3' Reverse: 5' GATCAGAGTTTCCATCGGTGG 3'	NM_144831.2	60	109
Dusp11	Forward: 5' AACCAGCATTATGGCCGACAT 3' Reverse: 5' GGAGAT AGTCTTT CCACCTTT CG 3'	NM_028099.4	58	112
Elav14	Forward: 5' CCAATCACCATTGACGGGATG 3' Reverse: 5' CAGGGGACAGGTT AT AGACGAA 3'	NM_010488.4	60	97
Esr1	Forward: 5' CACTTT GATCCACCT GAT GGC 3' Reverse: 5' GGCACAACGTTCTTGCATTTC 3'	NM_007956.5	58	153
Esr2	Forward: 5' GAAAGTGACGCGATACACCGT 3' Reverse: 5' CCT GT GAGGTAGGAAT GCGAA 3'	NM_010157.3	58	150
Khdrbs2	Forward: 5' GGGGT GAATAT GAT GACCAGACC 3' Reverse: 5' CCT CGTTTACT CCAT GACCGT AG 3'	NM_133235.3	59	105
Nanos2	Forward: 5' CATGGACCGTGTGAGTCTTG 3' Reverse: 5' TTCTTGGCAGTGACGATCAG 3'	NM_194064.2	58	197
Ncbp2	Forward: 5' T GT ACT GGAAGCCACCT CT G 3' Reverse: 5' TGTTGCCTGGGTAGAGCTTT 3'	NM_026554.4	58	126
Ppargc1b	Forward: 5' TCCTGTAAAAGCCCGGAGTAT 3' Reverse: 5' GCT CT GGTAGGGGCAGT GA 3'	NM_133249.2	58	174
Rbm12	Forward: 5' GTT AT GCCTT CGGTT GGAAA 3' Reverse: 5' GGCCATTT CCAAAGTT CT GA 3'	NM_029397.3	55	130
Rbm48	Forward: 5' AAACCTGGTGGCGTATTCGAT 3' Reverse: 5' ACGCT CAACTAATT CCTT CAT GG 3'	NM_172991.4	58	177
Rpl13a	Forward: 5' ATGAGGTCGGGTGGAAGTACC 3' Reverse: 5' CAGGAGT CCGTT GGT CTT GAG 3'	NM_009438.5	62	179
Sfpq	Forward: 5' GGAGTTCCACCAGCAACCAT 3' Reverse: 5' CTGCCCAAAGCGCTCAGT 3'	NM_023603.3	60	69
Srsf3	Forward: 5' TGAGGATCCCCGAGATGCT 3' Reverse: 5' CTTACACGGCAGCCACACAGT 3'	NM_013663.5	60	72
Srsf10	Forward: 5' GCGT CAGATT GAAAT CCAGTT CG 3' Reverse: 5' CGAGAGCGTCTAT AT CGGT CAT 3'	NM_010178.3	60	126
Strbp	Forward: 5' GT GT GGT GTAAT GAGGATT GG 3' Reverse: 5' TTTCCGTGGGTTTGTCTTTGC 3'	NM_009261.4	58	101
Tdrd3	Forward: 5' AGTGACATCAACGGTGGAAAG 3' Reverse: 5' GCTTGCGATTCCTCGTTATCCT 3'	NM_001253755.1	58	107
Thoc3	Forward: 5' AT CT GGGATGT GAGGACT ACAA 3' Reverse: 5' T GT GT CTT GGCGT CAAT GAAA 3'	NM_028597.3	59	143
Tut1	Forward: 5' GATT CGGACGTT GT AT CGCT G 3' Reverse: 5' CTCGGAGTTGCACCAAATCC 3'	NM_197993.3	59	130
Xist	Forward: 5' GCT GGTT CGT CTAT CTT GT GGG 3' Reverse: 5' GGAT CCT GCACT GGAT GAGT 3'	NR_001463.3	58	225

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TABLE 3.

Mean (± SE) body weights (g) of the mice used for Experiments 1, 2, and 3.

Experiment 1: Effect of sex and age					
			Sex		
		N	***Female***	***N***	***Male***
Age(days) *	*0*	16	1.25 ± 0.03 g	20	1.26 ± 0.03 g
	7	11	3.78 ± 0.14 g	11	3.62 ± 0.16 g
	14	8	7.64 ± 0.49 g	8	7.18 ± 0.38 g
	21	8	8.35 ± 0.56 g	8	8.84 ± 0.24 g
Experiment 2: Effect of prenatal TP					
			Sex		
		N	***Female***	***N***	***Male***
Treatment	Vehicle	10	1.29 ± 0.04 g	10	1.29 ± 0.03 g
	TP	10	1.24 ± 0.02 g	10	1.26 ± 0.02 g
Experiment 3: Effect of postnatal TP					
			Sex		
		N	***Female***	***N***	***Male***
Treatment	Vehicle	11	7.59 ± 0.47 g	8	8.23 ± 0.65 g
	TP	11	8.09 ± 0.20 g	11	9.20 ± 0.34 g

N: number of mice in each group; TP: testosterone propionate;

*indicates significant difference between

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TABLE 4.

Effect of prenatal exposure to vehicle (V) and testosterone propionate (TP, 100 μg/0.05 ml) on expression of *Ar*, *Cyp191a*, *Esr1*, and *Esr2* mRNA in the female (F) and male (M), neonatal mouse cortex/hippocampus.

Group	N[a]	*Ar*	N	*Cyp19a1*	N	*Esr1*	N	*Esr2*
V-F	10	1.03 ± 0.07[b]	9	1.10 ± 0.16	10	1.06 ± 0.13	10	1.04 ± 0.10
V-M	10	0.90 ± 0.07	10	1.01 ± 0.20	10	0.82 ± 0.05	10	0.79 ± 0.05
TP-F	10	0.82 ± 0.04	10	1.29 ± 0.22	10	0.86 ± 0.03	10	0.81 ± 0.06
TP-M	10	1.06 ± 0.09	10	1.09 ± 0.20	10	0.91 ± 0.03	10	0.80 ± 0.06

[a]N: number of mice in each group;

[b]Relative mRNA levels of each gene were calculated and expressed as relative to the average of vehicle-treated females.