Molecular Brain Research 118 (2003)
www.elsevier.com/locate/molbrainres
doi> 10.1016/S0169-328X(03)00339-5

**Research report**

# Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation

**Phoebe Dewing[a], Tao Shi[a,b], Steve Horvath[a,b], Eric Vilain[a,c,d,*]**

[a]*Department of Human Genetics, University of California, Los Angeles, CA, USA*
[b]*Department of Biostatistics, University of California, Los Angeles, CA, USA*
[c]*Department of Pediatrics and Mattel Children's Hospital, University of California, Los Angeles, CA, USA*
[d]*Department of Urology, University of California, Los Angeles, CA, USA*

* Corresponding author. Department of Human Genetics, UCLA, 695 Charles E. Young Drive South, Gonda 6357, Los Angeles, CA 90095, USA. Tel.: +1-310-267-2455; fax: +1-310-794-5446. *E-mail address:* evilain@ucla.edu (E. Vilain).

Accepted 31 July 2003

---

**Abstract**

The classic view of brain sexual differentiation and behavior is that gonadal steroid hormones act directly to promote sex differences in neural and behavioral development. In particular, the actions of testosterone and its metabolites induce a masculine pattern of brain development, while inhibiting feminine neural and behavioral patterns of differentiation. However, recent evidence indicates that gonadal hormones may not solely be responsible for sex differences in brain development and behavior between males and females. Here we examine an alternative hypothesis that genes, by directly inducing sexually dimorphic patterns of neural development, can influence the sexual differences between male and female brains. Using microarrays and RT-PCR, we have detected over 50 candidate genes for differential sex expression, and confirmed at least seven murine genes which show differential expression between the developing brains of male and female mice at stage 10.5 days post coitum (dpc), before any gonadal hormone influence. The identification of genes differentially expressed between male and female brains prior to gonadal formation suggests that genetic factors may have roles in influencing brain sexual differentiation.

*Theme:* Development and regeneration
*Topic:* Developmental genetics

*Keywords:* Brain sexual development; Microarray; Sex differences

---

## 1. Introduction

The central dogma of sexual differentiation contends that sexual dimorphisms of somatic tissues are dependent primarily on testicular secretions from the developing fetus [27, 28]. The primary event of sexual development in mammals is the development of the gonadal sex from a bipotential and undifferentiated gonad into either a testis or an ovary. This process, known as sex determination, is triggered by the actions of the testis-determining gene *SRY*, a transcription factor located on the Y chromosome [30, 46]. Once sex is determined, the developing testes produce hormones, such as testosterone and Müllerian Inhibiting Substance (MIS), which subsequently influence the phenotypic sex of the individual. It was established that this concept of hormonal control of sexual differentiation could also be applied to brain sexual development, where testosterone induces masculine patterns of neural and behavioral development, while preventing feminine patterns of differentiation [35, 52].

A great deal of experimental evidence supports the concept that gonadal steroids are solely responsible for inducing brain sexual differentiation. By acting during critical periods of neural development, testosterone and its metabolites cause male and female brains to develop differently [12, 25]. These differences manifest themselves in a variety of ways, such as sizes of particular regions of the brain, number of nerve cells, distribution of neuro-transmitters, and even in development of behavior [12, 19, 25, 45]. In particular, the roles of testosterone, acting directly on the brain by itself or after its conversion to estradiol, have been well studied and shown to have considerable effects on the development of sex-specific structures by organizing neural circuitry to promote behavior more typical of males [3, 36, 41, 52]. Their sites and modes of action have also been delineated [32, 34] as well as their masculinizing actions [20].

However, studies conducted primarily in mammals and birds have challenged the classic theory of brain differentiation and suggested that not all brain sexual dimorphisms are attributable to gonadal hormones. When rat embryonic mesencephalic or diencephalic neurons are harvested and cultured in vitro prior to the detection of differences in levels of testosterone between the sexes (14 days post coitum (dpc)), female cultures tend to harbor more neurons that express tyrosine hydroxylase, an enzyme involved in the biosynthesis of catecholamines [6, 35, 38]. This suggests that the signal for initiating this sexually dimorphic neuronal growth cannot be exclusively dependent on the differences in male and female hormonal action. Another exception arises from thorough endocrine manipulations in zebra finch, whose neural song circuit is sexually dimorphic; males sing while females do not. When females are treated with fadrozole, an aromatase inhibitor, ovarian development is blocked allowing testicular development to progress. However, despite the presence of functional testicular tissue and secretion of androgens, these female birds still have a feminine pattern of neural development [51]. This result suggests that gonadal secretions cannot solely be responsible for differences in male and female brain sexual differentiation.

Recently, a surge of research has begun to explore the prospects that differences in gene expression may result in sex differences in brain development and function. One focus has been on comparing differences in the expression of genes located on sex chromosomes [53], while another has been in developing a mouse model system to study the effects of sex chromosomes on sexually dimorphic neural and behavioral traits [13]. By using this system, DeVries and colleagues have already shown that when comparing XY male mice to XX male mice, as well as XY female mice to XX female mice, their vasopressin fiber densities in the lateral septum are significantly different. In addition, differences in brain cell phenotypes have been noted [9] as well as observational differences

in masculine sexual behaviors and social exploration between male groups of mice differing only in the form of their Sry gene [13]. We propose to examine whether there is the possibility of a direct genetic influence on brain sexual differentiation by determining if there are any genes differentially expressed in male and female brain prior to the influences of gonadal hormones. By using microarray analysis, we have found 51 gene candidates for differential expression between male and female murine brain, suggesting that these genes may have potential roles in causing sex differences in neural function and/or development.

## 2. Materials and methods

### *2.1. Embryo collection and RNA isolation*

Timed matings of CD1 male and female breeding pairs were used to generate staged mouse embryos. Vaginal plugs were checked and considered day 0.5 of gestation (0.5 dpc). At 10.5 dpc, embryos were dissected from pregnant mice and placed in 1 × phosphate-buffered saline (PBS) until head dissections were performed. Whole heads of embryos were collected into separate tubes containing Trizol reagent (Life Technologies) until sexes of embryos were determined by PCR [1]. Primers for SMCY/X were used in PCR where male samples amplified two bands and female samples amplified only one band. PCR conditions were as follows: 94°C for 4 min; 94°C for 30 s, 58°C for 30 s, 72°C for 30 s for 35 cycles; and 72°C for 7 min. Same sex embryos of the same litter were pooled, and brain tissues homogenized. Litter sizes ranged from 7 to 16 embryos. Total RNA was isolated as per manufacturer's recommended protocol, and quantity and quality were determined by spectrophotometry using 260/280 absorption ratios.

### *2.2. Microarray probe labeling and hybridization*

The probe labeling and microarray hybridization were performed by the UC Irvine Microarray Core Facility, where 10μg of the total RNAwas used to produce double-stranded cDNA by reverse transcription using a cDNA synthesis kit (SuperScript Double Stranded cDNA Synthesis Kit, Life Technologies) as per manufacturer's recommended protocol. The cDNA product was then purified (Phenol:Chloroform:I-soamyl Alcohol, Sigma) and ethanol precipitated. Labeled cRNA was generated from the cDNA sample by an in vitro transcription reaction (HighYield RNA Transcript Labeling Kit T7, Enzo Diagnostics) and subsequently purified using RNeasy spin columns (Qiagen, Valencia, CA). Fifteen micrograms of each cRNA sample was fragmented at 94°C for 35 min and used to prepare a hybridization mixture containing a mixture of control cRNAs (Affymetrix, Santa Clara, CA) for comparison of hybridization efficiency between arrays and for relative quantitation of measured transcript levels. Aliquots of each sample were hybridized to Affymetrix U74Av2 GeneChips at 45°C for 16 h, stained with streptavidin–phycoerythrin (Molecular Probes), stained with biotinylated anti-streptavidin IgG, followed by a second staining with streptavidin –phycoerythrin, and washes using non-stringent wash buffer (6 × SSPE, 0.01% Tween 20) between each staining. Microarray experiments were conducted a total of five times and hybridized in pairs with five different sets of littermate RNA samples.

#### *2.2.1. Gene filtering*

In order to obtain normalized gene expression values, we used the widely used dChip software [31], which we (unpublished data) and others [26] have found to be a superior data pre-processing tool. All arrays were normalized against the array with median overall intensity. To identify genes differentially expressed between male and female mice, we used a ''gene filtering criterion'' which has been recommended by Li and Wong (dChip manual). We computed a lower boundary of the fold change (90% confidence interval) and filtered out those genes that passed the threshold of 1.1. Next we computed a permutation test *p*-value for each of the resulting genes by randomly permuting the gender labels. We found 51 genes differentially expressed that were statistically significant at a level

of $p<0.05$.

*2.3. RT-PCR and quantitative RT-PCR*

One microgram of total RNA from male and female littermates was used as template to perform the reverse transcription-polymerase chain reaction (RT-PCR) in a total volume of 25µl. Reverse transcription reactions were primed using both 1µl of oligo d(T) and 1µl of random hexamer primers and incubated at 65°C for 10 min. Reaction tubes were subsequently placed on ice for 2 min followed by the addition of the SuperScript II RNase $H^-$ reverse transcriptase (Life Technologies) and incubated at 42°C for 60 min. cDNA was then used to amplify brain genes with fragment sizes typically between 150 and 800 bp (see Table 1). Glyceraldehyde-3-phophate dehydrogenase (G3PDH) was also amplified at 25 cycles to serve as a control yielding a fragment of 450 bp (CLONTECH Laboratories, Palo Alto, CA). PCR reactions were initiated by denaturing at 95°C for 4 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. After the last cycle, PCR products were allowed to extend at 72°C for 7 min.

The real-time PCR reactions were carried out in an ABI Prism 7700 (PE, Applied Biosystems, Foster City, CA). Amplicons were designed to amplify between 100 and 200 bp in length. QuantiTect SYBR Green RT-PCR kits were used to set up one-step RT-PCR reactions (50 Al total volume) containing 150ng of total RNA as template (Qiagen). According to manufacturer's protocol, reverse transcription was conducted at 50°C for 30 min followed by an initial activation at 95°C for 15 min. PCR annealing temperature was 58–60°C for a total of 40 cycles. All samples were tested in triplicate with the reference gene G3PDH for normalization of data to correct for variations in RNA quality and quantity. Direct detection of PCR products was monitored by measuring the fluorescence produced by SYBR Green I dye binding to double-stranded DNA after every cycle. These measurements were then plotted against cycle numbers. The parameter Ct (threshold cycle) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Fold changes in relative gene expression were calculated by $2^{-\Delta(\Delta Ct)}$ where $\Delta Ct$ = Ct (target) - Ct (G3PDH) and $\Delta(\Delta Ct) = \Delta Ct$ (male) - $\Delta Ct$ (female) (Applied Biosystems).

**3. Results**

*3.1. Differential gene expression between male and female embryonic brains*

Analysis of gene expression between male and female brains at 10.5 dpc, just before the first sign of sexual differentiation of embryonic murine genital ridges [29], revealed the differential expression of a large number of genes. Affymetrix microarray chips U74A, version 2, which consists of all sequences (9268) in the current mouse UniGene database (Build 120) that have been functionally characterized, as well as 3220 EST clusters, detected 36 genes with expression levels enhanced in female brain (Table 2). One of these genes, Inactive X specific transcript (Xist), exhibited the highest fold change (18.5) compared to its expression level in male brains. The other genes enhanced in female brain had fold change values ranging between 1.23 and 2.4 compared to their expression levels in male brain. Affymetrix chips also detected 18 genes with higher expression levels in male brain (Table 3). Two genes, both located on the Y chromosome, DEAD box polypeptide (Dby) and eukaryotic translation initiation factor 2,Y (Eif2s3y), have been detected with fold changes of 10.0 and 8.8, respectively. Other genes with male brain enhanced expression have fold change values ranging between 1.25 and 2.46.

*Table 1*
PCR primers used for RT-PCR and real-time RT-PCR

| Gene name | Primers (forward and reverse) | Product size (bp) |
|---|---|---|

| | | |
|---|---|---|
| AW121876 | F5V-TACAAGATCATCTTCTACAAGTG-3V R5V-CCCTGGTTTCCCCAAGTTCTC-3V | 725 |
| Cyp7b | F5V-GCCGATTATCAGCGAAAGCC-3V R5V-GTGATTTTTAGCAGTTGGGAC-3V | 169 |
| Dby | F5V-CGACCATATCTTCCATTTTCC-3V R5V-GCCTGGACCAGCAATTTGTTG-3V | 752 |
| Eif2s3x | F5V-GACCAGAATGTTACAGATCGTG-3V R5V-CCGCATCCATCACTGCTGCAC-3V | 170 |
| Eif2s3y | F5V-GCCATTTCTGGTGTTCACACTG-3V R5V-CATAAGCTTCCCTTCTCCGTC-3V | 752 |
| Rora4 | F5V-GTCATTACGTGTGAAGGCTGC-3V R5V-CTTGACAGCATCTCGAGACATC-3V | 173 |
| Xist | F5V-ACTGAAAGAAACCACTAGAGG-3V R5V-GTGAGTTATTGCACTACCTGG-3V | 762 |

*Table 2*
Female-enhanced gene expression in brains at 10.5 dpc

| **Gene name** | **Accession number** | **Chromosomal location** | **Fold change** | **One-sided p-value** |
|---|---|---|---|---|
| Inactive X specific transcript (Xist) | L04961 | X | 18.5 | 0.0088 |
| Uridine monophosphate synthetase (Umps) | M29395 | 3 | 2.4 | 0.0144 |
| EST | C76020 | unknown | 2.2 | 0.0173 |
| Prodynorphin | AF026537 | 2 | 2.2 | 0.0313 |
| Cyclin-dependent kinase inhibitor 2B | AF059567 | 4 | 2.2 | 0.0245 |
| EST | AI121305 | unknown | 2.1 | 0.0146 |
| Anti-DNA antibody Ig kappa chain mRNA | U30629 | unknown | 2.0 | 0.0071 |
| Sulfate anion transporter 1 | BC022130 | unknown | 1.8 | 0.0311 |
| EST | AW121876 | unknown | 1.8 | 0.0167 |
| GA binding protein (GABP-alpha subunit) | M74515 | 16 | 1.8 | 0.0066 |
| Eukaryotic initiation factor 4G | 20534506 | unknown | 1.8 | 0.0169 |
| Interleukin 7 receptor | M29697 | 3 | 1.7 | 0.0091 |
| GLI-Kruppel family member GLI3 | X95255 | 13 | 1.7 | 0.0319 |
| DNA polymerase delta 1 | 20827934 | unknown | 1.7 | 0.0078 |

| | | | | |
|---|---|---|---|---|
| EST | AA691059 | unknown | 1.7 | 0.0165 |
| EST | AV359917 | unknown | 1.7 | 0.0078 |
| Zinc finger protein regulator of apoptosis | X95503 | 10 | 1.7 | 0.0317 |
| EST | AW047616 | unknown | 1.7 | 0.0333 |
| Nuclear receptor-binding SET-domain protein | AF064553 | 5 | 1.6 | 0.0144 |
| Thyroid peroxidase | X60703 | 12 | 1.6 | 0.0445 |
| Keratin complex 2 (Krt2-2) | M24151 | 15 | 1.5 | 0.0218 |
| UDP-galactose ceramide galactosyltransferase | U48896 | 4 | 1.5 | 0.0087 |
| Ectodermal-neural cortex 1 | U65079 | 13 | 1.5 | 0.0169 |
| Galactocerebrosidase | D38557 | 12 | 1.5 | 0.0095 |
| Euk translation initiation factor 2,X (Eif2s3x) | AJ0065817 | X | 1.4 | 0.0075 |
| X–Y homologous gene (Utx) | AJ002730 | X | 1.4 | 0.0171 |
| p53-variant (p53) | U59758 | unknown | 1.4 | 0.0075 |
| Neuronal pentraxin 1 | U62021 | 17 | 1.4 | 0.0074 |
| AhR receptor nuclear translocator (Arnt2) | D63644 | 7 | 1.4 | 0.0073 |
| EST | AA674798 | unknown | 1.3 | 0.0245 |
| EST | C78787 | unknown | 1.3 | 0.0093 |
| Somatostatin receptor type 2 (sst2) | AF008914 | 11 | 1.3 | 0.0282 |
| Eukaryotic elongation factor-2 kinase | U93848 | 2 | 1.3 | 0.0094 |
| Cadherin 6 | D82029 | unknown | 1.2 | 0.0084 |
| Norepinephrine transporter gene (SLC6A5) | U92654 | 8 | 1.2 | 0.0069 |

*Table 3*
Male-enhanced gene expression in brains at 10.5 dpc

| **Gene name** | **Accession number** | **Chromosomal location** | **Fold change** | **One-sided p-value** |
|---|---|---|---|---|
| DEAD box polypeptide (Dby) | AJ007376 | Y | 10.0 | 0.0078 |
| Euk translation initiation factor 2, Y (Eif2s3y) | AJ006584 | Y | 9.0 | 0.0076 |

| | | | | |
|---|---|---|---|---|
| EST | AA516942 | unknown | 2.5 | 0.0240 |
| Complement component factor I | U47810 | 3 | 2.4 | 0.0427 |
| EST | AV347947 | unknown | 2.3 | 0.0078 |
| Cytochrome P450, 7b1 (Cyp7b) | U36993 | 3 | 2.1 | 0.0158 |
| S100 calcium binding protein A3 | AF004941 | 3 | 2.0 | 0.0064 |
| Yolk sac permease-like molecule 3 | AF058318 | 18 | 1.9 | 0.0080 |
| Nuclear orphan receptor ROR-alpha 4 (Rora4) | Y08640 | 9 | 1.8 | 0.0306 |
| CD1 antigen | M63695 | 3 | 1.7 | 0.0159 |
| Wnt10b | AF029307 | 7 | 1.6 | 0.0218 |
| Lymphoblastomic leukemia | X57687 | 8 | 1.5 | 0.0163 |
| EST | AA711704 | unknown | 1.4 | 0.0091 |
| EST | AW210346 | unknown | 1.4 | 0.0235 |
| Cbp/p300-interacting transactivator 1 | U65091 | X | 1.4 | 0.0236 |
| EST | AW122995 | unknown | 1.3 | 0.0076 |

*3.2. Quantification of differentially expressed genes*

We selected a small number of genes based on their known profiles of expression and potential roles in brain development for further investigation. Differential expression of these genes was first verified by classical RT-PCR and results showed profiles of expression consistent with that of the microarray data ( ). Of the female-enhanced genes, we verified the differential expression of three genes, Xist, EST AW121876, and eukaryotic translation initiation factor 2, X (Eif2s3x). Likewise, we see the same differential expression in male genes Dby, Eif2s3y, cytochrome *P*450, 7b (Cyp7b), and nuclear orphan receptor ROR-alpha 4 (Rora4), as predicted by the microarray, using RT-PCR.

In some cases, differences in expression were too difficult to interpret by RT-PCR. The process of exponential amplification associated with RT-PCR makes quantification of slight differences in expression difficult to visualize. Therefore, we employed real-time quantitative RT-PCR with SYBR Green I dye to validate some of our differentially expressed genes with more subtle differences in fold change ( ). The RT-PCR reactions were carried out simultaneously with a control, G3PDH, to normalize values for each reaction tube. Pooled litter-mate-matched RNA samples were used for these experiments. Ct values obtained from these experiments showed relative differences in gene expression between males and females for all of the genes chosen. When examining the data obtained for Cyp7b, male brains produced an average Ct value of 26.0, while female brains exhibit an average Ct value of 27.2. Thus, the level of Cyp7b is slightly higher in the male brain than in the female. Moreover, calculations (see Materials and methods) show an average fold change difference of 1.9 for Cyp7b specific to male brain versus the fold change of 2.1 obtained by microarray. Real-time RT-PCR was performed four times, with fold changes ranging between 1.4 and 2.5, using different pairs of male and female littermate RNA samples. Similarly, real-time RT-PCR was performed multiple times for Ror-a4, Eif 2s3x, and EST AW121876 showing average fold changes of 1.8, 1.9, and 1.3, respectively, which closely correlated with the fold changes of 1.8, 1.4, and 1.8

obtained by the micro-array method.

*Fig. 1*. Validation of microarray results by RT-PCR analysis.
(a) Differential expression of seven genes, Xist, Dby, Eif2s3y, Eif2s3x, Cyp7b, Rora4 and AW121876, was detected from male and female brains at 10.5dpc.
(b) Relative expression levels were normalized in reference to the housekeeping gene G3PDH.

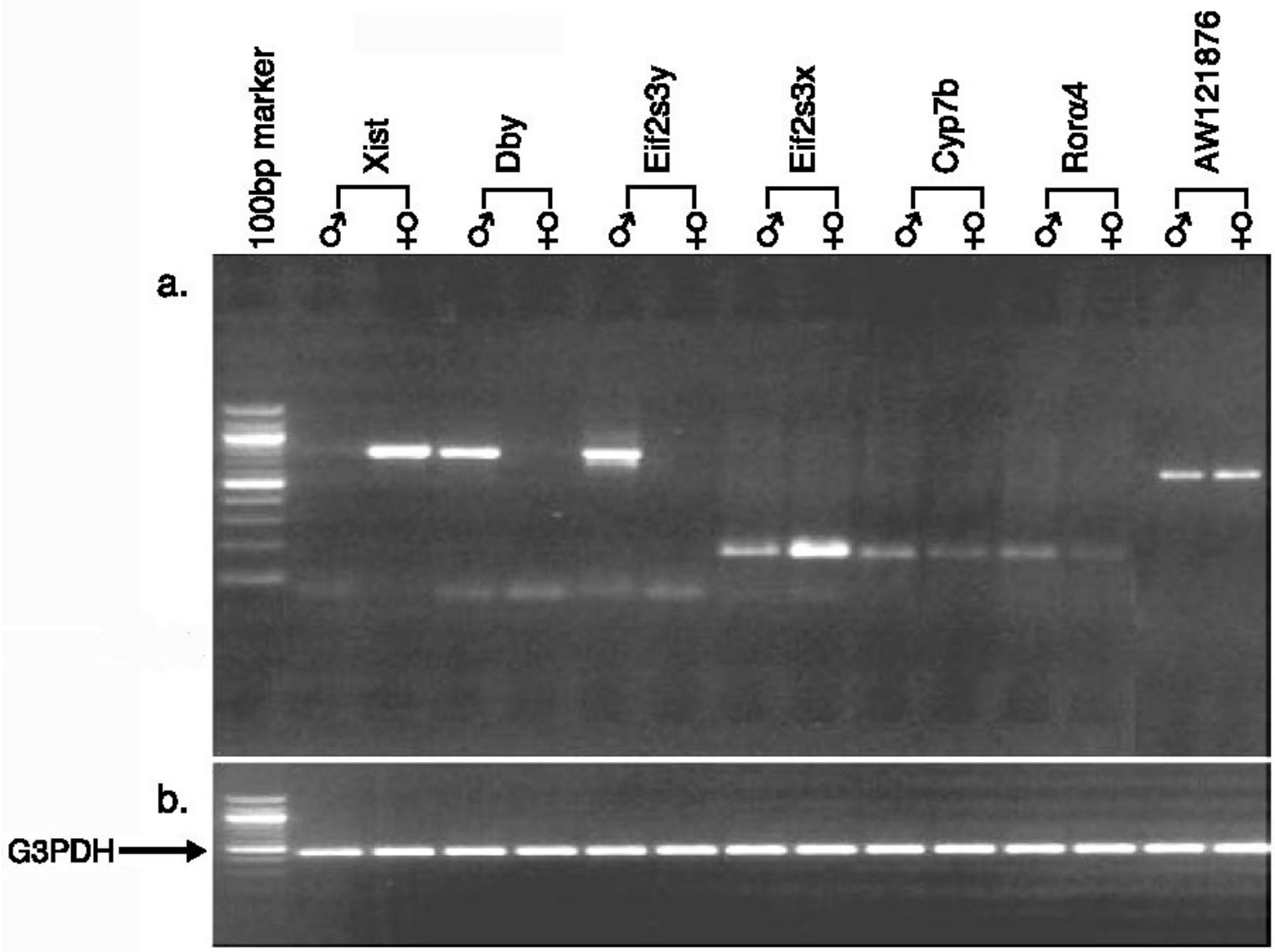
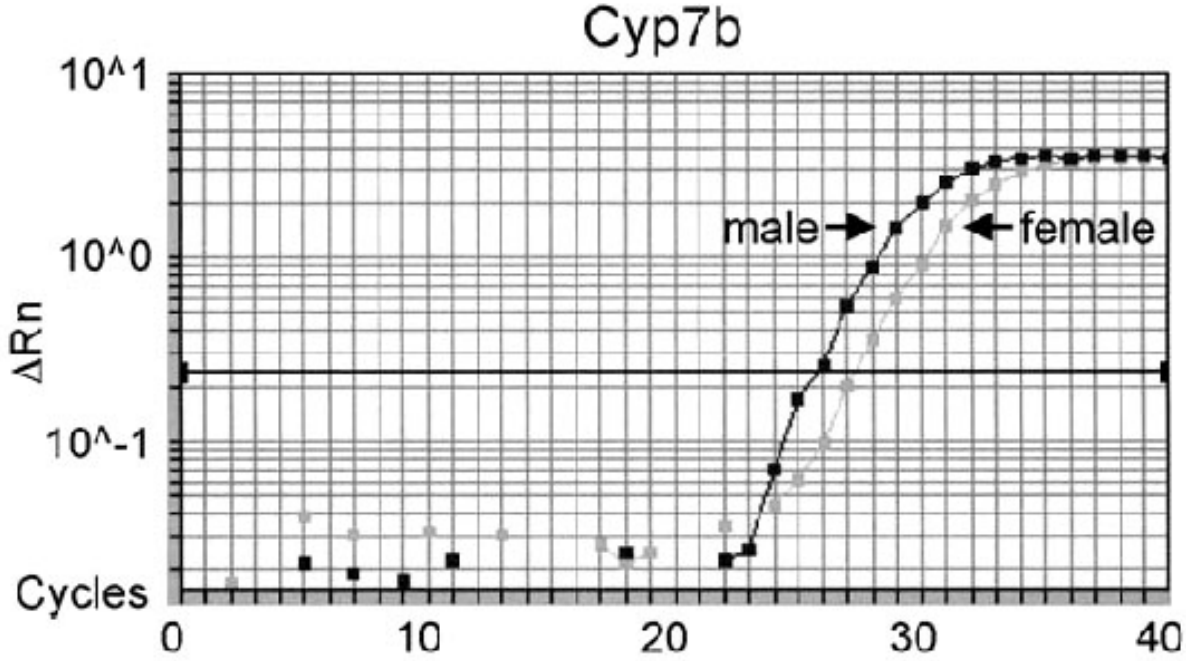
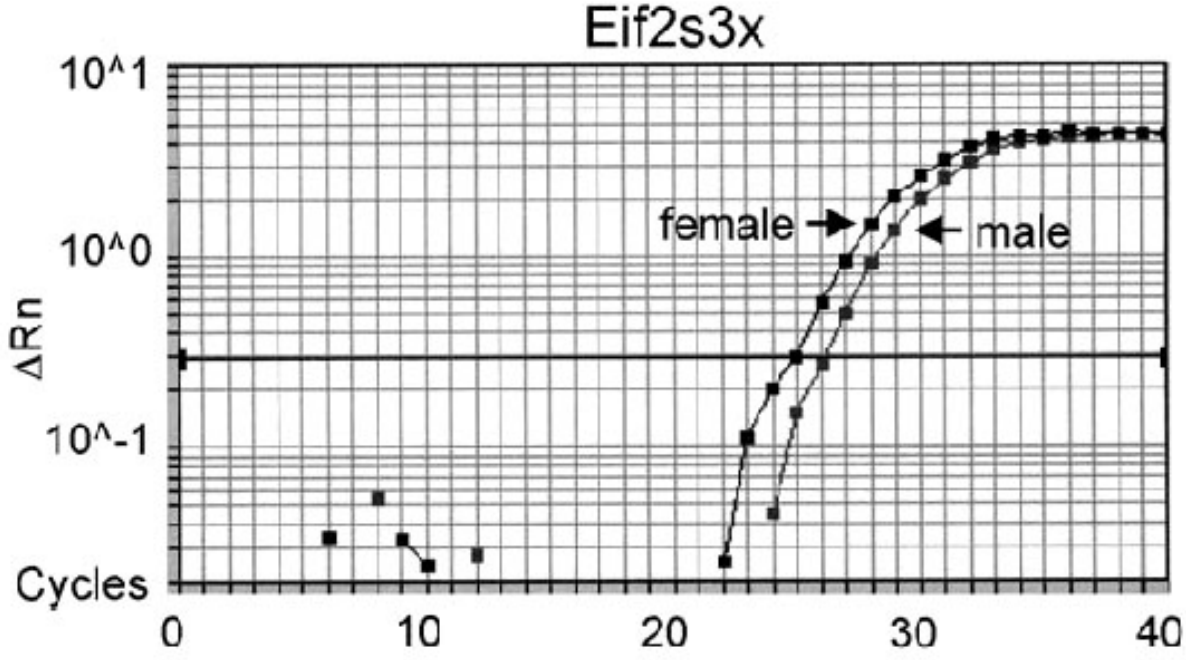
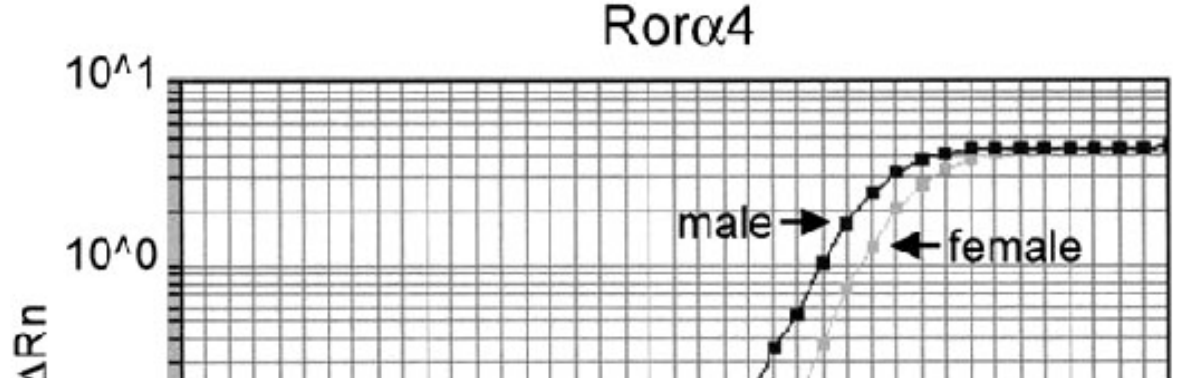
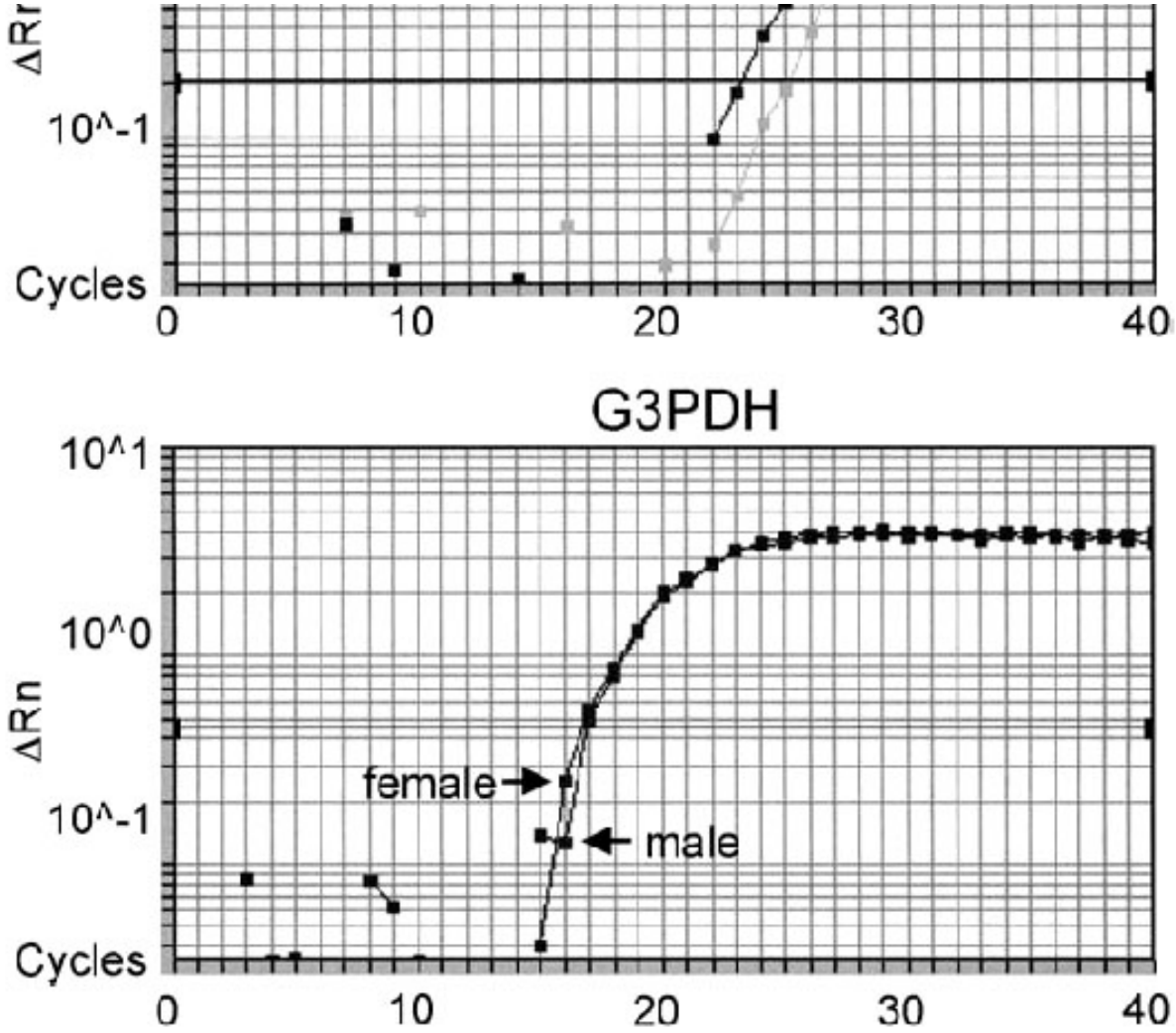


*Fig. 2.* Real-time RT-PCR analysis of Cyp7b, Eif2s3x, and Rora4. Each curve represents relative quantification of the gene of interest in reference to the housekeeping gene G3PDH. Each experiment was performed in triplicate though all curves are not shown in this figure. Each signal represents the total amplification of cDNA in that particular tube and Ct values exhibit differences in gene expression, which can further be translated into fold changes.






**4. Discussion**

Sexually dimorphic development of the brain has been shown to be caused by testosterone acting directly or indirectly, via conversion to estradiol, on male brain to induce masculine patterns of neural development [2, 3]. Since there are known morphological differences between male and female brains, we speculated that there may be direct genetic influences, aside from the influences of gonadal hormones, which result in these morphological differences. The current study has identified genes differentially expressed between male and female embryonic mouse brain before any embryonic gonadal secretion has taken place. This suggests that there are functional differences between male and female brains, which occur independently from hormonal influence. Moreover, these differentially expressed genes are good candidates for a role in brain sexual differentiation and sexual behavior.

Using microarray gene expression analysis and setting our fold change threshold value at 1.1, our analysis has detected a total of 51 genes with differential expression between males and females. The threshold value was arbitrarily set as low as 1.1 to avoid the risk of excluding genes which may have potential roles in brain differentiation yet only exhibit slight differences in gene expression between males and females. We considered five litters. For each litter, we pooled the RNA samples of the male mice and hybridized them onto Affymetrix gene chip arrays. We repeated the same for females. Then we used these separately pooled male and female samples to perform the gene differential analysis.

Of the genes obtained from our analysis, the majority of them with known biological functions fall into three main categories: (1) cellular differentiation and proliferation; (2) transcriptional regulators; and (3) signaling molecules. However, a considerable number of genes, about one-fourth, have unknown functions. From our list of 51 genes, seven were verified by multiple RT-PCR and real-time PCR analyses. Three of them, Xist, Eif 2s3x, and

EST AW121876 are female-enhanced and four genes, Dby, Eif 2s3y, Cyp7b, and Rora4 are male-enhanced. Two of these genes, Xist and Eif 2s3x, were located on the X chromosome, and two genes, Dby and Eif 2s3y, were located on the Y chromosome. The difference in expression level of the selected autosomal genes was no more than 2.1 times greater in one sex than the other, but was consistently confirmed by a series of RT-PCR and real-time RT-PCR experiments. RT-PCR reactions were performed multiple times for each gene using at least one other different set of littermate-matched RNA

Genes localized to sex chromosomes show marked differences in gene expression and their localization is consistent with a role in triggering brain sexual differentiation. According to the theory that mammalian sex genes evolved from a pair of autosomal chromosomes [21], we have to consider the dosage effects of genes expressed in the non-recombining regions of the X- and Y-chromosomes. From our microarray, Eif 2s3y, located on the non-recombining region of the Y-chromosome, was found to be expressed nine times higher in males than in females. In mammals, Eif 2s3y is not present in females, which simply suggests that Eif 2s3y might act in the brain to promote masculine patterns of neural and behavioral development. However, this idea is compounded by the existence of a closely related homologue, Eif 2s3x, which was found to be expressed only 1.4 times higher in females than in males. If Eif 2s3y and Eif 2s3x have remained functional homologues on the sex chromosomes, we might assume that they play equivalent roles in the brain. According to this view one would expect that in males, which have one copy each of Eif 2s3y and Eif 2s3x, their combined level of expression should be comparable to that of Eif 2s3x found in females. Eif 2s3x in the male would thus be half that found in females, since Eif 2s3x escapes inactivation [15], leaving the gene expressed essentially in a double dose. Yet our data do not support this hypothesis. From our data and data presented by Xu et al. [53], it appears that Eif 2s3y may not necessarily be functionally equivalent to its homologue, but rather have distinct and unknown roles in sexual differentiation. Differences in expression patterns of this pair of X and Y homologue genes suggest that they may be differentially regulated, resulting in different functional effects [53].

Another Y-linked gene, Dby, was highly differentially expressed between male and female brains. Dby encodes an RNA helicase with a conserved DEAD box motif that is localized to the azoospermia factor (AZF) region of the Y chromosome. AZF is frequently deleted in infertile males [17, 18], but the specific function of Dby remains unclear. Since the Y chromosome has been shown to have a direct role in male mouse aggression [33, 43, 47, 50], Y-linked genes with such sexually dimorphic patterns of expression are candidates for susceptibility factors of aggressive behavior. Furthermore, studies comparing mouse strains [22, 24, 33] have implicated that the Y-chromosome encodes genes that may play a role in specific sexually dimorphic behaviors or neural phenotypes, such as hippocampal asymmetry [49] and differences in hippocampal mossy fiber distribution. Moreover, the presence of such high expression levels of Dby and Eif 2x3y may support the hypothesis that Y chromosome dosage or Y chromosome genes have significant effects on the development of male neural and behavioral phenotypes [37].

Xist, one of the female-enhanced genes with the largest fold difference, is an RNA transcript localized to the inactive X chromosome in females, with no apparent protein-coding capacity [8]. Fluorescence in situ hybridization studies have shown that in males, Xist expression is very low at all stages studied [23], whereas Xist expression in female embryos is seen at measurable levels starting at the late two-cell stage and rapidly accumulates until the morula stage. Xist is well known for its actions in transcriptional silencing and is required during early embryogenesis. Thus, its expression profile depicted by the microarray is consistent with earlier findings and serves as an internal control of the microarray method. Since there are two copies of the X chromosome found in females, we speculate that the large gene expression level obtained by Xist may be a result of dose effect. However, whether or not Xist is a strong candidate for brain sexual differentiation is still unknown.

The majority of the remaining genes found differentially expressed in this study were localized to autosomal chromosomes. Surprisingly, some of these genes exhibited fold changes as great as 2.5 between males and females. This difference in expression, albeit moderate, may have subsequent effects on development of the neural system and behavior. Cytochrome P450, 7b, a gene localized to chromosome 3, belongs to a diverse family of heme-

containing monooxygenases (CYPs) that catalyzes oxidative interconversions of steroids. Cyp7b has previously been found to be highly expressed in mouse corpus callosum and rat hippocampus [39] and has been implicated as a neurosteroid hydroxylase because it catalyzes the synthesis of neurosteroids 7a-hydroxy dehydroepiandrosterone and 7a-hydroxypregnenolone [40]. The expression of Cyp7b has also been shown as early as 12.5 dpc throughout the CNS with strongest expression levels in the neocortex [5] but the sexes of the embryos studied were not mentioned. Its role and actions in neurosteroid metabolism may have considerable impact on the differentiation of specific regions of the developing brain since neurosteroids have been implicated in modulating cognitive function, behavior, and synaptic plasticity [4, 11].

Orphan nuclear receptor, Rora4, another gene localized to an autosomal chromosome, has been shown to bind as a monomer to a conserved AGGTCA motif located in the a-fetoprotein (AFP) enhancer [7]. AFP is a liver protein that is produced in abundance during the neonatal period. It is known to sequester estradiol and allows only testosterone to enter the neurons of the brain where it is aromatized into estrogen by a process that may affect brain differentiation [44]. The expression of the Rora gene, which exists in four isoforms, has been localized to the CNS to the cerebellar Purkinje cells (PCs), the thalamus, the suprachiasmatic nuclei, and retinal ganglion cells [44]. Moreover, a Rora null-mutant mouse has been generated by gene targeting and its cerebral and behavioral phenotypes include abolishment of cerebellar PC development and severe cerebellar ataxia suggesting an essential role of Rora in PC differentiation [48].

We speculate that the genes reported in this study may be fundamental factors that trigger differences between male and female brain development prior to the production of gonadal steroid hormones. Furthermore, these differences are present at 10.5 dpc in mice, when the adrenals, the other main source of hormonal steroidogenesis, have not yet differentiated [54]. Thus we can assume that the influences of gonadal or adrenal steroid hormones are quite minimal. In addition, while maternal placental hormones may influence brain sexual differentiation, we suggest that the effects of these hormones will be equalized between male and female embryos as long as they are from the same litter, which was the case in this study. Intrauterine positioning of male and female fetuses in rodents could also affect morphology, physiology and behavior, resulting from testosterone transfer from the developing male to neighboring female fetuses [16]. However, this environmental effect is irrelevant at 10.5 dpc since gonadal hormones have yet to be secreted.

We suggest that some of the differences seen in gene expression are involved in the development of brain sexual dimorphisms. They may contribute functionally to the well-documented differences in task performance or even the stereotypical behaviors seen between males and females [10, 14, 42]. The mechanisms by which these genes act on neural development and behavior, either independently or synergistically with sex hormones, are still to be determined. This data argues for a shift of paradigm from the classic hormone-dependent theory of brain differentiation to one including direct genetic effects. With more evidence suggesting a direct role for genes in brain sexual differentiation, using genetically modified animal models will be the next step in allowing the characterization of genes influencing neural and behavioral phenotypes, as well as promote the understanding of the non-hormonal mechanisms which induce the sexually dimorphic neural development between males and females.

## Acknowledgements

We thank J. Denis Heck and Kim T.T. Nguyen at the UCI DNA MicroArray Facility for all their help on this project. We are also deeply grateful to Dr. Art Arnold for his insightful comments and discussions. P.D. is supported by the Laboratory of Neuroendocrinology NIH training grant. T.S. is a predoctoral trainee supported by the UCLA IGERT Bioinformatics Program funded by NSF DGE 9987641.

## References

1. A.I. Agulnik, G. Longepied, M.T. Ty, C.E. Bishop, M. Mitchell, Mouse H-Y encoding Smcy gene and its X chromosomal homolog Smcx, *Mamm. Genome* 10 (1999) 926-929
2. A.P. Arnold, Genetically triggered sexual differentiation of brain and behavior, *Horm. Behav.* 30 (1996) 495-505.
3. A.P. Arnold, R.A. Gorski, Gonadal steroid induction of structural sex differences in the central nervous system, *Annu. Rev. Neurosci.* 7 (1984) 413-442.
4. E.E. Baulieu, Neurosteroids: a new function in the brain, *Biol. Cell* 71 (1991) 3-10.
5. R. Bean, J.R. Seckl, R. Lathe, C. Martin, Ontogeny of the neuro-steroid enzyme Cyp7b in the mouse, *Mol. Cell. Endocrinol.* 174 (2001) 137-144.
6. C. Beyer, C. Pilgrim, I. Reisert, Dopamine content and metabolism in mesencephalic and diencephalic cell cultures: sex differences and effects of sex steroids, *J. Neurosci.* 111 (1991) 325-1333.
7. B. Bois-Joyeux, C. Chauvet, H. Nacer-Cherif, W. Bergeret, N. Mazure, V. Giguere, V. Laudet, J.L. Danan, Modulation of the far-upstream enhancer of the rat a-fetoprotein gene by members of the RORa, Rev-erba, and Rev-erbh groups of monomeric orphan nuclear receptors, DNA *Cell Biol.* 19 (2000) 589-599.
8. N. Brockdorff, A. Ashworth, G.F. Kay, V.M. McCabe, D.P. Norris, P.J. Cooper, S. Swift, S. Rastan, The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus, *Cell* 71 (1992) 515-526.
9. L.L. Carruth, I. Reisert, A.P. Arnold, Sex chromosome genes directly affect brain sexual differentiation, *Nat. Neurosci.* 10 (2002) 933-934.
10. E. Choleris, M. Kavaliers, Social learning in animals: sex differences and neurobiological analysis, *Pharmocol. Biochem. Behav.* 64 (1999) 767-776.
11. E. Costa, J. Auta, A. Guidotti, A. Korneyev, E. Romeo, The pharmacology of neurosteroidogenesis, *J. Steroid Biochem. Mol. Biol.* 49 (1994) 385-389.
12. M.D. De Bellis, M.S. Keshavan, S.R. Beers, J. Hall, K. Frustaci, A. Masalehdan, J. Noll, A.M. Boring, Sex differences in brain maturation during childhood and adolescence, *Cereb. Cortex* 11 (2001) 552-557.
13. G.J. DeVries, E.F. Rissman, R.B. Simerly, L.-Y. Yang, E.M. Scordalakes, C.J. Auger, A. Swain, R. Lovell-Badge, P.S. Burgoyne, A.P. Arnold, A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits, *J. Neurosci.* 20 (2002) 9005-9014.
14. S.J. Duff, E. Hampson, A sex difference on a novel spatial working memory task in humans, *Brain Cogn.* 47 (2001) 470-493.
15. I.E. Ehrmann, P.S. Ellis, S. Mazeyrat, S. Duthie, N. Brockdorff, M.G. Mattei, M.A. Gavin, N.A. Affara, G.M. Brown, E. Simpson, M.J. Mitchell, D.M. Scott, Characterization of genes encoding translation initiation factor eIF-2gamma in mouse and human: sex chromosome localization, escape from X-inactivation and evolution, *Hum. Mol. Genet.* 7 (1998) 1725-1737.
16. M.D. Even, M.G. Dhar, F.S. vom Saal, Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats, *J. Reprod. Fertil.* 96 (1992) 709– 716.
17. C. Foresta, A. Ferlin, E. Moro, Deletion and expression analysis of AZFa genes on the human Y chromosome revealed a major role for DBY in male infertility, *Hum. Mol. Genet.* 9 (2000) 1161– 1169.
18. C. Foresta, E. Moro, A. Ferlin, Y chromosome microdeletions and alterations of spermatogenesis, *Endocr. Rev.* 22 (2001) 226– 239.
19. J.M. Goldstein, L.J. Seidman, N.J. Horton, N. Makris, D.N. Kennedy, V.S. Caviness Jr., S.V. Faraone, M.T. Tsuang, Normal sexual dimorphism of the adult human brain assessed by in vivo magnetic resonance imaging, *Cereb. Cortex* 11 (2001) 490– 497.
20. R. Gorski, Sexual differentiation of the endocrine brain and its control, in: M. Motta (Ed.), *Brain Endocrinology, 2nd ed.*, From Raven Press, New York, 1991.

21. J.A.M. Graves, The origin and function of the mammalian Y chromosome and Y-borne genes—an evolving understanding, *BioEssays* 17 (1995) 311-319.
22. P.V. Guillot, M. Carlier, S.C. Maxson, P.L. Roubertoux, Intermale aggression tested in two procedures, using four inbred strains of mice and their reciprocal congenics: Y chromosomal implications, *Behav. Genet.* 25 (1995) 357-360.
23. C. Hartshorn, J.E. Rice, L.J. Wang, Developmentally-regulated changes of Xist RNA levels in single preimplantation mouse embryos, as revealed by quantitative real-time PCR, *Mol. Reprod. Dev*. 61 (2002) 425-436.
24. R.A. Hensbroek, F. Sluyter, P.V. Guillot, G.A. Van Oortmerssen, W.E. Crusio, Y chromosomal effects on hippocampal mossy fiber distributions in mice selected for aggression, *Brain Res.* 682 (1995) 203-206.
25. J.B. Hutchison, Gender-specific steroid metabolism in neural differentiation, *Cell. Mol. Neurobiol.* 17 (1997) 603-626.
26. R.A. Irizarry, B.M. Bolstad, F. Collin, L.M. Cope, B. Hobbs, T.P. Speed, Summaries of Affymetrix GeneChip probe level data, *Nucleic Acids Res.* 31 (2003) e15.
27. A. Jost, Reserches sur la differenciation sexuelle de l'embryon de lapin. III, *Arch. Anat. Microsc. Morphol. Exp.* 36 (1947) 271-315.
28. A. Jost, Hormonal factors in the sex differentiation of the mammalian foetus, Philos. Trans. R. Soc. Lond., *B Biol. Sci.* 259 (1970) 119-130.
29. P. Koopman, A. Mu¨ nterberg, B. Capel, N. Vivian, R. Lovell-Badge, Expression of a candidate sex-determining gene during mouse testis differentiation, *Nature* 348 (1990) 450-452.
30. P. Koopman, J. Gubbay, N. Vivian, R. Lovell-Badge, Male development of chromosomally female mice transgenic for Sry, *Nature* 351 (1991) 117-121.
31. C. Li, W.H. Wong, Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 31-36.
32. N.J. MacLusky, F. Naftolin, Sexual differentiation of the central nervous system, *Science* 211 (1981) 1294-1303.
33. S.C. Maxson, Sex differences in genetic mechanisms for mammalian brain and behavior, *Biomed. Rev.* 7 (1999) 85-90.
34. B.S. McEwen, Neural gonadal steroid actions, *Science* 211 (1981) 1303-1311.
35. C.H. Phoenix, R.W. Goy, A.A. Gerall, W.C. Young, Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig, *Endocrinology* 65 (1959) 369-382.
36. C.H. Pilgrim, I. Reisert, Differences between male and female brains—developmental mechanisms and implications, *Horm. Metab. Res*. 24 (1992) 353-359.
37. S.G. Ratcliffe, G.E. Butler, M. Jones, Edinburgh study of growth and development of children with sex chromosome abnormalities, in: J.A. Evans, J.L. Hamerton, A. Robinson (Eds.), *Children and Young Adults with Sex Chromosome Aneuploidy: Follow-up*, Wiley, New York, 1999, pp. 1-44.
38. I. Reisert, C. Pilgrim, Sexual differentiation of monoaminergic neurons— genetic or epigenetic, *TINS* 14 (1991) 467-473.
39. K.A. Rose, G. Stapleton, K. Dott, M.P. Kieny, R. Best, M. Schwarz, D.W. Russell, I. Bjorkhem, J. Seckl, R. Lathe, Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7a-hy-droxy dehydroepiandrosterone and 7A-hydroxy pregnenolone, *Proc. Natl. Acad. Sci. U. S. A.* 94 (1997) 2925-2930.
40. K. Rose, A. Allan, S. Gauldie, G. Stapleton, L. Dobbie, K. Dott, C. Martin, L. Wang, E. Hedlund, J.R. Seckl, J.A. Gustafsson, R. Lathe, Neurosteroid hydroxylase Cyp7b, J. *Biol. Chem.* 276 (2001) 23927-23944.
41. C.E. Roselli, S.E. Abdelgadir, J.A. Resko, Regulation of aromatase gene expression in the adult rat brain, *Brain Res.* 44 (1997) 351-357.

42. M. Sakthivel, P.E. Patterson, C. Cruz-Neira, Gender differences in navigating virtual worlds, *Biomed. Sci. Instrum.* 35 (1999) 353-359.
43. N.K. Sandnabba, Selective breeding for isolation-induced intermale aggression in mice: associated responses and environmental influences, *Behav. Genet.* 26 (1996) 477-488.
44. S. Sashihara, P.A. Felts, S.G. Waxman, T. Matsui, Orphan nuclear receptor ROR alpha gene: isoform-specific spatiotemporal expression during postnatal development of brain, *Mol. Brain Res.* 42 (1996) 109-117.
45. S. Segovia, A. Guillamon, M.C. del Cerro, E. Ortega, C. Perez-Laso, M. Rodriguez-Zafra, C. Beyer, The development of brain sex differences: a multisignaling process, *Behav. Brain Res.* 105 (1999) 69-80.
46. A.H. Sinclair, P. Berta, M.S. Palmer, J.R. Hawkins, B.L. Griffiths, M.J. Smith, J.W. Foster, A.M. Frischauf, R. Lovell-Badge, P.N. Goodfellow, A gene from the human sex-determining region encodes a protein with homology to a conserved DNA binding motif, *Nature* 346 (1990) 240-244.
47. F. Sluyter, G.A. Van Oortmerssen, A.J.H. De Ruiter, J.M. Koolhaas, Aggression in wild house mice: current state of affairs, *Behav. Genet.* 26 (1996) 489-496.
48. M. Steinmayr, E. Andre, F. Conquet, L. Rondi-Reig, N. Delhaye-Bouchaud, N. Auclair, H. Daniel, F. Crepel, J. Mariani, C. Sotelo, M. Becker-Andre, Staggerer phenotype in retinoid-related orphan receptor a-deficient mice, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 3960-3965.
49. J.H. van Abeelen, C.J. Janssens, W.E. Crusio, W.A. Lemmens, Y-chromosomal effects on discrimination learning and hippocampal asymmetry in mice, *Behav. Genet.* 19 (1989) 543-549.
50. G.A. Van Oortmerssen, F. Sluyter, Studies on wild house mice. Aggression in lines selected for attack latency and their Y-chromosomal congenics, *Behav. Genet.* 24 (1994) 74-78.
51. J. Wade, A.P. Arnold, Functional testicular tissue does not masculinize development of the zebra finch song system, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 5264-5268.
52. R.E. Whalen, Differentiation of the neural mechanisms which control gonadotrophin secretion and sexual behavior, in: M. Diamond (Ed.), *Reproduction and Sexual Behavior*, Indiana Univ. Press, Bloomington, 1968, pp. 303-340.
53. J. Xu, P.S. Burgoyne, A.P. Arnold, Sex differences in sex chromosome gene expression in mouse brain, *Hum. Mol. Genet.* 11 (2002) 1409-1419.
54. J.T. Yarrington, Adrenal cortex, in: U. Mohr (Ed.), *Pathobiology of the Aging Mouse, vol. 1*, ILSI Press, Washington, DC, 1996, pp. 125-127.