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Sex Differences in the Cerebellum and Frontal Cortex: Roles of Estrogen Receptor Alpha and Sex Chromosome Genes

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Autism · X inactivation · Sex differences · Calbindin · Fragile X

Abstract
Most neurobehavioral diseases are sexually dimorphic in their incidence, and sex differences in the brain may be key for understanding and treating these diseases. Calbindin (Calb) D28K is used as a biomarker for the well-studied sexually dimorphic nucleus, a hypothalamic structure that is larger in males than in females. In the current study weanling C56BL/6J mice were used to examine sex differences in the Calb protein and message focusing on regions outside of the hypothalamus. A robust sex difference was found in Calb in the frontal cortex (FC) and cerebellum (CB; specifically in Purkinje cells); mRNA and protein were higher in females than in males. Using 2 mouse lines, i.e. one with a complete deletion of estrogen receptor alpha (ERα) and the other with uncoupled gonads and sex chromosomes, we probed the mechanisms that underlie sexual dimorphisms. In the FC, deletion of ERα reduced *Calb1* mRNA in females compared to males. In addition, females with XY sex chromosomes had levels of *Calb1* equal to those of males. Thus, both ERα and the sex chromosome complement regulate *Calb1* in the FC. In the CB, ERα knockout mice of both sexes had reduced *Calb1* mRNA, yet sex differences were retained. However, the sex chromosome complement, regardless of gonadal sex, dictated *Calb1* mRNA levels. Mice with XX chromosomes had significantly greater *Calb1* than did XY mice. This is the first study demonstrating that sex chromosome genes are a driving force producing sex differences in the CB and FC, which are neuoranatomical regions involved in many normal functions and in neurobehavioral diseases.

Introduction

Sex differences in behaviors such as cognition, mood, and motor skills are well documented [1–3]. Understanding the underlying neural mechanisms regulating sex differences can offer important insights into neurobehavioral diseases with strong sex biases. The roles of gonadal steroids and their receptors in the development of sexually dimorphic hypothalamic structures are well established [4, 5], but few sexual dimorphisms have been characterized outside of the hypothalamus [6]. Moreover, while many sex differences can be attributed to developmental exposure to hormones, a more novel hypothesis, i.e. that sex chromosome genes have a direct action on neural sexual dimorphism, is gaining momentum [7, 8]. The current studies specifically address nonhypothalamic sex differences and examine the role of estrogen recep-

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tor alpha (ERα) and the sex chromosome complement on Calbindin-D28K (Calb) expression.

Calb, a member of the EF-hand calcium-binding protein family, is highly expressed in the brain and plays an important role in maintaining Ca^{2+} homeostasis within neurons (for review see Kojetin et al. [9] and Bastianelli [10]). Calb immunoreactivity has been routinely used as a biomarker for the sexually dimorphic nucleus (SDN) in the hypothalamus (males > females) [11–13]. The greatest concentrations of Calb are in the cerebellum (CB), where it is expressed exclusively in Purkinje cells [10], and in the cortex; these are regions that play an important role in normal cognition, motor skills, and emotional processing [14–16]. This protein has multiple functions. Studies with Calb knockout (KO) mice report distinct deficits in circadian rhythms, motor skills, coordination, and spatial learning in the Morris water maze [17–20]. In addition, long-term potentiation in the hippocampus is not stable, and in Purkinje cells the time course and amplitude of calcium transients are significantly altered while spine lengths are increased [20–23]. Finally, in Purkinje cell-specific Calb KO mice motor coordination and visual and vestibular processing are markedly impaired [21].

The current study is the first to show that the Calb gene and protein are expressed in a sexually dimorphic manner outside of the hypothalamus. In particular, sex differences (female > male) in Calb were observed in normal juvenile mouse brains in the CB and the frontal cortex (FC). We capitalized on 2 mouse models to assess the underlying mechanisms regulating sex differences in Calb. Here we examined *Calb1* in ERα KO (ERαKO) and wild-type (WT) littermates to determine if this steroid receptor was responsible for the observed sex differences. Next, the roles of the sex chromosomes were evaluated using a mouse model that isolates the effects of gonadal sex and the sex chromosome complement (table 1) [8].

Table 1. Comparison of normal and FCG mice

Mouse cross	Nomenclature	Genotype	Gonadal phenotype	X	Y
WT	F	XX	ovaries	2	0
	M	XY	testes	1	1
FCG	XXF	XX	ovaries	2	0
	XYF	XY–	ovaries	1	1
	XXM	XX*Sry*	testes	2	0
	XYM	XY*Sry*	testes	1	1

In the FCG, numbers of X versus Y chromosomes can be separated from gonadal effects.

Methods

Animals

All mice were in a C57BL/6J background and were born, reared, and housed at the University of Virginia School of Medicine Animal Facility in Jordan Hall on a 12:12-hour light:dark cycle (lights on at 6:00 a.m.). Food (Harlan Teklad Mouse/Rat Sterilizable Diet No. 7012) and water were provided ad libitum. Three 'lines' of mice were used, all in the C57BL/6J background. These lines were: normal C57BL/6J, ERαKO [24], and the 4 core genotypes (FCG) [8]. All animals were weaned at 20–21 days of age and sacrificed between 21–25 days of age. These young animals were used to compare sexes at a time when gonadal hormone levels were uniformly low in males and females. We have examined testosterone levels and target organ weights in animals from the FCG at this age and there are no genotypic differences [Cox and Rissman, unpubl. data]. The University of Virginia Animal Use and Care Committee approved all animal procedures.

Genotyping

DNA was extracted from mouse ear clippings using a Tissue DNA Extraction and Amplification kit from SIGMA (XNATR). To screen for the presence or absence of the deletion of the ERα gene *(Esr1)*, 2 PCR reactions were run. To screen for the presence or absence of the Y chromosome, the YMT2/B sequence (a member of the *Ssty* gene family present on the long arm of the Y chromosome) was used. An amplification control myogenin primer set was also used. All of the PCR products were run on a 2% agarose gel.

Brain Tissue Preparation for in situ Hybridization and Protein and RNA Extraction

For in situ hybridization (ISH) studies, normal C57BL/6J (PN21–25) mice were anesthetized using a lethal dose of Euthanasol, and they were transcardially perfused with 4% paraformaldehyde (Polysciences, Inc., Warrington, Pa., USA) in 0.1 M phosphate buffer, pH 7.4. After perfusion, the brains were postfixed overnight prior to vibrotome sectioning (50-μm coronal sections). The sections for ISH were stored in cryoprotectant [30% sucrose, 30% ethylene glycol, and 1% polyvinylpyrrolidone (PVP-40)] at –20°C until they were processed. For protein and RNA collection, mice were sacrificed, their brains were rapidly removed, and the FC, CB, hypothalamus, hippocampus, and amygdala were carefully dissected on ice, frozen on dry ice, and stored at –80°C until further processing for protein and RNA extraction.

Synthesis of Calbindin RNA Probes

T3- or T7-labeled RNA probes were prepared from a PCR-generated *Calb1* DNA template with the T7 and T3 promoter in the 5′ ends of the gene. Specifically, a sense primer (Invitrogen, Carlsbad, Calif., USA) containing the T3 promoter (in bold) and *Calb1* gene (bp 109–129) (**CAGAGATGCAATTAACCCTCACTAAAGGGAGA**CATCTCTGATCACAGCCTCACAG) and an antisense primer (Invitrogen) containing the T7 promoter (in

Table 2. Forward and reverse nucleotide sequences of β-actin and Calbindin D28k used to generate primers for qPCR

Gene	Accession No.	Nucleotide sequence (start location)
β-Actin (Actb)	NM_007393	forward: 5′-CCAGATCATGTTTGAGACCTTCAA (439) reverse: 5′-CCAGAGGCGTACAGGGATAGC (519)
Calbindin (Calb1)	NM_009788	forward: 5′-ACTCTCAAACTAGCCGCTGCA (110) reverse: 5′-TCAGCGTCGAAATGAAGCC (212)

bold) and *Calb1* gene (bp 638–659) (**CCAAGCTTCTAATACGACTCACTATAGGGAGA**TTCCTGCACTGGTAGTAACCTGG) produced a DNA template with both promoters. DNA sequencing prior to use in an in vitro transcription assay verified the template. Digoxigenin-labeled probes were generated and incorporation was measured according to protocols and reagents provided by Roche Applied Science.

In situ Hybridization

Both prehybridization and posthybridization were done following a published protocol [25]. Sections were prehybridized for 60 min at room temperature (RT) followed by 1 h at 37°C in a hybridization mixture containing 0.6 M NaCl, 0.1 M Tris-Cl pH 7.5, 10 mM EDTA, 0.05% NaPP, 0.5 mg/ml yeast total RNA, 0.05 mg/ml yeast tRNA, 1× Denhardt's BSA, 50% formamide, 5% dextran sulphate, 10 μm of the 4 rNTPs, 0.5 mg/ml salmon sperm DNA, and 10 mM DTT. Labeled probe (800–1,000 ng) was added to the hybridization buffer and sections were incubated overnight at 55°C. Posthybridization consisted of washing the sections in 4× SSC/10 mM NaTS at 37°C followed by treatment with RNAse A and RNAse T1 in NTE (0.5 M NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0) for 30 min at 37°C. Sections were then rinsed in a series of SSC buffers of decreasing molarity for 20 min each at 37°C followed by a final wash in 0.1× SSC for 60 min at 55°C. For antibody incubation, sections were rinsed in TBST (0.15 M NaCl, 0.1 M Tris-Cl pH 7.5, and 0.1% Tween-20) at RT, incubated in blocking solution (2% Roche Blocking Reagent and 20% heat-inactivated goat serum in TBST), and then incubated with primary antibody digoxigenin-AP (Roche Applied Science) at 1:1,000 in blocking solution for 1 h at RT followed by overnight incubation at 4°C on a shaker. Secondary antibody application consisted of TBST and NTMT [0.1 M NaCl, 0.1 M Tris-Cl (pH 9.5), 50 mM $MgCl_2$, and 0.1% Tween-20] washes followed by incubation in NBT and BCIP (Roche Applied Science) for 30 min to several hours in the dark at RT. The reaction was stopped with 10 mM Tris-Cl pH 7.5 and 1 mM EDTA; sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. The sections were examined with an Olympus BX60 microscope fitted with a Photometrics CoolSNAP charge-coupled device video camera. Four brains from each sex were examined.

Protein Extraction and Immunoblotting

For protein extraction, brain tissues were thawed and homogenized in RIPA buffer. After centrifugation, the total lysate protein concentrations were determined with a bicinchoninic acid protein assay (Pierce Chemical Co.). Proteins were separated on 16% polyacrylamide-SDS gels and transferred to nitrocellulose membranes. After transfer, membranes were blocked and incubated with a polyclonal calbindin antibody (1:20,000; Chemicon) overnight at 4°C. After rinsing, blots were incubated for 1 h with HRP-conjugated anti-rabbit IgG secondary antibody (1:5,000; Vector Laboratories) followed by detection on X-ray film (X-OMAT) with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). The same blots were reprobed with the monoclonal antibody against β-actin at 1:50,000 (Sigma-Aldrich Corp.). The intensities of Calb and β-actin on individual films were measured and analyzed by densitometry with ImageQuant (GE Healthcare Life Sciences). Levels of Calb were normalized to those of β-actin in each sample and the protein amount was expressed as the ratio of calbindin to β-actin. Five brains from each sex were used for these blots.

Quantitative Real-Time PCR

Total RNA was isolated from brain tissues using an RNeasy® Lipid Tissue Mini kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of the RNA was determined using a Bio-Rad SmartSpec Plus spectrophotometer. The cDNA templates were prepared using an AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, La Jolla, Calif., USA). The reverse transcription reaction consisted of 0.8 μg total RNA, 0.3 μg random primers, 2× First Strand Master Mix, and AffinityScript RT/RNAse block enzyme mixture in a total volume of 20 μl. Primer annealing occurred at 25°C for 5 min followed by cDNA synthesis for 45 min at 42°C and heat inactivation for 5 min at 95°C. cDNA templates were stored at –20°C prior to use. For calbindin gene expression, real-time PCR was performed using an ABI Prism 275® 7300 Real-Time PCR System with Sequence Detection Software version 1.2.3 (Applied Biosystems, Foster City, Calif., USA). In a 25-μl PCR reaction volume, 5 ng cDNA was mixed with iTaq™ SYBR® Green Supermix with ROX (Bio-Rad) or RT² Real-Time™ SYBR Green/ROX PCR Master Mix (SABiosciences) and 500 nM primers. Separate β-actin endogenous control reactions were used to normalize the RNA input. Oligonucleotide primers were designed using a consensus sequence from the NCBI genomic alignment database and Primer Express version 2.0 and, they were synthesized by Invitrogen as detailed in table 2. The real-time PCR conditions were 95°C for 3 min (iTaq SYBR Green Supermix) or 10 min (RT² Real-Time SYBR), followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After the last PCR cycle, a dissociation melting curve stage was run according to software protocol. Target and endogenous control genes were measured in triplicate for each cDNA sample dur-

ing each real-time run to avoid intersample variance. For each RNA sample a no-reverse transcriptase reaction was run in parallel to cDNA synthesis and it was measured by quantitative real-time PCR (qPCR) to control for contamination and genomic amplification. Each qPCR reaction was verified for a single PCR product of an expected size with the disassociation melting curve stage. Normalization and quantifications of the *Calb1* and *β-actin* mRNA were performed using the comparative cycle thresholds (C_T) method as described in the ABI PRISM 7700 sequence detection system (user bulletin No. 2). Validation experiments were conducted to test for equally efficient target and endogenous control gene amplifications as described in the user bulletin. Both primers were between 90 and 110% efficient for all amplifications.

For estrogen receptor alpha *(Esr1)*, estrogen receptor beta *(Esr2)*, parvalbumin *(Pvalb)*, and neuronal calcium sensor-1 *(Ncs-1)* gene expression, qPCR was performed using an ABI StepOnePlus real-time PCR system. The following TaqMan® Gene Expression assays from ABI were used to detect the PCR products of interest: *Esr1* (Mm00433149_m1*), *Esr2* (Mm00599821_m1*), *Pvalb* (Mm00443100_m1*), and *Ncs-1* (Mm00490552_m1*; frequenin homolog). In a 20-μl PCR reaction volume, 5 ng cDNA was mixed with 1 μl of 20× the TaqMan Gene Expression assay of interest and 10 μl of 2× TaqMan Fast Advanced Master Mix. The real-time PCR conditions were 95°C for 20 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Separate β-actin endogenous control reactions (ABI TaqMan Gene Expression assay Mouse ACTB 20×) were run to normalize the RNA input for each cDNA template. Target and endogenous control genes were measured in triplicate for each cDNA sample during each real-time run to avoid intersample variance and a no-reverse transcriptase reaction was run in parallel to cDNA synthesis and was measured by qPCR to control for contamination. Normalization and quantification of the genes of interest and β-actin mRNA were analyzed with StepOne™ software using the comparative cycle thresholds (C_T) method. We assayed between 5 and 10 animals of each sex and genotype for these experiments.

Statistical Analyses

Comparisons of Calb protein and mRNA, *Pvalb*, and *Ncs-1* gene expression in brains of C57BL/6J males versus females were conducted with Student's t tests. We used 2-way ANOVA followed by Fisher's LSD tests to compare specific differences between *Esr1* and *Esr2* in males versus females. We employed 2-way ANOVA and Fisher's LSD tests to compare Calb1 with sex and genotype as the 2 factors in the KO and transgenic mouse experiments. In the ERαKO study the 2 factors were gonadal sex and the presence or absence of ERα. In the FCG experiments gonadal sex and the sex chromosome complement were the 2 factors.

Results

Calbindin mRNA Is More Robust in Females than in Males

Qualitative sex differences in expression were observed in the amount, but not in the location, of *Calb1* mRNA throughout the brain. Distinct populations of neurons with robust *Calb1* mRNA were noted in regions of the FC, thalamus, hypothalamus, hippocampus, amygdala, and CB. Hippocampal *Calb1* mRNA was predominantly expressed in the dentate gyrus and moderate levels were expressed in CA1 neurons. Expression in the CB was particularly robust when compared to other brain regions and, as reported in rats, was specifically confined to the Purkinje neurons [26–28]. These findings confirm earlier studies mapping the distribution of *Calb1* expression in the rat brain [26, 29–32], but the current study is the first to characterize *Calb1* mRNA expression in both sexes of juvenile mouse brain.

The neuroanatomical localization pattern of *Calb1* mRNA expression did not differ between the sexes. However, the amount of mRNA expression, as indicated by the intensity of staining, differed in a regionally specific manner between males and females, particularly in the CB and FC. Examples of *Calb1* in these areas in females versus males are shown in figure 1. Although the hybridization patterns are similar in both sexes, the *Calb1* signal was stronger in females when compared to males in both the FC and the CB. In contrast, hypothalamic areas such as the SDN of the medial preoptic area (MPOA) exhibit a more robust mRNA signal in male brains when compared to female brains. This corroborates previous findings using immunoreactivity measurements in mice [12]. Other areas of the brain did not reveal marked differences in the intensity of *Calb1* mRNA expression in male versus female brains.

Calbindin mRNA Quantified with qPCR Confirms Sex Differences

In the FC, hippocampus, MPOA/hypothalamus, amygdala, and CB (table 3) qPCR was used to quantify differences in *Calb1* mRNA levels between males and females. *Calb1* mRNA levels in the FC and CB were significantly higher in females when compared to males ($p < 0.05$). Sex differences were not evident in the MPOA/hypothalamus, the hippocampus, or the amygdala. It is likely that *Calb1* mRNA levels in the MPOA/hypothalamus were not dimorphic because the tissue used extended beyond the small confines of the SDN which likely diluted the sex differences.

To determine whether the sex difference in calbindin expression was a feature shared by other EF-hand calcium-binding proteins in the brain, we examined the mRNA expression of parvalbumin and neural calcium sensor-1 in the CB and FC using qPCR. Both of these proteins are expressed in the FC and in Purkinje cells of the CB [10, 33]. The relative mRNA expression of these 2 messages was not significantly different between males and

Table 3. Sex comparison of *Calb1* levels in brain regions

Area of the brain	Sex	Mean relative mRNA expression ± SEM
CB[a]	female	2.17 ± 0.28
	male	1.23 ± 0.21
FC[a]	female	1.55 ± 0.23
	male	0.84 ± 0.09
Hippocampus	female	1.02 ± 0.24
	male	0.61 ± 0.12
Amygdala	female	1.40 ± 0.10
	male	1.28 ± 0.19
Hypothalamus	female	1.31 ± 0.23
	male	1.29 ± 0.09

Mean (±SEM) relative *Calb1* mRNA levels across brain regions and sexes. n = 5–10 mice per region, with equal numbers of males and females.

[a] Significantly greater levels of *Calb1* mRNA in females vs. males in these areas ($p < 0.05$).

females in either the CB or FC (data not shown). Thus, sex differences in expression are not necessarily a feature of all EF-hand calcium-binding proteins.

Females Have More Calbindin Protein than Males Do in the CB and Cortex

Calbindin protein concentrations in the FC and CB were quantified using Western blot followed by densitometry (fig. 2). Significantly higher levels of calbindin protein were observed in females when compared to males ($p < 0.05$).

Esr1 and Esr2 Are Expressed at Low Levels in the CB and FC

Because the *Calb1* gene has an estrogen-responsive element (ERE) [34] and it is well accepted that estradiol orchestrates many sex neural differences, we examined the expression profile of both *Esr1* and *Esr2* in the CB and FC. As shown in table 4, both receptors are expressed in the CB and FC of juvenile mice. However, there was no significant difference in expression between males and females in either region. Interestingly, *Esr1* showed 7- to 8-fold more expression than *Esr2* did in both the CB ($F_{1, 25} = 12.13$, $p < 0.002$) and the FC ($F_{1, 25} = 31.88$, $p < 0.001$). This is in agreement with previously reported immunoreactivity data for both receptors collected in mouse brain [35, 36]. Because the abundance of these messages was so low, we also assayed testes and ovaries, organs known to have higher concentrations of both receptors, as additional controls (table 4).

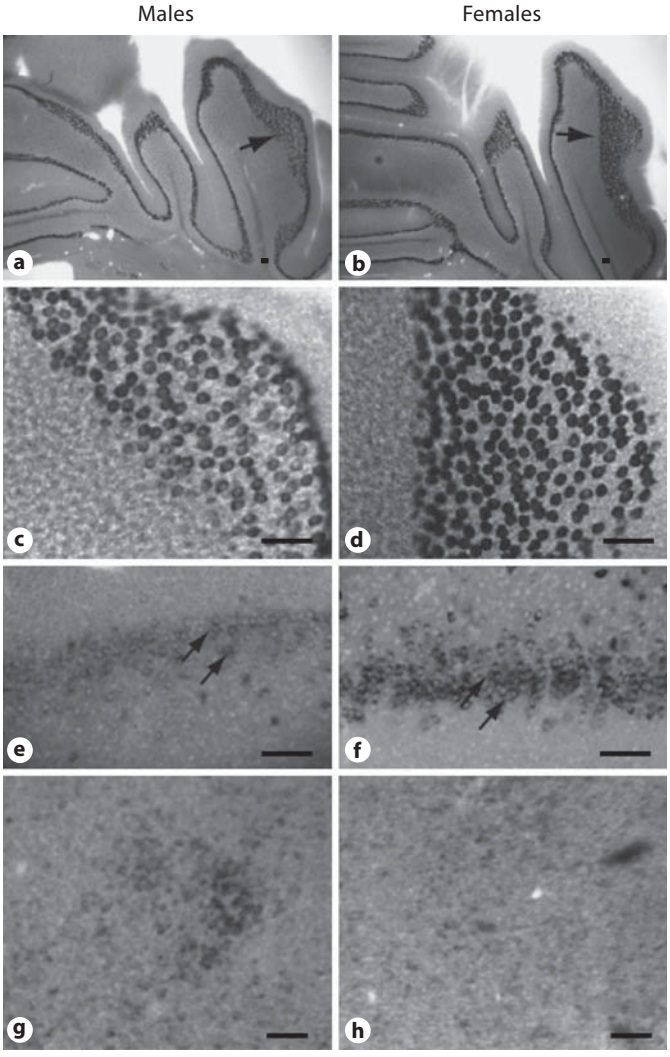

Fig. 1. ISH of calbindin mRNA shows sex differences in the CB (**a–d**), the FC (**e**, **f**), and the SDN of the hypothalamus (**g**, **h**). Bright-field photomicrographs of coronal sections show *Calb1* in juvenile mouse CB in low (**a**, **b**) and high (**c**, **d**) magnifications. Photographs of males (**a**, **c**, **e**, **g**) and females (**b**, **d**, **f**, **h**). Single arrows in **a** and **b** show regions pictured in **c** and **d**. Double arrows indicate neurons in the dorsal peduncular area of the FC (bregma 1.42 mm). Scale bars = 100 μm.

Calbindin mRNA in the FC Is Regulated by ERα and the Sex Chromosome Complement

In the FC, ERαKO mice had reduced expression of *Calb1* mRNA in females when compared with WT mice (fig. 3); no differences between KO and WT males were

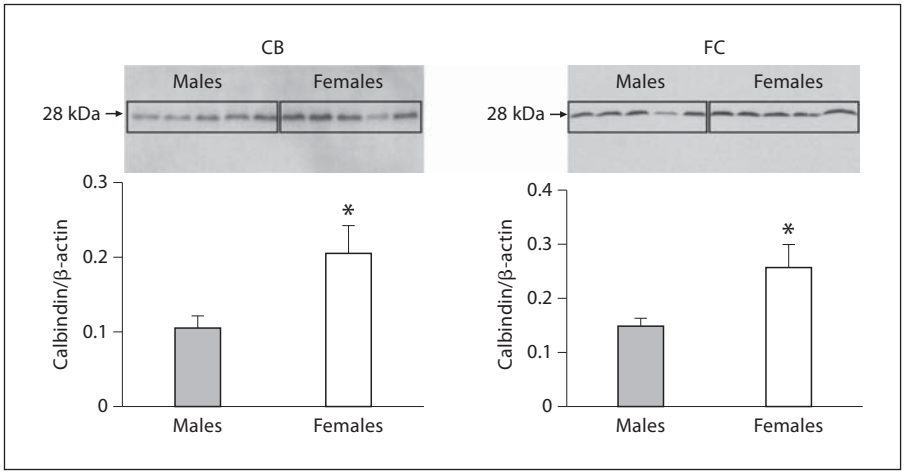

Fig. 2. Protein quantification of calbindin reveals sex differences in the cortex and CB. Calbindin protein quantification in CB and FC by Western blots. Each lane represents an individual animal. The ratio of calbindin to β-actin for each sample was measured (n = 5; animals were analyzed for each sex and region). Histograms represent the mean + SEM of the calbindin/β-actin ratio designating the protein level. * Females expressed significantly more calbindin protein ($p < 0.05$) than did males in both the CB and the FC.

Table 4. Relative mRNA expression of estrogen receptors *Esr1* and *Esr2* as a function of sex in the CB and FC

	CB		FC		Controls	
	females	males	females	males	testes	ovaries
Esr1	0.74 ± 0.19[a]	1.59 ± 0.38[a]	3.68 ± 1.00[a]	4.33 ± 0.78[a]	22.13	127.85
Esr2	0.15 ± 0.04	0.15 ± 0.03	0.49 ± 0.13	0.47 ± 0.06	1.00	457.00

Mean (±SEM) of the relative gene expression of *Esr1* and *Esr2* mRNA in the CB and FC. n = 5–10 mice per brain region. Estrogen receptor expression was also examined in the testes and ovaries to serve as positive controls. All expression values were normalized to *Esr2* in testes.

[a] *Esr1* expression was significantly greater than *Esr2* expression in both the CB ($p < 0.002$) and the FC ($p < 0.0001$). No significant differences in *Esr1* or *Esr2* expression were noted between the sexes.

observed. In this region significant effects of gonadal sex ($F_{1, 21} = 5.85$, $p < 0.02$), genotype ($F_{1, 21} = 18.1$, $p < 0.0005$), and an interaction between these factors ($F_{1, 21} = 9.2$, $p < 0.007$) were observed. Planned comparisons revealed that WT females had higher levels of *Calb1* mRNA expression than did animals in any of the other experimental groups ($p < 0.05$).

A similar pattern was noted in the FC from the FCG mice. A significant main effect of gonadal sex on *Calb1* expression ($F_{1, 22} = 10.8$, $p < 0.004$) was found. No main effect of the sex chromosome complement was detected, although a trend towards an interaction between gonadal sex and genotype was observed ($F_{1, 22} = 3.8$, $p = 0.07$). Females had higher levels of *Calb1* mRNA expression than did males ($p < 0.05$). In addition, XX females had higher levels of *Calb1* mRNA in the FC than did brain tissues from any of the other experimental groups. These results implicate both ERα and the chromosomal complement as factors associated with *Calb1* regulation in the FC of females.

In the CB ERα Affects the Amount of Calb1 mRNA Expressed

In the CB, expression of *Calb1* mRNA in ERαKO and WT mice (fig. 4) was affected by both gonadal sex ($F_{1, 21} = 7.5$, $p < 0.015$) and genotype ($F_{1, 22} = 9.5$, $p < 0.007$), but there was no interaction effect ($F = 0.65$) between the variables. The effect of gonadal sex was apparently due to overall higher *Calb1* mRNA expression in female as compared to male CB. However, both female and male WT mice demonstrated more than 2-fold higher *Calb1* mRNA levels in the CB when compared to their ERαKO littermates. Because the sex difference was maintained in ERαKO males and females we conclude that ERα is not critical for the observed sexual dimorphism in the CB.

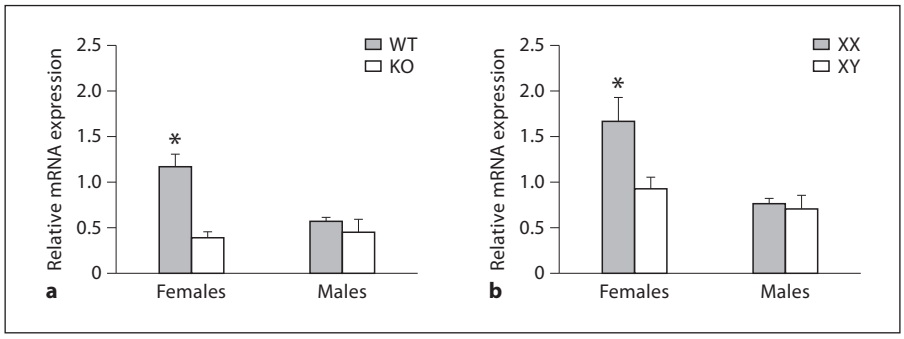

Fig. 3. In the FC, ERα and the sex chromosome complement influence *Calb1* mRNA in females. Mean + SEM *Calb1* in the FC of WT and ERαKO (KO) mice (**a**) and in FCG mice (**b**). n = 5–6 mice per group. * Significantly different from all other groups ($p < 0.05$).

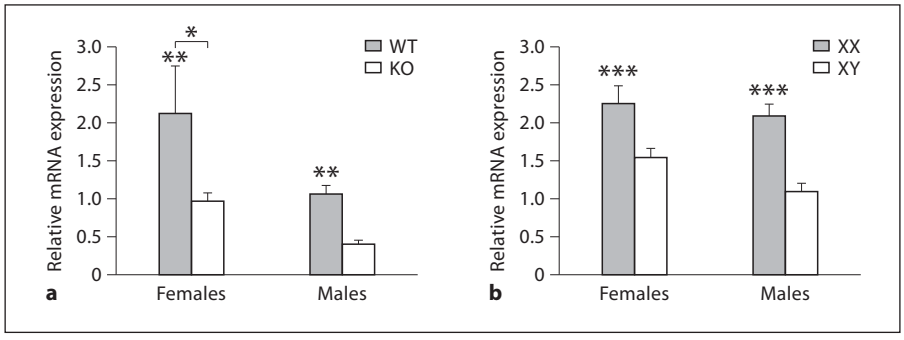

Fig. 4. In the CB, ERα modulates *Calb1* mRNA in both sexes, but sex chromosome genes modulate the sexual dimorphism. Mean + SEM *Calb1* in the CB of WT and ERαKO (KO) mice (**a**) and FCG mice (**b**). n = 5–6 mice per group. * Females had significantly greater concentrations of *Calb1* mRNA than did males ($p < 0.015$). ** WT littermates had significantly more *Calb1* mRNA than did ERαKO mice ($p < 0.007$). *** XX mice had significantly more *Calb1* than did XY mice ($p < 0.0001$).

The Sex Chromosome Complement Impacts Sexual Dimorphic Calb1 in the CB

The major determinant of the sexual dichotomous expression of *Calb1* in CB was the sex chromosome complement. A significant main effect of the sex chromosome genotype was noted ($F_{1, 22} = 21.7$, $p < 0.0001$) (fig. 4). Specifically, XX females and males showed significantly higher levels of *Calb1* than did XY individuals of either sex. This effect was so pronounced that gonadal sex was not a significant factor ($F = 2.73$), and no interaction between gonadal and chromosomal sex was found ($F = 0.65$). This result indicates that gonadal sex was not the critical determining factor, but the sex chromosome complement fully explains the observed *Calb1* mRNA sex difference in the CB.

Discussion

Robust Sexual Dimorphism Outside the Hypothalamus

Here we show that *Calb1* mRNA and protein are sexually dimorphic in 2 nonhypothalamic regions, i.e. the FC and CB. This is the first report that this protein is expressed in a sexually dimorphic manner outside of the hypothalamus, and the direction of this sex difference (female > male) is actually opposite to the higher expression of Calb typically observed in the SDN of male, rather than female, rodents [11–13]. There are 2 reported sex differences in genes and/or proteins in rat CB. At PN10 female CB had more mRNA for nerve growth factor than male CB did [37]. In adult rats, Foxp2 immunoreactivity was quantified in Purkinje cells, and a small but significant difference in optical density and cells numbers (males > females) was reported [38]. However, hormone levels were not controlled in this study; thus, it is unclear whether the sex difference was simply a reflection of differences in circulating hormone levels.

Our study specifically identified a novel organizational factor: sex chromosome genes. This finding could be functionally important since several cognitive behaviors are sexually dimorphic in humans and rodents, and both the FC and the CB mediate many complex processes [2, 39]. A prime example is the classically conditioned eye blink response which utilizes cerebellar circuitry [2]. In rats, females are faster to learn this response than males, and the Purkinje cells in the CB are essential for the conditioning of this learning [40]. Interestingly, gonadectomized adult mice demonstrate sex differences when performing some motor tasks; females outperformed males

on the rotarod and descended a pole more rapidly than males. Given that Calb is expressed only in Purkinje cells [26–28] and is associated with sexual dimorphism favoring females, it may play a key role in efficiency in acquiring the eye blink conditioning response and motor skills, and it likely impacts other cognitive and emotional tasks.

Sex Chromosomes Dictate Sex Differences in the CB and FC

The sex chromosome complement significantly impacts *Calb1* gene expression, and there are important differences in the way this factor regulates *Calb1* in the 2 brain regions under investigation. In the CB, XX females and XX males demonstrated significantly higher levels of *Calb1* mRNA compared to mice with an XY genotype; moreover, levels of mRNA did not significantly differ between the XX males and XX females. In contrast, *Calb1* mRNA expression in the FC was driven by the sex-chromosomal complement in females only, with XXF having more *Calb1* than XYF. In males, both XX and XY mice had low *Calb1* levels similar to those of XY females. Thus, the 'advantage' that the XX sex-chromosomal complement gave to females was also extended to males in the CB but not in the FC.

Several other neural sex differences have been attributed to the sex chromosome complement. For example, sex differences exist in prodynorphin mRNA in the mouse striatum (XX > XY) [41]. The reverse was noted in embryonic midbrain tyrosine hydroxylase-containing neurons, which are more plentiful in XY than in XX cell cultures [42]. Finally, the same XY > XX difference is apparent in the density of vasopressin in the lateral septum [8, 43]. On the other hand, the only cortical sex difference previously examined in FCG is cortical thickness, which is larger in males than in females. Using the FCG model, no-sex chromosome effects were found in this measure [44].

Interactions between ERα and Sex Chromosomes

Our results indicate that both ERα and sex chromosome genes regulate *Calb1* in the FC. In this area WT females in the ERαKO mice and XXF in the FCG have more *Calb1* than do females with other genotypes. In the CB, because the absence of functional ERα does not eliminate the sex difference, we believe sex chromosomes and ERα play different roles. ERα increases *Calb1* production likely via ERE and this effect is noted in both sexes. In addition, the data from the FCG mice suggest that the normal sex difference in CB is caused by the sex chromosome complement.

Vasopressin is produced in neurons in the bed nucleus of the stria terminalis and these cells project primarily to the lateral septum. Fiber density in the septum is sexually dimorphic with males showing more intense immunoreactivity compared to females [45, 46]. Studies of ERαKO mice have demonstrated that when ERα is functional WT males have more vasopressin in the septum than do KO males [47]. In the FCG, XY mice of both sexes have more vasopressin fibers than their XX counterparts with the same gonadal sex [8, 43]. Thus, as is the case for *Calb1*, both the sex chromosome complement and ERα likely underlie this sex difference. Interestingly, the sex differences are reversed in these two systems (Calb females > males and vasopressin females < males). We speculate that ERα interacts with different sex chromosomes in these two cases.

While FC and CB express ERα in juvenile brain, the timing and source of the ligand are unknown at this time. There are regional and sex differences in estradiol in the developing rat brain [48] such that plasma concentrations of steroids may not entirely reveal all actions of steroids on neural architecture. In fact, Purkinje cells are a major site of synthesis for the steroidogenic enzyme P450scc, which generates de novo estradiol (for review see Dean and McCarthy [6] and Tsutsui [49]). The enzyme is developmentally regulated in both sexes, but it shows increased gene expression in the CB of males when compared to females during the early neonatal period [50]. An analysis of neurosteroid concentrations in the CB of C57BL/6 pups showed that 17β-estradiol is higher in females than in males on PN5; the reverse was noted for testosterone and the inactive 17α-estradiol [51]. When local infusions are given to mice at PN5 and brains collected at PN15, ER antagonists reduce Purkinje cell numbers in females. A thorough determination of steroid production in developing mouse cortex and CB, and its association with the *Calb1* gene, is essential to unravel these complex effects.

Which Sex Chromosome Genes Regulate Calbindin in the CB?

When a sex chromosome effect is detected in the FCG, several underlying causes are possible. The sex difference may be due to the presence or absence of the Y genes, higher expression of X chromosome genes that escape inactivation in XX mice, or a paternally imprinted gene on the X which is only present in XX mice [7]. Our next challenge is to identify which sex chromosome is responsible for this effect in the CB. If the candidate chromosome is the X, there are several X genes known to escape inactivation in the brain [52]. These candidates, i.e. *Kdm6a,*

Kdm5c, Ddx3x, Usp9x, and *Eif2s3x*, show robust in situ expression in the CB [53] and studies with FCG mice show that *Kdm6a, Kdm5c, Usp9x,* and *Eif2s3x* are expressed at higher levels in brains from XX versus XY individuals of either sex [52]. Both *Kdm6a* and *Kdm5c* are histone demethylase enzymes [54]. Interestingly, in PN6 rat cultures from cerebellar cortex, the targeted knockdown of *Kdm5c* produces marked reductions selectively in dendritic lengths [55]. In progenitor neurons treated in culture, *Calb1* is subject to epigenetic regulation by the REST complex on an H3K9Me mark [56]. Therefore, it is possible that either *Kdm6a* and/or *Kdm5c* modify *Calb1* via similar mechanisms.

Implications for Human Neurobehavioral Disease

Striking sex differences in the incidence, age of onset, and/or severity of a number of neurobehavioral diseases have brought the study of sex differences into the forefront [1, 6]. For example, autism, Parkinson's disease and attention deficit hyperactivity disorder with impulsivity are more prevalent in males, and schizophrenia is typically more severe in males [57–61]. In contrast, Alzheimer's disease, depression and anxiety are more likely to affect women [62, 63]. Among the behavioral difficulties patients experience are issues involving executive function, emotionality, reasoning, and motor skills, all of which are mediated by the FC and/or CB functions [64, 65]. A reduction in *Calb1* gene and/or protein expression has been detected in brains of patients who suffered from an assortment of neurodegenerative diseases [66–68], and several neurobehavioral disorders are correlated with decreased size and/or numbers of Purkinje cells [69, 70]. Sex differences in these brain regions in normal animals, such as those reported here, could provide clues underlying the etiologies of these diseases.

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