Sex-Specific Expression of the X-Linked Histone Demethylase Gene Jarid1c in Brain

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Abstract

Jarid1c, an X-linked gene coding for a histone demethylase, plays an important role in brain development and function. Notably, JARID1C mutations cause mental retardation and increased aggression in humans. These phenotypes are consistent with the expression patterns we have identified in mouse brain where Jarid1c mRNA was detected in hippocampus, hypothalamus, and cerebellum. Jarid1c expression and associated active histone marks at its 5’ end are high in P19 neurons, indicating that JARID1C demethylase plays an important role in differentiated neuronal cells. We found that XX mice expressed Jarid1c more highly than XY mice, independent of their gonadal types (testes versus ovaries). This increased expression in XX mice is consistent with Jarid1c escape from X inactivation and is not compensated by expression from the Y-linked parologue Jarid1d, which is expressed at a very low level compared to the X parologue in P19 cells. Our observations suggest that sex-specific expression of Jarid1c may contribute to sex differences in brain function.

Introduction

Mutations in JARID1C are one of the leading causes of X-linked mental retardation [1,2]. Mouse Jarid1c and human JARID1C encode a highly conserved JmJ-C-domain protein that catalyzes the removal of methyl groups from tri- or di-methylated lysine 4 on histone H3 [3–5]. Since this histone modification is associated with enhanced gene activity, demethylation of H3 lysine 4 by JARID1C leads to transcriptional repression [6]. In particular, neuronal specific genes are repressed in stem cells and non-neural tissues in part due to JARID1C-mediated H3K4 demethylation at promoter sequences of these genes [7]. Besides its role in neuronal differentiation, JARID1C is involved in neuronal cell death and dendritic growth [3]. To better understand the pathophysiology of mental retardation and increased aggression caused by JARID1C mutations [1,2,8], it is important to determine where JARID1C accumulates in the brain. In the present study we determined the pattern of expression of Jarid1c in mouse brain sections by in situ hybridization.

In human and in mouse, the JARID1C/Jarid1c gene escapes X inactivation, i.e. it is expressed from both alleles in females [9,10]. Thus, it is not surprising that Jarid1c expression is higher in brains from adult females compared to males [11]. However, the role of sex hormones such as testosterone and estrogens, which could dramatically affect gene expression, was not investigated in our previous study. To simultaneously examine the effects of sex hormones (male versus female) and of the sex chromosome complement (XY versus XX), we have now used a transgenic mouse model, which consists of four genetically distinct types of mice: XX normal females, XY females (XY mice sex-reversed by deletion of Sry), XY/Sry males (XY mice with an Sry transgene to restore the male sex), and XX/Sry males (XX mice sex-reversed by insertion of an Sry transgene) [12,13].

An important question is whether the sex difference in Jarid1c expression is present in all tissues and developmental stages. The reported expression of Jarid1c from the inactive X ranges between 20% and 100% of that from the active X chromosome, depending on the tissue [14–16]. Furthermore, we have previously shown that Jarid1c is transiently silenced on the inactive X chromosome in early development, suggesting that Jarid1c may have a similar expression level between the sexes at certain developmental stages [16]. Therefore, we compared Jarid1c expression in neonates and adult mice. Since the higher expression of Jarid1c in females could theoretically be compensated for in males by expression from the Y-linked parologue, Jarid1d, we also examined expression of this gene. The two paralogues are highly similar in nucleotide and amino acid sequence and both function as histone demethylases [5,10]. However, it is plausible that the two paralogues differ in their expression patterns across tissues as a result of differences in their developmental regulation. For instance, Utx and Uty, another X–Y paralogous gene pair, appear to be differentially regulated and expressed in the brain [17]. We tested this possibility by in situ hybridization to brain sections. In addition, quantitative RT-PCR was done in male P19 embryonic carcinoma (EC) cells (hereafter P19 stem cells) that can be differentiated into neurons. This study was extended to four additional X/Y gene pairs. Transcriptional regulation of genes is modulated by histone modifications, DNA methylation, and non-coding RNA binding [18]. Active histone
marks include histone H3 and H4 acetylation at lysine residues (H3K4ac and H4K8ac), and H3 di- or tri-methylation at lysine 4 (H3K4me2 or H3K4me3; [6,19]). Using chromatin immunoprecipitation (ChIP), we tested three active chromatin modifications at the 5′ end of Jarid1c and Jarid1d in both P19 stem cells and P19 neurons.

Our results demonstrate that higher expression of Jarid1c in adult mouse brain is associated with the presence of two X chromosomes, regardless of phenotypic sex. We showed that the female bias in Jarid1c expression was apparently not present in neonatal brain and adult liver, suggesting that it may be tissue- and developmental stage-specific. We determined that expression from the Y-linked paralogue, Jarid1d, is very low in P19 stem cells and in differentiated neurons, suggesting that it does not compensate for the difference in Jarid1c levels between males and females in these cell types. The two paralogous genes may be similarly regulated across brain regions.

Results

Jarid1c is expressed in specific brain regions

Using in situ hybridization with an antisense riboprobe, Jarid1c mRNA was detected throughout brain sections from male and female adult mice. Higher expression levels relative to surrounding areas were found in the olfactory bulb, piriform cortex, habenula, hypothalamus (such as paraventricular nucleus [PVN], supraoptic nucleus [SON], ventromedial nucleus [VMH] and arcuate nucleus), hippocampus, and cerebellum (Fig. 1), all of which are brain regions with high cell densities. In addition, Jarid1c mRNA was detected in the triangular septal nucleus, anterior paraventricular thalamic nucleus, bed nucleus of the stria terminalis (BST), anteroventral thalamic nucleus, interstitial nucleus of Cajal, mammary nuclei, and pontine nuclei. In males, the Y-linked paralogue Jarid1d was transcribed in similar brain regions as Jarid1c, but at relatively lower levels, as revealed with in situ hybridization (data not shown), implying that the two paralogous genes may be similarly regulated across brain regions.

The female bias in Jarid1c expression in brain depends on the sex chromosome complement

We have previously shown that Jarid1c is expressed more highly in brains of female mice compared to male mice [11]. To test whether this difference was influenced by the sex chromosomes, sex hormones, or both, we compared Jarid1c expression in brains from adult XX females, XY females, XXδy males and XYδy males by Northern blot analyses. Three independent samples, each from two pooled adult brain samples were analyzed for each genotype. Jarid1c expression was normalized against that of Actb (β-actin gene) A two-way ANOVA revealed a main effect of the sex chromosome complement, with XX mice having higher levels than XY mice (Fig. 2; F[1,8] = 7.038; p<.05). There was no main effect of gonadal sex (male versus female) and no significant interaction. We conclude that the higher level of Jarid1c expression in females is mainly due to the presence of two active copies of the gene, consistent with escape from X inactivation.

We also tested Jarid1c expression in neonatal brains and adult brains, but found no significant sex difference in either (p>0.05 in both cases), suggesting the sex difference in Jarid1c expression found in adult brain might be tissue- and age-specific (Fig. 3). There were, however, noticeable variations among females in both neonatal brains and adult brains and some females did show a higher level of Jarid1c mRNA than males (Fig. 3). This is possibly caused by individual difference in transcription of escape genes from the inactive X chromosome. Similar observations have shown that in women the level of expression of escape genes from the inactive X chromosome varies between individuals and between tissues of the same individual [20].

Jarid1c expression is higher than that of Jarid1d in P19 neurons

Using quantitative RT-PCR, expression levels of Jarid1c and Jarid1d were measured in P19 stem cells and differentiated neurons. Three samples of each type were measured in duplicate. Jarid1c expression was about 280-fold higher than that of Jarid1d both in undifferentiated and neuron-differentiated P19 cells (Table 1). Four other X–Y gene pairs, Ddx3x/y, Eif2s3x/y, Usp9x/y, and Utxx/y were also tested for comparison. Interestingly,
all five X-linked genes, *Jarid1c, Ddx3x, Eif2s3x, Usp9x*, and *Utx*, were expressed consistently higher than their Y-linked paralogues, both in P19 stem cells and neurons (Table 1). Ratios between X-linked and Y-linked gene expression levels ranged between 150 and 1000 (Table 1).

**Figure 2.** *Jarid1c* expression level depends on the number of X chromosomes, not on gonadal sex. Example of a northern blot containing mRNA from brains of XX females, XY females, XXSry males and XY Sry males hybridized to a *Jarid1c* probe. Hybridization to a probe for Actb (Actin) was used as a loading control. The graph below shows the relative expression of *Jarid1c* versus Actb (β-actin) quantified by densitometry. XXSry males and XX females had significantly higher *Jarid1c* expression than XY Sry males and XY females, with no difference between mice possessing testes or ovaries.

**Figure 3.** Expression of *Jarid1c* in adult liver and neonatal brain is similar between males and females. Example of a northern blot containing mRNA from four neonatal brain samples and four liver samples from males and females hybridized to a probe for *Jarid1c*. Hybridization to a probe for Gapdh was used as a loading control.

**Table 1.** Expression (mean±SEM) of X- and Y-linked paralogues in P19 stem cells and neurons.

| Gene   | Cell type | X-linked paralogue | Y-linked paralogue | X / Y expression ratio |
|--------|-----------|--------------------|--------------------|------------------------|
| *Jarid1c – Jarid1d* | stem      | 28±3               | 0.1±0.006          | 280                    |
|        | neuron    | 63±15              | 0.3±0.06           | 277                    |
| *Ddx3x – Ddx3y*   | stem      | 826±134            | 2.0±0.2            | 413                    |
|        | neuron    | 1907±262           | 3.5±0.5            | 545                    |
| *Eif2s3x – Eif2s3y* | stem    | 676±67             | 4.5±0.1            | 150                    |
|        | neuron    | 1349±242           | 9.1±1.1            | 148                    |
| *Usp9x – Usp9y*   | stem      | 30±2               | 0.09±0.01          | 333                    |
|        | neuron    | 137±23             | 0.3±0.09           | 457                    |
| *Utx – Uty*       | stem      | 4.1±0.1            | 0.004±0.001        | 1025                   |
|        | neuron    | 9.7±2.2            | 0.01±0.004         | 970                    |
Chromatin remodeling at *Jarid1c* in P19 neural differentiation.

To examine the chromatin structure of *Jarid1c/Jarid1d* in P19 cells before and after differentiation, three sites located at the 5' end of *Jarid1c* (nt -657 to -465, nt67 to 195, and nt602 to 702) were examined for H3 acetylation, H3 di-methylation at lysine 4, and H4 acetylation at lysine 16 by ChIP. Each of these histone modifications, known to be associated with gene activation, was analyzed with two-way ANOVAs to test the effects of cell types and DNA sites. One site at the 5' end of *Jarid1d* (nt -790 to -594) was examined for these three histone marks. For *Jarid1c* all three histone marks showed a significant enrichment in P19 neurons relative to stem cells (p<0.05) [Fig. 4]. We were unable to detect any enrichment in these three histone marks on *Jarid1d* sequences, indicating the absence of these modifications on the Y-linked sequence. We further performed a ChIP-on-chip analysis by hybridization of the ChIP fraction from P19 neurons bound to an antibody to histone H4 acetylated at lysine 16 to a mouse tiling array (MM8_tiling set38) representing the second half of the mouse X and the entire Y chromosome. The array data showed a significantly higher accumulation of H4K16ac along *Jarid1c* gene body compared to *Jarid1d*, the latter showing signals no higher than background (data not shown).

**Discussion**

The high expression of *Jarid1c* that we found in the mouse hippocampus is consistent with the cognitive defects in human patients with *JARID1C* mutations [1,2]. Some of these patients also exhibit elevated aggression [2], an emotion governed by certain brain regions including the bed nucleus of the stria terminalis (BST) [21]. Interestingly, we detected relatively high levels of *Jarid1c* mRNA in the corresponding regions of the mouse brain, including the BST and anterior hypothalamus. These regions have also been implicated in aggressive behavior in rodents [21]. In adult mouse brain, the expression pattern of *Jarid1c* was generally in line with cell densities, i.e. brain regions with higher cell density showed higher levels of hybridization signals. However, some areas (including triangular septal nucleus, anterior paraventricular thalamic nucleus, BST, anteroventral thalamic nucleus, interstitial nucleus of Cajal, mammillary nuclei, and pontine nuclei) showed high expression despite low neuron densities, suggesting specifically enhanced expression in these cell types. We are currently carrying out detailed analyses to locate and quantify the transcripts inside specific cellular compartments in males and females.

*Jarid1c* encodes a histone demethylase specific for histone H3 where it converts tri-methylated lysine 4 to di- and mono-methylated forms [3,7]. This activity results in gene repression by removal of the active epigenetic mark. Although the complete list of genes targeted by *JARID1C* is not fully established, one group of neuronal genes that shares similar motifs at their promoter is regulated by this demethylase [7]. These genes are stably repressed in stem cells and non-neural tissues following binding of the REST complex that consists of several proteins including REST, *JARID1C* and other chromatin modifying enzymes [7]. Surprisingly, we found that *Jarid1c* was highly expressed in P19 differentiated neurons, a finding seemingly contradictory to *JARID1C*’s role as a powerful repressor of neuronal specific genes [7]. Our observations, which were obtained on fully differentiated neurons, support the hypothesis that *JARID1C* plays an additional role in neurite development. In a previous study a *Jarid1c* knockdown in cultured cerebellar neurons led to shorter neurites [3].

Using a mouse model to distinguish the effects of steroid hormones from those of the sex chromosome complement [22] we found that XX mice had a higher level of *Jarid1c* than XY mice, irrespective of whether they were phenotypic males with testes or females with ovaries. These findings are best explained by the fact that XX mice have two actively transcribed copies of *Jarid1c* due to escape from X inactivation. Although *Jarid1c* is transcribed from both copies in a XX mouse, the expression from the inactive X chromosome is often lower relative to the active X chromosome [14–16]. Although the parsimonious explanation for the sex difference in *Jarid1c* expression is that XX females have two actively transcribed copies relative to males having one copy, it is possible that the transcriptional activity of *Jarid1c* differs between one of the two copies in females and the single copy in males. For instance, there is a possible gonadal steroid effect on *Jarid1c* expression since we have not tested by manipulating steroid hormones’ levels in gonadectomized mice. Moreover, it is also possible that a Y-linked factor, such as JARID1D suppresses the expression of *Jarid1c* in XY mice. It is not certain whether the sex differences we observed for *Jarid1c* mRNA will necessarily result in a corresponding difference at the protein level. Indeed, *Eif2s3x*, another gene that escapes X inactivation in humans and mice,

![Figure 4. Expression of Jarid1c in P19 neurons is associated with active chromatin marks.](image-url)
displays sex specific expression at the mRNA level, but not at the protein level [23].

Expression of Y-linked paralogues in males could theoretically compensate for the high expression of genes that escape X inactivation in females [24]. However, we found that expression of all five mouse X-linked genes examined here was consistently higher — up to a thousand fold — than that of their Y paralogues. Thus, it appears that the Y-linked paralogues did not compensate for higher female-specific expression in these cultured cells. This is consistent with our previous observation in mouse brain that the summed expression of X- and Y-linked paralogues in males is less abundant than that of the two X paralogues in females, one escaping X inactivation [11]. If our observations are confirmed in humans, this could explain why males who carry JARID1C mutations are affected in spite of having a normal copy of the X-linked JARID1D gene. Alternatively, JARID1D, despite an apparently similar function as a histone demethylase [25], may have a different role from JARID1C. Among the five mouse X–Y gene pairs compared in our study, two (Utx/Uyp, Usp9y/Uspy) have a Y parologue with a separate function from the X-linked parologue [17,26–28], which is consistent with the differential expression of the paralogues in P19 cells. The lower expression of all Y paralogues may be due to their location in the generally heterochromatic Y chromosome and/or to the up-regulation of the active X chromosome [29].

Gene expression is tightly regulated by histone modifications. We found that three active histone marks, H3 lysine 4 dimethylation, H3 acetylation, and H4 acetylation at lysine 16, were enriched at the 5’ end of Jarid1c in P19 neurons compared to stem cells, which suggests a different chromatin conformation in neurons. Acetylation of histone H4 at lysine 16, which is usually enhanced throughout the body and 3’ end of expressed genes [30] was present along Jarid1c but absent on Jarid1d, as expected given the difference in expression of the paralogues. It will be interesting to further characterize changes in histone modifications at the Jarid1c/Jarid1d sequence in P19 neuronal differentiation, particularly in terms of repressive marks.

In summary, we found that Jarid1c was expressed in specific brain regions in adult mice and was up-regulated in mice with two X chromosomes versus those with one X chromosome. Expression of the Y parologue Jarid1d did not appear to compensate for the female bias. The expression patterns and differences in Jarid1c expression in brain between males and females may lead to sex differences in specific behavior, possibly including aggression, which will need to be further investigated.

Materials and Methods

Animals

Procedures for mouse use were approved by the UCLA Chancellor’s Animal Research Committee. Mice were bred from stocks obtained from Jackson Laboratories (C57BL/6j) or as a gift (MF1 mice) from Dr. P. Burgoyne (MRC National Institute for Medical Research, London). Conditions of mouse husbandry and tissue collection and the breeding paradigm to generate sex-reversed and control mice were previously described [11]. The four core genotypes included XX normal females, XY females (XY mice sex-reversed by deletion of Sry), XX Sry males (XY mice with an Sry transgene to restore the male sex), and XX Sry males (XX mice sex-reversed by insertion of an Sry transgene). All tissues were collected from BL/6 mice except in the Northern analysis of the four core mice which were from MF1 mice. Adult tissues were normally harvested from 8–10 months old mice, with the exception of the four-core mouse brains, which were from 12–14 months old animals.

Cell culture and neuronal induction

P19 EC cells were cultured in DMEM medium containing 10% fetal bovine serum. Neural differentiation was initiated by plating cells in medium containing 0.3 μM retinoic acid (RA; Sigma, St Louis, MO) in non-adhesive Petri dishes to promote the formation of aggregates. After a 4-day exposure to RA, aggregates were dispersed with trypsin (Invitrogen, Carlsbad, CA) and re-plated on cell culture dishes. Cytosine arabinoside (Ara-C; Sigma) was then added to the medium to inhibit proliferation of non-neuronal cells and to select for neurons, which were differentiated by day 6. Cells were collected on day 10 for mRNA and histone modification analyses. Three independent samples were tested for each cell type.

In situ hybridization

In situ hybridization of brain sections was carried out as described in [17,23]. The riboprobes were transcribed from linearized plasmids containing either a Jarid1c or Jarid1d cDNA insert. The Jarid1c cDNA was an IMAGE clone (clone ID: 6841578; Invitrogen) that contained a 473 bp long insert starting at position bp 4273 of the GenBank sequence AF127245. The Jarid1d riboprobe was transcribed from a cDNA clone that contained a 192 bp PCR product (bp 1165–1356 of sequence NM_011419) using a pCRScript kit (Stratagene, La Jolla, CA). The specificity of the Jarid1c and Jarid1d antisense riboprobes was verified with Northern blots [11]. When a sense strand riboprobe was used, no hybridization signal was detected (data not shown).

Northern blots

Northern blot hybridization was done as described previously [11]. The template for synthesis of the Jarid1c probe was a 234 bp RT-PCR product (bp 1109–1342 of sequence NM_013668). Quantification of band intensity was done using Gapdh as a control [11]. Actb mRNA was also measured as a loading reference, especially in cases when signals for Gapdh mRNA appeared to be saturated (Fig. 2). The two reference genes led to similar results in terms of expression of genes of interests between groups.

Quantitative RT-PCR

Total RNA isolated with an RNeasy kit (QIAGEN) was reverse-transcribed using a first-strand synthesis kit (Invitrogen). Expression of X- and Y-linked paralogues in P19 stem cells and neurons was determined on a LightCycler system (Roche, Indianapolis, IN). Forward and reverse primer sequences were obtained from the Primer Bank website (http://pga.mgh.harvard.edu/primerbank/; Supplementary Table S1). Expression of Gapdh was used as a reference. PCR measurements were repeated at least twice. Standard curves based on serial dilutions of samples were established to correct for differences in efficiencies between primers. The specificity of each primer set was confirmed by alignment of dissociation curves. Expression was compared between X–Y paralogues using a paired t-test in six P19 samples, i.e., for each sample, the X and Y paralogues were compared as a pair. For each gene pair, comparison was made across three undifferentiated and three P19 neuron samples.

ChIP assays

Chromatin was extracted from three P19 stem cell samples and three P19 neuron samples following the manufacturer’s instructions (EZ-ChIP kit; Upstate Biotechnology, Charlottesville, VA). Briefly, formaldehyde-fixed chromatin was incubated for 15 hr at 4°C with 5 μg of antibody (anti-acetylated H3 at lysine 9 and lysine 14, anti-dimethylated H3 at lysine 4, anti-acetylated H4 at lysine 16, Upstate Biotechnology). After a second incubation with
Analysis was done using the manufacturer software. Jarid1c was used in this study to closely compare the H4K16 acetylation on the second half of the X chromosome, which covers the second half of the X chromosome (ChrX:93,883,966-165,556,020, UCSC mouse genome Feb 2006 assembly) and the complete Y chromosome, was used in this study to closely compare the H4K16 acetylation pattern between Jarid1c and Jarid1d present on the same array. Analysis was done using the manufacturer software.

Supporting Information

Table S1 PCR primer sequences.

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Author Contributions

Conceived and designed the experiments: CD JX XD. Performed the experiments: JX XD. Analyzed the data: CD JX XD. Wrote the paper: CD JX.

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