Mutations of protocadherin 19 in female epilepsy (PCDH19-FE) lead to allopregnanolone deficiency

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Abstract

Protocadherin 19 (PCDH19) female limited epilepsy (PCDH19-FE; also known as epilepsy and mental retardation limited to females, EFMR; MIM300088) is an infantile onset epilepsy syndrome with or without intellectual disability (ID) and autism. We investigated transcriptomes of PCDH19-FE female and control primary skin fibroblasts, which are endowed to metabolize neurosteroid hormones. We identified a set of 94 significantly dysregulated genes in PCDH19-FE females. Intriguingly, 43 of the 94 genes (45.7%) showed gender-biased expression; enrichment of such genes was highly significant (P = 2.51E−47, two-tailed Fisher exact test). We further investigated the AKR1C1-3 genes, which encode crucial steroid hormone-metabolizing enzymes whose key products include allopregnanolone and estradiol. Both mRNA and protein levels of AKR1C3 were significantly...
decreased in PCDH19-FE patients. In agreement with this, the blood levels of allopregnanolone were also (P < 0.01) reduced. In conclusion, we show that the deficiency of neurosteroid allopregnanolone, one of the most potent GABA receptor modulators, may contribute to PCDH19-FE. Overall our findings provide evidence for a role of neurosteroids in epilepsy, ID and autism and create realistic opportunities for targeted therapeutic interventions.

Introduction

Protocadherin 19 (PCDH19) female limited epilepsy (PCDH19-FE; also known as epilepsy and mental retardation limited to females, EFMR; MIM300088) is an infantile onset epileptic encephalopathy (EIEE), with or without intellectual disability (ID) and autism (1,2). PCDH19-FE eluded genetic mapping due to its female-limited expression until 1997 when Ryan et al. (3) mapped the responsible gene, perhaps surprisingly, to the X-chromosome. It was another 10 years before systematic sequencing of X-chromosome exons revealed mutations of the protocadherin 19 (PCDH19) gene as the cause of the disorder (4). In general, the EFMR phenotype was restricted to females while males transmitting the mutations were apparently unaffected (1). However, serendipitous identification of a male with somatic mosaicism for PCDH19 deletion and a seizure disorder resembling Dravet syndrome led Depienne and colleagues (5) to identify PCDH19 mutations in small families and singletons with SCN1A negative Dravet-like infantile epileptic encephalopathy. Many reports of PCDH19-positive cases followed [see example in Ref. (6) for review] and the PCDH19-FE-related clinical spectrum broadened (6,7). Most typical characteristics are early-onset seizures (6–36 months) (1) followed by clusters of recurrent seizures throughout childhood. The severity and frequency of seizures, as well as the presence of other clinical features such as autistic and obsessive compulsive behavior, depression and schizophrenia, vary among the affected females (8). No obvious correlation between the type of PCDH19 mutation and the phenotype has been ascertained. The majority of mutations cluster in the extracellular domain of PCDH19 protein. Only frameshift mutations, but no missense mutations, have been described in the intracellular domain (6). Variable clinical expressivity was noted originally when comparing large EFMR pedigrees with PCDH19-FE-reproducing females (1), and when comparing re-producing females with severely affected singletons (5), twins (9), sibs (10) and mother–daughter pairs (11). The broad clinical variability of PCDH19 mutation expression, including non-penetrance, suggests the presence of environmental, genetic or stochastic (e.g. X-chromosome inactivation) modifiers. We postulate that interindividual variability in steroid and neurosteroid metabolism together with variation in X-chromosome inactivation may explain the variable penetrance of PCDH19-FE. Here, we provide evidence in support of a role for neurosteroids, allopregnanolone, in particular in the pathophysiology of PCDH19-FE.

Results

Expression profiling

Using genome-wide gene expression (Affymetrix Human Exon 1.0 ST arrays) analysis, we investigated two cohorts of PCDH19-FE patients. Cohort 1 (AF1-O) comprised older PCDH19-FE females, with a mean age of 25 years, the majority of whom no longer suffered from seizures (n = 6). Cohort 2 (AF2-Y) comprised young PCDH19-FE females, with a mean age of 8.8 years, the majority of whom were still experiencing seizures (n = 6). These females were from different families and had different PCDH19 mutations (see Table 1 for further detail and references). We also investigated transmitting males (TM; n = 3) skin cell lines from age and passage-matched, control males (n = 3) and females (n = 3). Comparison of gene expression between PCDH19-FE females and female controls (FC) identified 192 (AF1-O) and 140 (AF2-Y) significantly dysregulated genes (P < 0.05, fold change greater than ±2, one-way ANOVA; Fig. 1A), respectively. There were 94 dysregulated genes in common between AF1-O and AF2-Y, of which 73 were annotated (Fig. 1B, see full list of dysregulated gene in Supplementary Material, Table S2). Of the 73 genes, 92% are known to be expressed in the brain (Uni-gene). Comparison between the expression profiles of TM and female controls (MC) identified 136 significantly dysregulated genes (P < 0.05, fold change greater than ±2, one-way ANOVA; Fig. 1A), of which four genes (IL1R1, STEAP1, OSR1 and ASPA) were also identified in the 94 shared dysregulated genes from AF1-O/AF2-Y versus FC comparison.

The unusual, gender reversed X-chromosome inheritance of PCDH19-FE led us to speculate that genes with different expression between the two sexes, that is gender-biased genes, may play a role. To investigate this, we first generated a list of likely gender-biased genes by comparing genome-wide expression between FC and MC with 223 genes identified (Fig. 1A and Supplementary Material, Fig. S1). Subsequently, we compared this set of genes against the 94 significantly dysregulated genes from the affected female versus control female comparisons (see above). Interestingly, 43 of these 94 genes (68%) had gender-biased expression (Fig. 1B). This enrichment of gender-biased genes was statistically highly significant compared with the number of gender-biased genes in control skin fibroblast cells (P = 2.51 × 10^-17, two-tailed Fisher’s exact test; Fig. 1C). To further test this observation, we took advantage of publicly available (Array Express < EMBL—EBI—European Bioinformatics Institute) expression array data sets with larger numbers of control skin fibroblasts (25 females and 23 males) and generated refined gender-biased gene lists (Supplementary Material, Fig. S1). Despite the reduction of overall number (Supplementary Material, Fig. S1B), the enrichment of gender-biased genes in PCDH19-FE remained highly statistically significant (P = 1.58 × 10^-18 or P = 2.91 × 10^-18 when looking at two different data sets, respectively; Supplementary Material, Fig. S1B). Additionally, we performed an in silico permutation assay with randomly generated lists of 100 genes and assessed the enrichment of gender-biased genes. After 100 simulations, we found on average only one (minimum = 0, maximum = 5) gender-biased gene in each randomly generated gene list. The difference between the observed and the simulated enrichment of gender-biased genes was also statistically significant (observed = 43/94 versus simulated = 5/100, P = 7.8 × 10^-6, two-tailed Fisher’s exact test).

Pathway analysis and data validation

To investigate molecular pathways affected in PCDH19-FE skin fibroblasts, we assessed the significant annotated gene list (73 genes) using Database for Annotation, Visualization and Integrated Discovery (DAVID) pathway and Ingenuity Pathway Analysis (IPA). Highlighted by DAVID analysis were pathways that are highly relevant to PCDH19 protein function, including cell adhesion and integrin-related signaling (Supplementary Material, Table S3). IPA analysis showed similar pathway enrichment, with cell-to-cell signaling and interaction ranking the highest
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<th>AF2-Y (Cohort 2)</th>
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Figure 1. PCDH19-FE Affymetrix Human Exon 1.0 ST arrays analysis. (A) Comparison of number of dysregulated gene reported in each group. Fibroblast samples were grouped into affected female (Cohort 1; AF1-O; O = old) \( n = 6 \), affected female (Cohort 2; AF2-Y; Y = young) \( n = 6 \), female controls \( n = 3 \); FC), male controls \( n = 3 \); MC) and transmitting male \( n = 3 \); TM). All expression-profiled samples were imported and analyzed simultaneously in Partek Genomic suite V6.6. One-way ANOVA tests were performed to detect differentially expressed genes between selected status groups \( P \)-value < 0.05 and fold change greater than or equal to ±2). (B) Venn diagram illustrating the overlap of three genes lists. AF1-O and AF2-Y shared 94 differentially expressed genes compared with FC, in which 43 overlapped with the gender-biased gene list generated from comparing FC with MC. (C) Graph illustrating the percentage enrichment of gender-biased genes in each gene list generated from one-way ANOVA analysis of the microarray results. \( P \)-values were derived from Fisher's exact t-test with two-tailed analysis for each ratio of gender-biased genes in the gene lists compared with the ratio of gender-biased genes present in the fibroblast pool. These analyses showed significant enrichment of gender-biased genes \( P \)-value < 0.05). (D) Hierarchical clustering analysis using 43 significantly dysregulated genes originally identified by comparison of affected PCDH19-FE females against FC. Note that PCDH19-FE female’s cluster with TM and MC instead of FC.
(Supplementary Material, Table S3). None of the highlighted pathways showed enrichment of the dysregulated gender-biased genes. However, when we examined the upstream regulatory regions of the 73 annotated PCDH19-FE dysregulated genes (part of the IPA analysis), we found that 22% (16/73) of these genes are regulated by chorionic gonadotropin (Cg), progesterone and estrogen through their respective receptors, that is progesterone receptor (PGR) and estrogen receptor alpha (ESR1) (Fig. 2A). Cross-examination of these 16 genes with IPA pathway analysis, in particular molecular and cellular functions, showed that 56% of these were involved in cell-to-cell signaling and interaction and that all 16 genes are also bona fide targets of nuclear steroid hormone receptors (Supplementary Material, Table S3). We selected 5 (WISP2, OXTR, AKR1C3, APOD and GRIA1) for reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) validation and showed that all five genes had the same trend of dysregulation as identified by microarray expression analysis (Fig. 2B). To ascertain the biological relevance of this finding, additional controls (3 males and 3 females) were included. We validated 4/5 genes (WISP2, AKR1C3, APOD and GRIA1) (Supplementary Material, Fig. S2A). OXTR, while it did not validate across all PCDH19-FE individuals, showed statistically significant difference (P < 0.01) for the AF-O cohort (Supplementary Material, Fig. S2A). Intriguingly, when examining these and several other genes that we tested (data not shown), PCDH19-FE females showed expression levels more similar to control males than to control females. This observation was reinforced by supervised hierarchical clustering analysis (see Fig. 1D). This ‘male-like’ expression was further validated on skin fibroblast cell lines from additional six PCDH19-FE females (data not shown). When we performed RT-qPCR analysis of three genes (OXTR, AKR1C3 and GRIA1) in primary skin fibroblast cell line from the single reported affected mosaic male (5), we found that all three genes were dysregulated and were expressed at the levels more similar to FC than to MC (Fig. 2B). In addition to genes linked to hormone regulation, we selected six non-hormonal regulated genes with potential functional relevance to PCDH19-FE for RT-qPCR validation. In total, we validated >60% (technical) and 45% (biological) of these genes (Fig. 2B).

**Neurosteroid enzyme dysregulation and measurements**

Two genes, AKR1C2 and AKR1C3, were of particular interest among the significantly dysregulated genes. AKR1C2 and
AKR1C3 are members of the aldo-keto reductase 1C (AKR1C) family that consists of four members, AKR1C1-4, of which only AKR1C1-3 are expressed in brain. In mammalian brain, these enzymes are responsible for reducing or oxidizing steroid hormones into their downstream metabolites, namely neurosteroids. AKR1C3 has several enzymatic activities, including 20α-hydroxysteroid dehydrogenase (20α-HSD), 3α-HSD, 17β-HSD and 11-ketoprostaglandin reductase activity, indicating that it can coordinate ligand access to progesterone, androgen, estrogen and peroxisome proliferator-activated nuclear steroid receptors localized in tissues in which it is present (14). Together with AKR1C1 and AKR1C2, AK1RC3 is able to metabolize 5α-hydroxyprogesterone (5αDHP; immediate downstream product of progesterone) into allopregnanolone (3α-hydroxy-5α-pregn-20-one). Neurosteroids exert multiple biological activities, conventional genomic as well as various membrane receptor facilitated. Neurosteroids act not only on GABA<sub>A</sub>, but also on NMDA, kainate and AMPA glutamate receptors or glycine and serotonin receptors (15). Allopregnanolone acts as a positive allosteric modulator of GABA<sub>A</sub> inhibitory neurotransmitter receptors, causing prolonged hyperpolarization of neurons and hence inhibiting the over excitability of neurons associated with an epileptic seizure (16). However, allopregnanolone can also modulate glutamate release via presynaptic GABA<sub>A</sub> receptors (17). We confirmed significant downregulation of AKR1C3 mRNA in PCDH19-FE females by RT-qPCR (Figs 2B and 3A). AKR1C3 dysregulation was also confirmed at the protein level (Fig. 3B). AKR1C3 was confirmed to be expressed in a gender-biased manner (Fig. 3B). TM investigated showed slight upregulation of AKR1C3 (data not shown); the importance of this remains to be determined (e.g. whether potentially protective). AKR1C2 mRNA was significantly downregulated primarily in old (AF1-O) PCDH19-FE females (data not shown). We were unable to determine the protein level of AKR1C2 due to the lack of AKR1C2-specific antibody.

Given that AKR1C1-3 enzymes are crucial for allopregnanolone production, we speculated that PCDH19-FE females might be allopregnanolone deficient. We therefore recruited further 9 PCDH19-FE females, different to those 12, in whom skin fibroblasts were analyzed by microarrays. We measured their blood allopregnanolone using two alternative assays. Additional seven young PCDH19-FE and seven age-matched control females' blood allopregnanolone levels were measured by a third, gold standard test, radioimmunoassay (RIA). Altogether we tested 18 different PCDH19-FE females for AKR1C3 mRNA and protein levels in their skin fibroblasts and 16 PCDH19-FE females for blood allopregnanolone levels. While allopregnanolone levels vary with age (Supplementary Material, Fig. S3), our data using RIA, ELISA and high-pressure liquid chromatography-mass spectrometry showed significant downregulation of AKR1C3 mRNA in PCDH19-FE females by RT-qPCR (Figs 2B and 3A). AKR1C3 dysregulation was also confirmed at the protein level (Fig. 3B). AKR1C3 was confirmed to be expressed in a gender-biased manner (Fig. 3B). TM investigated showed slight upregulation of AKR1C3 (data not shown); the importance of this remains to be determined (e.g. whether potentially protective). AKR1C2 mRNA was significantly downregulated primarily in old (AF1-O) PCDH19-FE females (data not shown). We were unable to determine the protein level of AKR1C2 due to the lack of AKR1C2-specific antibody.

The microarray data showed significant downregulation of AKR1C3 mRNA in PCDH19-FE females (Fig. 3A). Western blot analysis confirmed the downregulation of AKR1C3 protein in PCDH19-FE females (Fig. 3B). The plasma allopregnanolone levels were measured by ELISA and high-pressure liquid chromatography-mass spectrometry (Fig. 3C). The data showed significant downregulation of allopregnanolone in PCDH19-FE females compared to control females. The data also showed a trend toward upregulation of AKR1C2 in PCDH19-FE females (Fig. 3B).

Figure 3. Downregulation of AKR1C3 mRNA and protein level in AF and involvement of AKR1C family in progesterone metabolism. (A) Biological validation of AKR1C3 expression. We detected lower mRNA level in AF (cohort AF1-O and AF2-Y) compared with FC (n = 6). In addition to the control samples used in the microarray, three other female and four MC were included. Error bar indicates the standard deviation. Student’s t-tests (two-tailed, unequal variance) showed significant differences between AF1-O and FC, while AF2-Y and FC yielded a P-value of 0.06. (B) Western blot. The results illustrate overall lower AKR1C3 protein level in AF (cohort 1 and 2) compared with FC. Samples were probed with the AKR1C3 antibody. β-Tubulin antibody (TUBB) (Abcam, ab6046) was used for loading control. (C) Plasma allopregnanolone levels. Allopregnanolone was measured by either ELISA or HPLC/MS assays showing lower metabolite level in AF. Plasma was isolated from blood collected from age-matched patients (n = 5) and controls (n = 9). Each sample was measured twice by both ELISA and HPLC/MS assays. ELISA measurement is plotted against the left Y-axis (Concentration ng/ml); HPLC/MS measurement is plotted against the right Y-axis (area ratio relative to D6-5αDHP). Error bars indicate the standard deviation of biological repeat. *P < 0.05 and **P < 0.01.
Mutations in PCDH19 are an important cause of epilepsy in females. The unusual X-chromosome inheritance of PCDH19-FE, with male sparing of the phenotype, variable seizure type and severity, the possibility of non-penetrance, and the frequent presence of neurological and neuropsychiatric comorbidities, has puzzled the scientist and clinician for years. Using primary skin fibroblasts from affected females, TM and the only known affected mosaic male (5), we have shown that steroid metabolism, enzyme activities and their dysregulation in the regions of the DHP to allopregnanolone. While we cannot comment on AKR1C enzymes, there is evidence that skin has the neurosteroidogenic enzymatic apparatus (15,23), which underscores the relevance of these genes (and their encoded enzymes) in regulating neurosteroid metabolism and seizure control in PCDH19-FE. We have identified that skin fibroblasts of PCDH19-FE females have dramatically altered gene expression, favoring, at least for a specific set of genes, a male-like expression pattern. Recent advances in genome-wide systematic expression profiling using arrays and RNA-Seq technologies reveal considerable differences in gene expression between males and females (for review, see Ref. (17)). These gender-biased genes are under evolutionary selection and may explain differences in fitness between males and females (19,20). Of the 73 significantly dysregulated annotated genes in PCDH19-FE females, 43 showed male–female biased expression. We took specific interest in the AKR1C2 and AKR1C3 genes, which code for crucial neurosteroid-metabolizing enzymes. These genes are among a family of four paralogous genes, AKR1C1-4, that cluster on chromosome 10p14-p15 and all four share high similarity in amino acid sequence (21).

A role for these genes (and their encoded enzymes) in regulating gene expression in a sex-specific manner has recently been reinforced by the identification of mutations in AKR1C2 and AKR1C4 in 46, XY male patients with disordered sexual development (DSD) (22). Minimum activity of AKR1C2 has been detected in all patients, suggesting that low enzymatic activities of AKR1C2 together with alternative splicing of AKR1C4 result in a dose-dependent 46, XY DSD with ambiguous genitalia (22). We observed that fibroblasts from PCDH19-FE females have lower expression level of AKR1C3, at both mRNA and protein, and considerable downregulation of AKR1C2. Although we are yet to determine the underlying mechanism(s) of AKR1C gene dysregulation in PCDH19-FE, we show that AKR1C gene downregulation leads to allopregnanolone deficiency in the blood of all PCDH19-FE females tested so far. This would be expected, as one of the main enzymatic activities of AKR1C2/AKR1C4 is conversion of 5α-DHP to allopregnanolone. While we cannot comment on AKR1C enzyme activities and their dysregulation in the regions of the brain involved in PCDH19-FE seizures or related cognitive performance, there is evidence that skin has the neurosteroidogenic enzymatic apparatus (15,23), which underscores the relevance of our findings for PCDH19-FE.

Fluctuations in sex steroid hormone levels have been linked to epilepsy in the past. Neurosteroids have anticonvulsant and proconvulsant properties mediated by their effect as allosteric modulators of neurotransmitter ion channel receptors (23). A

**Figure 4.** Seizure onset and offset in PCDH19-FE females coincides with period of low sex hormones during human maturation. This figure shows the age of seizure onset and offset of PCDH19-FE females, plotted on a diagram of hormonal fluctuation during human maturation reproduced from Ober et al. (18). Data from over 150 PCDH19-FE patients were collected from publicly available resources (see references in Supplementary Material, File). The diagram shows that seizure onset occurs after mini-puberty, which seems to start after the fall in in utero sex steroid levels. The median age of onset was 8 months old, while the median age of offset was 12 years old, when sex steroid hormones are elevated in association with puberty.
good example is catamenial epilepsy (24), which is characterized by increased seizure sensitivity in women at different times of the menstrual cycle due to hormone fluctuations. In the perimenstrual form (most common), the seizures often occur at the time of menstruation when circulating estradiol: progesterone ratio is high (24,25). This has parallels with the observation that the seizure-active phase in PCDH19-FE females falls between post mini-puberty and pre-puberty, a developmental window during which sex hormone levels are low (18). This suggests that for a period of time, levels of particular effector steroids in PCDH19-FE females are low, confirmed by our detection of reduced allopregnanolone levels in blood of seizure-active PCDH19-FE girls. Neurosteroids, and allopregnanolone in particular, are known to have anticonvulsive effects (23). Supplementation with ganaxolone, a synthetic methyl analog of allopregnanolone, was able to rescue audiogenic seizures in the fragile X syndrome (Fmr1) knockout mouse model (25). Ganaxolone also had promising outcomes for a group of epilepsy patients, who were likely to have had heterogeneous etiologies, as genetic and non-genetic cases were not distinguished (26–28). More recently, allopregnanolone was used successfully to treat status epilepticus in both adults and children (29–31). These antiepileptic properties of allopregnanolone and its analog ganaxolone underline the importance of investigating involvement of AKR1C2 and AKR1C3 in PCDH19-FE epileptogenesis.

We are yet to address the question of how mutations of the PCDH19 gene converge on the regulation of steroid-metabolizing enzymes and whether PCDH19 gene itself is regulated by steroids. The role of cell adhesion molecules in sexual differentiation of the brain, and their regulation by sex steroids, has been observed before. Two members of the focal adhesion complex, FAK and paxillin, have been implicated in feminization of the brain. These genes are highly expressed in the hypothalamus of females compared with males, and in a study using rats treated with estradiol, an effector of masculinization in the brain, their levels were decreased to that of their male counterparts (32). Similarly the expression of N-cadherin, a known interactor of PCDH19 (33), has been shown to be upregulated by androgens, testosterone and dihydroprogesterone in motor neurons of rats (34).

Our data do not resolve the proposed mechanism of cellular interference of the PCDH19-FF (4,5), however, it is tempting to speculate that the two different PCDH19-positive and PCDH19-deficient cell populations, as a consequence of random X-chromosome inactivation in the affected females (4) or somatic mosaicism in the affected males (5), present with two different expression profiles. Such expression differences, for example in genes like AKR1C genes, GRIA1 or OXTR, might then drive developmental, structural and most importantly functional, including network, differences in the brain, which likely underpin the clinical presentations of PCDH19-FF.

Taken together, our findings on AKR1C gene dysregulation and subsequent allopregnanolone deficiency suggest that steroids and in particular neurosteroids (e.g. allopregnanolone) play an important role in PCDH19-FF and represent a realistic therapeutic target.

Materials and Methods

Patients

Clinical presentations of patients investigated in this study have been reported previously (1,12,13).

Human materials

Primary skin fibroblast cell lines

Skin biopsies were collected and primary skin fibroblast cultures established and cultured as previously described (4). Collections of skin biopsies were coordinated by I.E.S., S.F.B., R.S.M., C.D. and C.M. The relevant institutional human research ethics committee has approved the protocol for skin biopsy collection. Informed consent has been obtained from all participants. Blood: Patient bloods were obtained under approved consent and coordinated by J.M. and L.S. De-identified age-matched female control bloods were kindly provided by Adelaide SA Pathology’s core laboratory.

Sample sizes and ages

Gene expression analysis: (i) Affected female 1-O (old), n = 6, mean age = 25 years; (ii) AF2-Y (young), n = 6, mean age = 8.8 years; (iii) TM, n = 3, mean age = 51.3 years; (iv) FC, n = 3, age > 20 years and (v) MC, n = 3, age > 20 years.

ELISA and HPLC/MS: (i) Young female patients (patient 13–17), n = 5, age as per indicated in the Supplementary Material, Figure S3 and (ii) Female control group (n = 3, 5-, 8- and 12-year-old group). Five-year-old group, n = 6, mean age = 5 years; 8-year-old group, n = 3, mean age = 8 years and 12-year-old group, n = 2, mean age = 12 years.

RIA assay: (i) Young female patients (patient 18–24), n = 7, mean age = 7 years and (ii) Female control, n = 7, mean age = 8 years.

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis were performed as previously described (35).

Reverse transcriptase-quantitative polymerase chain reaction

RT-qPCR was performed as previously described (36). All primers were designed to generate a specific 70–250 bp amplicon (see primer sequences in Supplementary Material, Table S1).

Western blotting

Protein extraction: Skin fibroblast cells were collected as mentioned above. Cells were lysed with 1× lysis buffer [50 mM Tris-HCl pH7.5; 250 mM NaCl; 1 mM EDTA; 50 mM NaF; 1× Complete Protease Inhibitor, EDTA-free (Roche, REF 11873580001); 1% Triton-X-100 and 0.1 mM Na3VO4], and proteins were extracted by two rounds of low-frequency sonication for 5 s, followed by quick centrifugation to remove cell debris.

Immunoblotting: Western blot was performed as previously described (36). Polyclonal anti-AKR1C3 (Abcam; ab27491) used as primary and polyclonal Rabbit anti-goat conjugated to HRP (Dako; PO44901-2) as secondary antibody. The blots were re-probed with rabbit polyclonal β-tubulin antibody (Abcam, ab6046) as primary and polyclonal goat anti-Rabbit conjugated to HRP (Dako; PO44701-2) as secondary antibody.

Expression arrays

Affymetrix Human Exon 1.0 ST array (Affymetrix, Santa Clara, CA, USA) has been used. The analysis was performed using Partek® software, version 6.3 Copyright© 2012 Partek Inc., St Louis, MO, USA. Data files were imported as CEL files simultaneously, and quality control check was performed under default settings. Samples were grouped according to status, separated into...
affected female, transmitting male, control female or control male. Differential gene expression due to batch effect was dealt with using ‘Remove Batch Effect’ tool. Principal component analysis (PCA) graphs were generated from the QA/QC option in the gene expression workflow menu to aid visualization of sample clustering. A gene summary was then created to enable one-way ANOVA tests to detect for differentially expressed genes between selected status groups. Gene lists were created by setting a threshold P-value of <0.05 and fold change greater than ±2.

Allopregnanolone measurements

Steroid hormone extraction: Plasma was obtained by centrifugation of blood samples at 3 000g at 4°C for 15 min. Plasma’s steroid hormones were extracted by subjecting them to vigorous mixing with hexane:isopropanol (4:1, v/v) and subsequent centrifugation at 3000g for 15 min. Supernatant was air-dried (nitrogen gas and heat block) before resuspension in 100% methanol. D6-5αDHP (6 deuterium-labelled 5αDHP; Steraloids Inc.) was added to each plasma sample prior to extraction and served as internal standard.

HPLC/MS: Extracted samples were subjected to a C8 HPLC column (Luna 3 micron, 50 x 3.00 mm) before analysis in API 5000 LC/MSMS machine. The mobile phase was composed of 100% acetonitrile + 0.1% formic acid (v/v) (A) and 50% acetonitrile + 0.1% formic acid (v/v) (B) using the following gradient program: 0–1 min, isocratic at 50% (A and B); 1.01–12 min, isocratic at 51% (A) to 49% (B); 12.01–15 min, isocratic at 100% (B); 15.01–20 min, isocratic at 50% (A and B). The flow rate was 0.25 ml min⁻¹. Each sample ratio (μl μl⁻¹) was generated by comparing the intensity (cps; count per second) of allopregnanolone peak (m/z = 319.1/161.1) divided by intensity of an internal standard (m/z = 323.1/95.1).

ELISA: ELISA measurement was performed according to manufacturer’s instruction (USCN Life Science Inc.) with some modifications. Plasma volume used was 100 μl, and the lowest standard curve concentration was reduced to 0.41 ng ml⁻¹ to increase the detection range of plasma allopregnanolone.

Supplementary Material

Supplementary material is available at HMG online.

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Conflict of Interest statement. None declared.

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References