Reduced steroidogenesis in patients with PCDH19-female limited epilepsy

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Summary

Patients affected by protocadherin 19 (PCDH19)–female limited epilepsy (PCDH19-FE) present a remarkable reduction in allopregnanolone blood levels. However, no information is available on other neuroactive steroids and the steroidogenic response to hormonal stimulation. For this reason, we evaluated allopregnanolone, pregnanolone, and pregnenolone sulfate by liquid chromatographic procedures coupled with electrospray tandem mass spectrometry in 12 unrelated patients and 15 age-matched controls. We also tested cortisol, estradiol, progesterone, and 17OH-progesterone using standard immunoassays. Apart from estradiol and progesterone, all the considered hormones were evaluated in basal condition and after stimulation with adrenocorticotropic hormone (ACTH). A generalized decrease in blood levels of almost all measured neuroactive steroids was found. When considering sexual development, cortisol and pregnenolone sulfate basal levels were significantly reduced in postpubertal girls affected by PCDH19-FE. Of interest, ACTH administration did not recover pregnenolone sulfate serum levels but restored cortisol to control levels. In prepubertal girls with PCDH19-FE, by challenging adrenal function with ACTH we disclosed defects in the production of cortisol, pregnenolone sulfate, and 17OH-progesterone, which were not apparent in basal condition. These findings point to multiple defects in peripheral steroidogenesis associated with and potentially relevant to PCDH19-FE. Some of these defects could be addressed by stimulating adrenocortical activity.

Key Words: Adrenocorticotropic hormone, Allopregnanolone, Cortisol, Female-limited epilepsy, PCDH19, Pregnenolone sulfate.

Allopregnanolone (AP) belongs to the family of neuroactive steroids, peripherally born molecules that are converted by the nervous tissue into other active steroidal compounds or that, alternatively, are directly synthesized in the brain. In this latter case, they are usually referred to as “neurosteroids.”1–3 Notably, there is considerable...
published evidence pointing to AP as the most potent positive modulator of γ-aminobutyric acid receptor A (GABA_A) enhancing both tonic and phasic GABA_A-dependent inhibitory currents. This biologic activity of AP has been addressed in various seizure and epilepsy models, leading to the suggestion that AP is a neuroprotective, antiepileptic agent. In contrast, sulfated neuroactive steroids such as pregnenolone sulfate (PS) negatively modulate GABA_A-mediated inhibitory currents, thereby promoting seizures.

Recently, it has been found that girls with mutations in the protocadherin 19 (PCDH19) gene, known to cause female-limited epilepsy (PCDH19-FE), have significantly reduced levels of AP in their blood. In agreement with this result, AKR1C1-3 genes were dysregulated in the PCDH19-FE girls and were likely responsible for the AP deficiency. These genes have multiple other activities in the synthesis of a broad range of steroids, and it is plausible that the PCDH19-FE patients are deficient for a range of neuroactive steroids. This observation led us to postulate that the remarkable deficit of production of AP and other neurosteroids could be responsible for an imbalance in the ratio of anticonvulsant and proconvulsant hormones, so to favor the occurrence of recurrent, difficult to treat seizures in PCDH19-FE patients.

To address these important issues, we aimed to investigate the production of a variety of neuroactive steroids in the PCDH19-FE patients. In particular, we replicate AP deficiency, and further the study into other neuroactive steroids such as PS, pregnanolone, progesterone, 17OH-progesterone, and cortisol, both at the basal level as well as after stimulation with adrenocorticotropic hormone (ACTH).

**Methods**

**Experimental design**

**PCDH19-FE group**

We prospectively enrolled 12 unrelated patients affected by PCDH19-FE from April 2014 to May 2015. Genetic report assessing PCDH19 gene mutations was available for all patients. The age of PCDH19-FE patients was 8.3 ± (standard error of the mean) 5.7 years (range 2.5–18.9). Other than epilepsy, six patients had intellectual disability. Relevant clinical details of all patients studied are reported in Table S1.

**Clinical control group**

The controls (n = 15) were a group of individuals evaluated for adrenogenital syndrome within the same period. All controls were age-matched females (9.2 ± 4.0 years; range 6.1–17.9) with a normal cognitive profile. Nine of these individuals received a diagnosis of precox puberty and six of hyperandrogenism, but their hormonal profile was completely within the normal range (Table S2).

**Treatment**

Blood samples of the PCDH19-FE patients and controls were obtained twice. First at 9 a.m., which represented the basal levels (T0), and second at 60 min (T1) after a bolus of 0.25 mg of ACTH delivered intravenously. For postpubertal patients, the test was performed from the fifth to the ninth days of the menstrual cycle, to minimize hormonal variations. No seizure was observed in the 48 h preceding the test, or during the test. Blood samples were maintained at room temperature for 30 min and then centrifuged at 27,627 g for 10 min to collect sera, which were then stored at −80°C. Standard immunoassays were used to test for estradiol, progesterone, 17OH-progesterone, and cortisol levels. After that, the samples were shipped on dry ice from the Bambino Gesù Children’s Hospital in Rome to the Laboratory of Experimental Epileptology in Modena for analytic determination of the AP, PS, and pregnanolone levels.

The research protocol applied was approved by the Bambino Gesù Hospital Institutional Review Board according to the local regulations. Informed written consent was obtained from all studied subjects or their relatives.

**Quantitative analysis of AP, PS, and pregnanolone in serum by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS)**

**Chemicals and reagents**

Allopregnanolone and PS standards were purchased from Sigma (St. Louis, MO, U.S.A.). Internal standards with isotope labeling were the following: 5α-pregn-3α-ol-20-one-17α,21,21,21-d4 (AP-D4) and sodium pregnenolone-17α,21,21,21-d4 sulfate (PS-D4), purchased from CDN Isotopes (QC, Canada). All solvents for high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) were liquid chromatography-mass spectrometry (LC-MS) purity grade, whereas other solvents used for sample preparation were analytical grade (Sigma-Fluka, St. Louis, MO, U.S.A.).

**LC-MS/MS analysis**

The chromatographic separation was performed on an Agilent 1200 Series Binary Pump (Agilent, Waldbronn, Germany). Mass spectrometric detection was performed using an Agilent QQQ-MS/MS (6410B) triple quadrupole mass analyzer equipped with an ESI ion source (Agilent), operating in the positive mode, as described previously.

**Statistics**

Data were compared using a repeated-measures three-way analysis of variance (ANOVA), considering as factors the time interval, sexual maturation, and disease; groups were compared with post hoc Holm-Sidak test. The only exception to this design was for estradiol and progesterone, which were analyzed at T0 with the Student’s t-test. All statistical analyses were carried out using SigmaPlot 11 (Systat...
Software, San Jose, CA, U.S.A.). Data are presented as mean ± standard error of the mean (SEM), and were regarded significantly different at p < 0.05.

RESULTS

Consistent with our previous findings, chromatographic assessment of AP, PS, and pregnanolone allowed detection of these analytes in all samples (see Fig. S1A, in which typical AP peaks follow pregnanolone peaks and are indicated by an arrow, and Fig. S1B showing isolated PS peaks). These neuroactive steroids were quantified along with hormones measured by standard immunoassays, for which in some cases it was not possible to obtain consistent results. For this reason, estradiol and progesterone were analyzed in only the PCDH19-FE postpubertal group (n = 4) in basal condition and compared to controls (n = 5).

Although no statistically significant differences were found for estradiol (not shown), progesterone was lower in PCDH19-FE girls (0.355 ± 0.083 vs. 0.740 ± 0.115 in controls; p < 0.05, Student’s t-test). For the other hormones, a complete set of data was available and a main effect of puberty was evident for AP (p < 0.05, three-way ANOVA) and PS (p < 0.01). In the case of PS, a significant (p < 0.05) interaction between puberty and disease was also found. At variance, cortisol levels were not influenced by puberty and a trend was found for 17OHP progesterone (p = 0.07) only, but for this latter hormone, the interaction between puberty and ACTH administration was significant (p < 0.05). Main effects of disease were present for AP, PS, 17OHP progesterone, and cortisol (p < 0.001 for all of them), whereas the effect of ACTH administration was significant (p < 0.001) for PS, 17OHP progesterone, and cortisol but not for AP. No differences were observed for pregnanolone (not shown).

The multiple comparison test showed interesting differences in the two subgroups of prepubertal (Table 1) and postpubertal girls (Table 2). Specifically, in prepubertal girls, no statistically significant differences were evident in the basal condition for the investigated hormones. However, when considering the results of adrenocortical stimulation, the increases in PS and cortisol levels were more sustained in controls (see Table 1, T1 in controls vs. T1 in PCDH19-FE). In addition, ACTH did not induce significant changes in PS levels, whereas it elevated this same steroid in the control group (p < 0.001, Holm-Sidak test; see Table 1, T0 vs. T1 in controls). Although 17OHP progesterone and cortisol were significantly increased by ACTH administration in both groups, the stimulated cortisol levels in PCDH19-FE girls were lower than in controls (p < 0.05, see T1 vs. T1 in Table 1).

In postpubertal girls, differences in PS and cortisol serum levels were observed also in absence of adrenocortical stimulation (p < 0.01 for PS; p < 0.05 for cortisol, see T0 vs. T0 in Table 2). Following ACTH, PS increased significantly in both subgroups and, this time, in the same manner (Table 2). No effects of ACTH administration were observed for AP and 17OHP progesterone.

DISCUSSION

We aimed to investigate neuroactive steroids in PCDH19-FE patients, at baseline as well as after stimulation of adrenal steroidogenesis. We found that AP levels were reduced, both at baseline as well as after stimulation with ACTH, thus confirming the previous findings and suggesting that PCDH19-FE patients present a reduced ability to synthesize AP. We determined that this defect was not

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**Table 1. Determination of hormonal serum levels (ng/ml) for allopregnanolone (AP), pregnenolone sulfate (PS), 17OH-progesterone (17P), and cortisol (Cort), in prepubertal patients affected by protocadherin 19 mutation associated with female-limited epilepsy (PCDH19-FE) and their respective controls, investigated for presumptive adrenogenital syndrome but found to be healthy**

| Hormone   | Controls (T0) | Controls vs. PCDH19 (T0 vs. T0) | PCDH19-FE (T1) |
|-----------|---------------|---------------------------------|----------------|
| AP        | 0.15 ± 0.03   | NS T1 vs. T0                    | 0.08 ± 0.01    |
| PS        | 54.61 ± 11.80 | NS p < 0.001                    | 5.42 ± 1.13    |
| 17P       | 0.59 ± 0.08   | NS p < 0.001                    | 0.30 ± 0.14    |
| Cort      | 85.83 ± 8.86  | NS p < 0.05                     | 30.34 ± 8.81   |

Hormonal levels were determined in basal condition (T0) or 60 min (T1) after adrenocorticotropic hormone (ACTH) administration. Statistical analysis was performed with a repeated-measure three-way analysis of variance (factors: disease, ACTH administration, puberty) followed by Holm-Sidak test. Few samples were missing for technical reasons. NS, not significant.
limited to AP, but involved other neuroactive steroids, such as PS, 17OH-progesterone, progesterone, and cortisol. Overall, our investigations provide evidence for a significantly compromised ability to synthesize various neuroactive steroids in PCDH19-FE.

The finding of reduced PS levels is, however, against our initial hypothesis of a possible imbalance in the AP/PS ratio leading to seizure facilitation in PCDH19-FE. Surprisingly, PS production was reduced even more than that of AP. This left open an important question of whether and how these neuroactive steroids play a role in the seizures observed in patients with PCDH19-FE. Although the role of PS in the human brain has still to be clearly defined, a few cases of successful use of allopregnanolone for the treatment of status epilepticus in children suggests that AP potently modulates seizures. Thus it can be hypothesized that a therapeutic approach aimed at restoring steroidogenesis in patients affected by PCDH19-FE could result in beneficial effects. Of interest, such a goal could be partially attained by administering ACTH. Indeed, corticosteroid administration has been reported to be efficacious in some pilot studies of PCDH19-FE.

Restoring adrenal steroidogenesis in patients affected by PCDH19-FE can be beneficial for the expected increase in cortisol production that we found to occur in postpubertal PCDH19-FE girls. It is a common observation that exposure to stress may facilitate the occurrence of seizures, especially in patients affected by epilepsy. It has recently been shown that patients who present an association between acute stress and seizures are characterized by reduced adrenal response when compared to those less sensitive to stress. Thus, a reduced capability to produce cortisol apparently results in enhanced seizure susceptibility to environmental challenges. Although studies on the adrenal response to acute stress in patients with PCDH19-FE are lacking, the observed response to ACTH in prepubertal PCDH19-FE girls suggests that also this type of epilepsy may be aggravated by exposure to stressors, thus indicating that attainment of a normal adrenocortical function could be a therapeutic goal in this condition.

A limitation of this study was the small sample size. However, we found multiple, striking differences between the two groups, even when considering sexual development, and also in agreement with previously published work on a separate PCDH19-FE cohort. A second limitation of the study is the controls: we enrolled only individuals who underwent ACTH stimulation, thus reducing the size of control group. Elevated AP levels have been reported in patients affected by disorders of sexual development, such as in the case of precocious puberty. For this reason, we could have overestimated the deficit in AP production occurring in our PCDH19-FE patients. On the other hand, we also found that adrenal PS production did not completely recover after ACTH stimulation, suggesting a reduced capacity for hormonal synthesis in the PCDH19-FE in the first instance. In addition, cortisol levels were also different in the two considered groups, definitely indicating that steroidogenesis was dysfunctional in at least the adrenal glands of patients with PCDH19-FE.

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### Disclosure

All authors declare no conflicts of interest. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
REFERENCES

1. Mellon SH, Griffin LD. Neurosteroids: biochemistry and clinical significance. Trends Endocrinol Metab 2002;13:35–43.
2. Melcangi RC, Panzica GC. Allopregnanolone: state of the art. Prog Neurobiol 2014;113:1–5.
3. Biagini G, Panuccio G, Avoli M. Neurosteroids and epilepsy. Curr Opin Neurol 2010;23:170–176.
4. Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the GABA A receptor. Nat Rev Neurosci 2005;6:565–575.
5. Kokate TG, Juhng KN, Kirkby RD, et al. Convulsant actions of the neurosteroid pregnenolone sulfate in mice. Brain Res 1999;831:119–124.
6. Harteneck A. Pregnenolone sulfate: from steroid metabolite to TRP channel ligand. Molecules 2013;18:12012–12028.
7. Tan C, Shard C, Ranieri E, et al. Mutations of protocadherin 19 in female epilepsy (PCDH19-TE) lead to allopregnanolone deficiency. Hum Mol Genet 2015;24:5250–5259.
8. Meletti S, Lucchi C, Monti G, et al. Decreased allopregnanolone levels in cerebrospinal fluid obtained during status epilepticus. Epilepsia 2017;58:e16–e20.
9. Schumacher M, Liere P, Akwa Y, et al. Pregnenolone sulfate in the brain: a controversial neurosteroid. Neurochem Int 2008;52:522–540.
10. Broomall E, Natale JE, Grimason M, et al. Pediatric super-refractory status epilepticus treated with allopregnanolone. Ann Neurol 2014;76:911–915.
11. Higurashi N, Takahashi Y, Kashimada A, et al. Immediate suppression of seizure clusters by corticosteroids in PCDH-19 female epilepsy. Seizure 2015;27:1–5.
12. Bertani G, Spagnoli C, Iodice A, et al. Steroids efficacy in the acute management of seizure clusters in one case of PCDH19 female epilepsy. Seizure 2015;32:45–46.
13. van Campen JS, Jansen FE, de Graan PN, et al. Early life stress in epilepsy: a seizure precipitant and risk factor for epileptogenesis. Epilepsy Behav 2014;38:160–171.
14. van Campen JS, Jansen FE, Pet MA, et al. Relation between stress-precipitated seizures and the stress response in childhood epilepsy. Brain 2015;138:2234–2248.
15. Iughetti L, Predieri B, Cobellis L, et al. High serum allopregnanolone levels in girls with precocious puberty. J Clin Endocrinol Metab 2002;87:2262–2265.

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1. Clinical, electrophysiologic, and genetic features of patients with PCDH19-female limited epilepsy.
Table S2. Age and hypothesized pathologic disorder illustrated for each subject enrolled in the control group.
Figure S1. Allopregnanolone, pregnanolone, and pregnenolone sulfate chromatograms.