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The complex role of protocadherin-19 in brain function: a focus on the oxytocin system

Sara Mazzoleni, Marta Busnelli, Silvia Bassani*

Mutations in the protocadherin-19 (*PCDH19*) gene (Xq22.1) cause the X-linked syndrome known as developmental and epileptic encephalopathy 9 (DEE9, OMIM # 300088) (Dibbens et al., 2008).

DEE9 is characterized by early-onset clustering epilepsy associated with intellectual disability ranging from mild to profound, autism spectrum disorder, and other neuropsychiatric features including schizophrenia, anxiety, attention-deficit/ hyperactivity, and obsessive or aggressive behaviors. While seizures may become less frequent in adolescence, psychiatric comorbidities persist and often worsen with age (Dibbens et al., 2008; Kolc et al., 2020).

Females with heterozygous mutations and males with postzygotic mutations (bona fide mosaic patients) share the typical DEE9 clinical profile. In contrast, males with hemizygous mutations are generally healthy, although some of them may exhibit autistic traits or autism spectrum disorder (Kolc et al., 2020; Chouery et al., 2023). This unique inheritance pattern for an X-linked gene highlights the role of cellular mosaicism, i.e., the coexistence of cells expressing either the wild type or mutant form of PCDH19, as a crucial, though probably not unique, determinant of DEE9 pathogenesis (Dibbens et al., 2008).

The calcium-dependent cell adhesion protein PCDH19 is highly expressed in neurons, particularly in the cortex and structures of the limbic system, including the hippocampus, amygdala, and hypothalamus (reviewed in Gerosa et al., 2018). PCDH19 consists of an extracellular domain with cadherin repeats that mediate adhesion, a transmembrane region, and an intracellular C-terminus (Dibbens et al., 2008; Gerosa et al., 2018).

The identification of the synapse-to-nucleus signaling mediated by PCDH19 intracellular C-terminus following its proteolytic cleavage has brought attention to the gene-expression regulatory role of PCDH19 (Gerosa et al., 2022). A comprehensive dataset of PCDH19 gene targets is lacking but, according to the available data, it includes immediate early genes in hippocampal neurons (Gerosa et al., 2022) and estrogen receptor α-regulated genes in MCF-7 cell line (Pham et al., 2017).

One of the estrogen receptor α target genes is the oxytocin neuropeptide (OXT) receptor (*OXTR*), which is highly expressed in the brain and regulates neuronal processes underlying neurodevelopment and socio-emotional behaviors (Chini et al., 2016). *OXTR* was found to have altered expression in the human epithelial-like cell line MCF-7 transfected with PCDH19 (Pham et al., 2017), as well as in primary skin fibroblasts from DEE9 patients (Tan et al., 2015).

In particular, MCF-7 breast cancer cells transfected with wild-type PCDH19 or with a pathogenic variant containing an extracellular missense mutation (Asn557Lys) showed a tendency to increase or decrease *OXTR* expression, respectively, compared to control cells (Pham et al., 2017).

In fibroblasts from DEE9 patients, *OXTR* expression was not consistently affected (Tan et al., 2015). Two cohorts of female patients were examined. These cohorts differed in age, with a mean age of 8.8 years for the younger group and 25 years for the older group. In addition, most patients in the younger group were still experiencing seizures, unlike most of the patients in the older group who were seizure-free. A statistically significant increase in *OXTR* mRNA was observed in older DEE9 female patients compared to controls, but not in younger patients. Fibroblasts from a single male mosaic patient were also analyzed and showed a lower level of *OXTR* mRNA than controls (Tan et al., 2015).

Altogether, these findings suggest a potential crosstalk between PCDH19 and the OXT system, the dysregulation of which may contribute to the etiology of DEE9, if reconfirmed in neurons.

It is worth mentioning that the dysregulation of the OXT system in preclinical animal models has been associated with neuronal excitation/inhibition imbalance, increased seizure susceptibility, and autistic-like symptoms (Sala et al., 2011; Chini et al., 2016). Consistently, aberrant levels of OXT and *OXTR* gene polymorphisms linked to specific social traits and behaviors have been found in patients diagnosed with autism spectrum disorder and various neurodevelopmental disorders. OXT supplementation is a promising treatment for these patients (Sannino et al., 2017).

To provide a more comprehensive picture, we decided to test the hypothesis of an interplay between PCDH19 and the OXT system in the brain of a mouse model of DEE9.

We characterized the content of OXT and *Oxtr* gene expression in the brain of *Pcdh19* conditional knock-out (cKO) mice, which reproduce key features of DEE9, including susceptibility to seizures and behavioral deficits (Giansante et al., 2024).

The crossbreeding of *Pcdh19* floxed mice (*Pcdh19*$^{fl/fl}$) with Syn1-Cre mice resulted in the generation of *Pcdh19* cKO mice (*Pcdh19*$^{fl/y}$ Syn1-Cre, *Pcdh19*$^{fl/x}$ Syn1-Cre) that are characterized by mosaic deletion of PCDH19 irrespective of sex, as we previously reported (Giansante et al., 2024). Littermates that did not inherit the Syn1-Cre transgene (*Pcdh19*$^{fl/y}$, *Pcdh19*$^{fl/x}$) exhibit normal *Pcdh19* expression levels (Giansante et al., 2024) and were used as controls.

Male and female mice were analyzed at the end of early postnatal life (postnatal days 21–28) when the OXT system reached maturation (Sannino et al., 2017).

We analyzed the hypothalamic paraventricular nucleus (PVN), where most OXT is synthesized, and two brain regions that express the *Oxtr* receptor, the hippocampus and cortex. These regions are implicated in DEE9 and receive projections from PVN neurons (Chini et al., 2016; **Figure 1A**).

Because the number of OXT-positive (OXT$^+$) neurons was found to be reduced in mouse models of neurodevelopmental disorders such as Fragile X and Schaaf-Yang syndromes (Chini et al., 2016), and a role for PCDH19 in regulating neurogenesis has been reported (reviewed in Gerosa et al., 2018), we first quantified OXT$^+$ neurons in the PVN of *Pcdh19* cKO mice and their sex-matched control littermates. To this end, we stained coronal brain slices with an anti-OXT antibody and the nuclear marker DAPI and acquired confocal immunofluorescence images in which we counted OXT$^+$ and DAPI$^+$ cells of the PVN (**Additional materials and methods**). We found comparable numbers of cells (DAPI$^+$) in *Pcdh19* cKO and controls of both sexes, with the same proportion of OXT$^+$ neurons among them. Therefore, we concluded that there was no overall loss of PVN cells or a specific loss of OXT$^+$ neurons in *Pcdh19* cKO mice (**Figure 1B** and **Additional Table 1**).

Next, we measured the content of OXT in the PVN, as the activation and downstream signaling of OXTR depends on the availability of its ligand. For this purpose, we used a highly specific and sensitive radioimmunoassay (refer to **Additional materials and methods**). However, the results indicate that the OXT content in the PVN of *Pcdh19* cKO is not statistically different from that of controls (**Figure 1C** and **Additional Table 1**). Overall, immunofluorescence and radioimmunoassay data indicate that OXT availability in the PVN of prepubescent *Pcdh19* cKO mice of both sexes is preserved.

Finally, we measured *Oxtr* mRNA by RT-PCR in two of the most prominent OXTR-expressing brain regions, i.e. the hippocampus and cortex. We also tested the receptor of the closely related arginine-vasopressin hormone, specifically the *Avpr1a* subtype (see **Additional materials and methods**), as its hippocampal expression has been shown to slightly increase in *Oxtr* full knock-out mice (Sala et al., 2011). We found no significant difference in *Oxtr* and *Avpr1a* expression in *Pcdh19* cKO compared to control mice. In accordance with studies showing sex differences in *Oxtr* expression (Gigliucci et al., 2023), we observed higher expression of *Oxtr* in the hippocampus of female mice compared to male mice, regardless of genotype (*Pcdh19* cKO or controls) (**Figure 1D** and **Additional Table 1**).

Here we provided the first characterization of the OXT system in the brain of a DEE9 mouse model. Based on this collective evidence, we concluded that the major source of OXT and the hippocampal and cortical expression of *Oxtr* are preserved in *Pcdh19* cKO mice of both sexes.

Therefore, this study suggests that deficits in the OXT system are not directly responsible for the pathological phenotype observed in the *Pcdh19* cKO mice.

However, since our research focused on a specific postnatal developmental period and selected brain regions, and considering the high plasticity of the OXT system (Chini et al., 2016), we cannot rule out the possibility of dysregulation in the OXT system during other developmental stages or in different brain areas.

Similarly, the findings in cells of human origin (Tan et al., 2015; Pham et al., 2017) should be approached with caution due to the small sample size and limited number of replicates. If confirmed, the observed correlation between *OXTR* expression and patients' sex and age, as well as with the natural course of the disease characterized by seizure onset and offset (Tan et al., 2015), warrants further investigation.

In conclusion, the data collected so far in non-neuronal cells and in the brain of the *Pcdh19* cKO mouse model depict a complex scenario, with evidence both supporting and challenging the involvement of the OXT system in the pathogenesis of DEE9.

Further research, including studies on neurons derived from human induced pluripotent stem cells, will be crucial in determining whether the proposed interplay between PCDH19 and OXTR observed in non-neuronal cells could extend to neurons under specific conditions.

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Presentation at a meeting: Some of the unpublished data in Figure 1 (panel B: the immunofluorescence images and a preliminary quantification of OXT+ cells from a reduced subset of samples; panel C: dosage of OXT) were presented in a poster at the XXI Scientific Convention of Telethon Foundation; Organization: Fondazione Telethon; Place: Riva del Garda; Date: March 13–15, 2023.

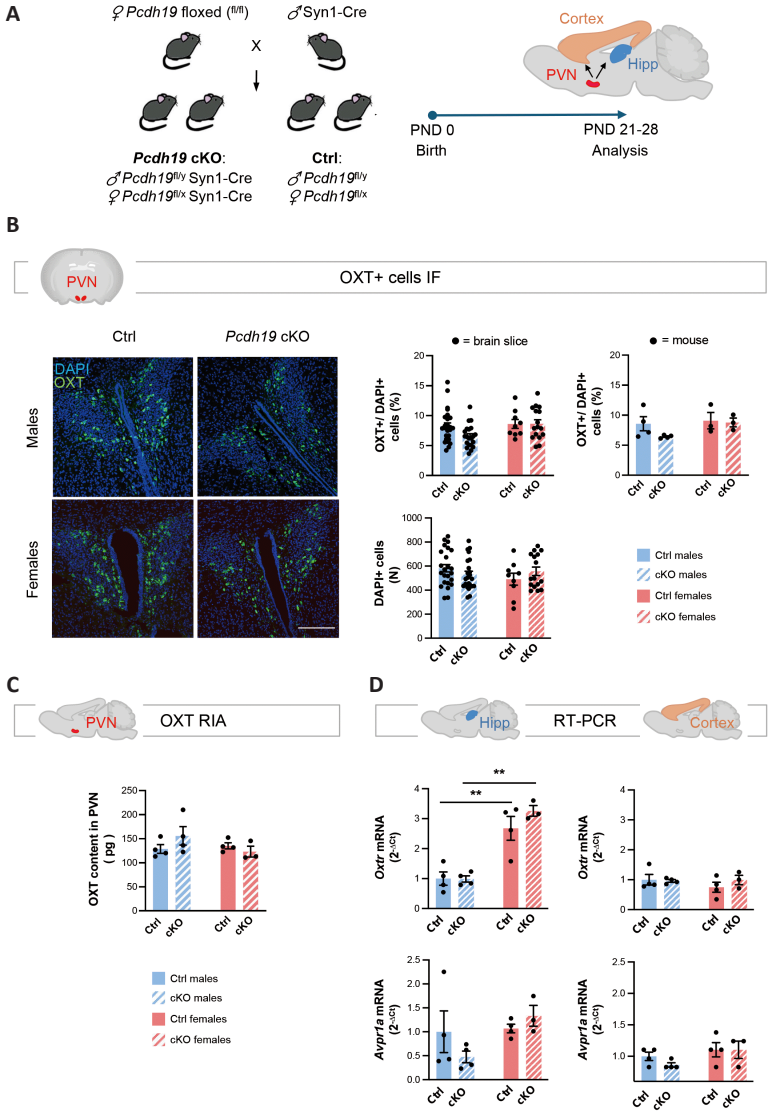

Figure 1 | **Prepubescent *Pcdh19* cKO mice display normal levels of OXT and hippocampal and cortical *Oxtr* mRNA.** (A) Schematic diagram of experimental workflow. *Pcdh19* floxed female mice (*Pcdh19*$^{fl/fl}$) were crossed with Syn1-Cre male mice to obtain *Pcdh19* cKO of both sexes (*Pcdh19*$^{fl/x}$ Syn1-Cre, *Pcdh19*$^{fl/y}$ Syn1-Cre) and control littermates (*Pcdh19*$^{fl/x}$, *Pcdh19*$^{fl/y}$) (Ctrl). Selected brain regions (PVN; cerebral cortex, Cortex; hippocampus, Hipp) of *Pcdh19* cKO mice and Ctrl mice were analyzed at PND 21–28. (B) Left: Representative immunofluorescence images of OXT-expressing neurons in coronal PVN sections from *Pcdh19* cKO mice and controls of both sexes at PND 21–28. Cell nuclei are stained with DAPI. Scale bar: 100 µm. Right: Quantification of OXT-immunopositive (OXT$^+$) PVN cells expressed as the % of DAPI-positive (DAPI$^+$) cells, and of total DAPI$^+$ cells in immunofluorescence images. Each dot corresponds to 1 brain slice or the average of the brain slice values from a single mouse, as indicated. Number of brain slices: 2–6 per mouse. Number of mice, males: 4 in Ctrl and 4 in *Pcdh19* cKO; females: 3 in Ctrl and 3 in *Pcdh19* cKO. Two-way ANOVA, not significant (n.s.). (C) Dosage by radioimmunoassay of OXT content in the PVN of mice as in (A) at PND 27. Number of mice, males: 4 in Ctrl and 4 in *Pcdh19* cKO; females: 4 in Ctrl and 3 in *Pcdh19* cKO. Two-way ANOVA, not significant (n.s.). (D) Quantification of *Oxtr* and *Avpr1a* mRNA by RT-PCR in the hippocampus (left histograms) and cerebral cortex (right histograms) of mice as in (A) at PND 27. mRNA values were normalized on those of *Gapdh*. Number of mice, males: 4 in Ctrl and 4 in *Pcdh19* cKO; females: 4 in Ctrl and 3 in *Pcdh19* cKO. Two-way ANOVA and Tukey's multiple comparisons *post hoc* test, $^{**}P < 0.01$. All data were presented as means ± SEM. The mice in A were drawn using Microsoft 365 PowerPoint and Adobe Photoshop CS6; the brain sections were drawn using Microsoft 365 PowerPoint. Unpublished data. ♀: Female; ♂: male. *Avpr1a*: Arginine vasopressin receptor 1A; cKO: conditional knock-out; Ctrl: control; DAPI$^+$: 4′,6-diamidino-2-phenylindole (DAPI)-positive; fl: floxed; Hipp: hippocampus; OXT$^+$: Oxytocin (OXT)-positive; *Oxtr*: Oxytocin receptor; PND: postnatal day; PVN: paraventricular nucleus; Syn1: synapsin1.

Sara Mazzoleni, Marta Busnelli, Silvia Bassani*
CNR Institute of Neuroscience, Vedano al Lambro, Italy (Mazzoleni S, Busnelli M, Bassani S)
NeuroMi Milan Center for Neuroscience, Milan, Italy (Busnelli M, Bassani S)
***Correspondence to:** Silvia Bassani, PhD, silvia.bassani@in.cnr.it.
https://orcid.org/0000-0003-0553-7168 (Silvia Bassani)

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Additional files:
Additional file 1: *Open peer review report 1.*
Additional file 2: *Materials and Methods.*
Additional Table 1: *Mean values, SEM, number of samples (n), and P values of data displayed in Figure 1.*

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