CDKL5 ensures excitatory synapse stability by reinforcing NGL-1–PSD95 interaction in the postsynaptic compartment and is impaired in patient iPSC-derived neurons

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Mutations of the cyclin-dependent kinase-like 5 (CDKL5) and netrin-G1 (NTNG1) genes cause a severe neurodevelopmental disorder with clinical features that are closely related to Rett syndrome, including intellectual disability, early-onset intractable epilepsy and autism. We report here that CDKL5 is localized at excitatory synapses and contributes to correct dendritic spine structure and synapse activity. To exert this role, CDKL5 binds and phosphorylates the cell adhesion molecule NGL-1. This phosphorylation event ensures a stable association between NGL-1 and PSD95. Accordingly, phospho-mutant NGL-1 is unable to induce synaptic contacts whereas its phospho-mimetic form binds PSD95 more efficiently and partially rescues the CDKL5-specific spine defects. Interestingly, similarly to rodent neurons, iPSC-derived neurons from patients with CDKL5 mutations exhibit aberrant dendritic spines, thus suggesting a common function of CDKL5 in mice and humans.

Rett syndrome (RTT) is a severe neurodevelopmental disorder and is the most common genetic cause of intellectual disability in females1,2. The disorder is mainly caused by mutations in the methyl-CpG-binding protein 2 (MECP2) gene3,4. In addition to classical RTT, several atypical forms exist, including the Hanefeld variant, which is caused by mutations in the X-linked CDKL5 gene. Patients present with early-onset intractable seizures starting before the age of 6 months and also show some typical symptoms of RTT although the clinical spectrum is heterogeneous5–11. This suggests that CDKL5 mutations cause an early-onset neurodevelopmental disorder12. In a small number of patients, CDKL5 deletions have been reported13–15. These very probably caused loss of CDKL5 function, similarly to what has previously been found in patients with a chromosome translocation that disrupted CDKL5 (refs 8,9). CDKL5 encodes a serine/threonine kinase whose catalytic domain shares homology with members of the cyclin-dependent kinase family and mitogen-activated protein kinases16,17. Interestingly, we showed that CDKL5 and MeCP2 are both induced at high levels during neuronal maturation and synaptogenesis. In addition, CDKL5 binds and phosphorylates MeCP2 in vitro18. Despite this molecular interplay, it is now well established that CDKL5 exerts its own functions, regardless of MeCP2 (refs 19–22). However, the functional role of CDKL5 is unknown at present and, thus, the question of how CDKL5 mutations affect neuronal development and function and contribute to the pathophysiology of RTT remains to be answered.

Herein, we describe that CDKL5 silencing leads to severe deficits in spine density and morphology. Notably, similar alterations were found in neurons established from patient fibroblast-derived pluripotent stem cells (iPSCs). Furthermore, we identified the netrin-G1 ligand (NGL-1, also known as LRRC4C) as a direct interactor and substrate of CDKL5. Importantly, NGL-1 phosphorylation strengthens the NGL-1–PSD95 interaction. Our findings demonstrate a role for CDKL5 in spine development and synapse morphogenesis.

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Figure 1 CDKL5 localizes in dendrites and gathers at excitatory synapses both in vitro and in vivo. (a) Primary hippocampal neurons immunolabelled at DIV15 with an antibody against CDKL5. The arrowheads point to intranuclear CDKL5 immunolocalization. The arrows point to CDKL5 immunolabelling in neuronal dendrites. (b,c) Primary hippocampal neurons at DIV15 co-immunostained for CDKL5 and MAP2. The right panel in c shows a higher magnification of the left panel. The arrowheads point to CDKL5 dendritic puncta localized to dendritic spines. (d-f) Primary hippocampal neurons immunolabelled at DIV15 with antibodies against CDKL5 and PSD95. The bottom panels in d and e show higher magnifications of the top panels. The inset in the bottom panel of e and the arrowheads in f show domains of co-localization between CDKL5 and PSD95. (g) Immunostaining of hippocampal neurons with CDKL5 and VGLUT1. The arrowhead indicates co-localization of CDKL5 and VGLUT1. The arrowhead indicates co-localization of CDKL5 and VGLUT1. (h,i) Immunostaining of CDKL5 in hippocampal neurons at DIV15 shows CDKL5 clustering at excitatory synapses co-localizing with PSD95 and apposed to VGLUT1. (j) Quantification of the mean percentage of co-localization (±s.e.m.) of endogenous CDKL5 with PSD95, Shank, NR2 and VGLUT1. (k-o) Immunolocalization of CDKL5 in mouse brain also shows CDKL5 clustering at excitatory synapses, as shown by apposition with PSD95 (l) or Shank (m) in P15 mouse cortex and with VGLUT1 in P45 mouse hippocampus (n-o). The right panels in l and m show a higher magnification of area outlined in the left panel. The arrows in l (right), m and o (right) point to a region of co-localization of CDKL5 with either PSD95, Shank or VGLUT1. (p-r) Immunostaining with CDKL5 and either gephyrin or VGAT antibodies both in vitro (p,q) and in vivo (r). The inset in q and the right panel of r highlight separate protein domains between CDKL5 and Gephyrin, respectively. (s) Quantification of the mean percentage of co-localization (±s.e.m.) of endogenous CDKL5 with gephyrin and VGAT. n = 10 neurons for each. n is derived from three experiments. (t) CDKL5 is detected in the synaptosomal fraction (Syp) and is enriched in PSD fraction I (PSD I). Note that CDKL5 is also detected in PSD fractions II and III (PSD II and PSD III). PSD95 and synaptophysin (Syn) were used as a control. 1-Trition/2-Trition represents extraction with Triton X-100 once/twice. Scale bars, 100 μm (n); 10 μm (a, b, d (top), h, k, p, r (left)); 5 μm (d (bottom), f, l (right), o (right)); 3 μm (e (right), g, m); 1 μm (r (right)). Uncropped images of blots are shown in Supplementary Fig. S8.
RESULTS

CDKL5 is enriched at the PSD of glutamatergic synapses

In the mouse, CDKL5 levels were highest in brain (Supplementary Fig. S1a,b). In postnatal day (P) 21 brain, CDKL5 immunoreactivity was evident in a punctate pattern in cell bodies as previously described\(^{(39)}\) (Fig. 1a), and also along dendrites (Fig. 1b,c). The expression of CDKL5 steadily increased during early postnatal brain development and, likewise, during maturation of in vitro cultured neurons (Supplementary Fig. S1c–f). Interestingly, some of the CDKL5 dendritic puncta localized to dendritic spines (Fig. 1c, right).

Subsequently, we investigated whether CDKL5 is present at the postsynaptic density (PSD) and found that a large number of CDKL5 puncta (61 ± 3%) co-localized with PSD95 in neurons at 15 days in vitro (DIV15; Fig. 1d–f). Likewise, CDKL5 staining closely matched the pattern of other PSD markers (Fig. 1j). Consistent with a postsynaptic localization, CDKL5 immunolabelling was closely juxtaposed with presynaptic VGLUT1 (Fig. 1g). Triple staining for CDKL5, PSD95 and VGLUT1 confirmed CDKL5 localization at excitatory synapses (Fig. 1h,i). CDKL5 puncta coincided with the dot-like immunostaining of shRNAs; sh-CDKL5#1 and sh-CDKL5#2). In HEK293T cells, both shaped spines decreased (Fig. 2h,i). We obtained similar results for knockdown neurons showed a significant increase in protrusion density (Fig. 2a), and also along dendrites (data not shown). These results indicate that CDKL5 is required for ensuring a correct number of well-shaped spines. Conversely, we did not score any significant alteration in dendritic arborization (data not shown). Notably, some of these thin filopodia-like spines were particularly branched and exhibited an aberrant morphology (middle and bottom panel in the top right of Fig. 2b). Furthermore, the percentage of filopodia-like spines and thin-headed spines increased, whereas the percentage of stubby and mushroom-shaped spines decreased (Fig. 2h,i). We obtained similar results for neurons infected at DIV10 and analysed at DIV15 (data not shown). Conversely, we did not score any significant alteration in dendritic arborization (data not shown). These results indicate that CDKL5 is required for ensuring a correct number of well-shaped spines. Specificity was confirmed by the ability of a CDKL5 truncation of CDKL5\(^{K42R}\) mutant was similar to the phenotype of CDKL5-knockdown neurons, it is conceivable that CDKL5 promotes spine formation through a mechanism that is dependent on its kinase activity.

Next, we sought to confirm the significance of our findings in vivo by electroporating embryonic day (E)13.5 embryos in utero with a plasmid containing cassettes for independent expression of sh-CDKL5#1 and EGFP. Interestingly, at E18.5, CDKL5-downregulated neurons showed a delay in radial migration. On the contrary, at P11 their relative location within the cerebral cortex was comparable to that of controls, thus indicating a transient delay in the definitive neuronal positioning (Fig. 3a,b and data not shown). Imaging of the GFP-labelled dendritic protrusions at P11, a crucial period of synaptogenesis, showed numerous protrusions with a large fraction having a well-defined head structure, characteristic of mature spines. In agreement with the in vitro studies, sh-CDKL5#1-knockdown cortical pyramidal neurons showed a robust increase in the protrusion density with significantly longer and dysmorphic dendritic protrusions (Fig. 3c–h).

Next, we examined whether CDKL5 deficit might affect the number of functional excitatory synapses also in vivo. The density of VGLUT1 puncta identifying excitatory presynaptic buttons was significantly lower in sh-CDKL5#1 than in sh-control pyramidal neurons (Fig. 3e,f,i), confirming that CDKL5 is crucial for dendritic spine morphogenesis and synaptic contact maintenance in vivo.

NGL cell adhesion molecules interact with CDKL5 at the PSD

Next, we set out to identify new CDKL5-interacting partners within dendritic spines. On the basis of our previous results showing that truncation of NTTNG1 caused atypical RTT with early-onset seizures\(^{(23)}\), we considered the netrin-G1 receptor NGL-1 and the other members of the NGL family (NGL-2 and -3, also known as LRR4C and LRR4B), which play a crucial role in early synapse formation and subsequent
**Figure 2** CDKL5 knockdown alters spine morphology and synaptic activity. (a–i) Effects of CDKL5 knockdown on spine morphology. (a–c) Representative images of primary hippocampal neurons transfected at DIV7 with sh-vector, sh-control or sh-CDKL5#1 and labelled for GFP at DIV14. The bottom panels in a and b show a higher magnification of the panels above. The top right panels in b represent higher magnifications of a (top) and b (middle and bottom) and show the spine phenotype in single GFP-positive dendrites expressing either sh-vector or sh-CDKL5#1. The arrowheads point to dendritic protrusions. (d,e) Quantification of dendritic protrusion density (d) and length (e) of sh-vector-, sh-control- and sh-CDKL5#1-transfected neurons. The bars show mean ± s.e.m. n = 15 neurons for sh-vector and n = 18 neurons for sh-control and sh-CDKL5#1. n is derived from four independent experiments. (f,g) CDKL5 downregulation decreases spine width. The bars in g show mean ± s.e.m. n = 15 neurons for sh-vector and n = 18 neurons for sh-control and sh-CDKL5#1. n is derived from four independent experiments. (h) Schematic representation of dendritic protrusion morphology categories. (i) Quantification of the mean percentage of spines in each morphology category in neurons expressing sh-vector, sh-control or sh-CDKL5#1. The bars show mean ± s.e.m. n = 15 neurons for sh-vector and n = 18 neurons for sh-control and sh-CDKL5#1 (***P < 0.001, *P < 0.01, t-test). n is derived from four independent experiments. (j–s) CDKL5 knockdown affects excitatory synapse number. Primary hippocampal neurons were transfected at DIV7 with sh-control or sh-CDKL5#1 and immunostained at DIV14 with the indicated antibodies. I and o represent merged images. The arrowheads in j–l indicate co-localization of PSD95 with VGLUT1 at dendritic spines. The bars in r and s show mean ± s.e.m. n = 16 neurons for sh-control and sh-CDKL5#1 (**P < 0.01, t-test). n is derived from three independent experiments. (t–y) CDKL5 knockdown in DIV15 cultured neurons reduced the frequency (v–x) and the amplitude (v–y) of mEPSCs. n = 9 neurons for sh-control and n = 13 neurons for sh-CDKL5#1 (**P < 0.01, t-test). n is derived from two independent experiments. Data are presented as mean ± s.e.m. Scale bars, 10 μm (a (top and bottom), j, p, t); 5 μm (b (top right)).
In utero electroporation E13.5 → P11

Figure 3 CDKL5 knockdown impairs spine structure and excitatory synapse density in vivo. (a,b) Representative confocal images of coronal slices of P11 mouse brains transfected with sh-control or sh-CDKL5#1 by in utero electroporation at E13.5. Many transfected GFP-positive cells were detected in each transfection condition. (c,d) Representative images of single dendrites of GFP-positive cortex pyramidal neurons expressing sh-control or sh-CDKL5#1. The arrows and arrowheads point to morphologically normal and aberrant spines, respectively. (e,f) Representative confocal images of cortex pyramidal neurons transfected with either sh-control or sh-CDKL5#1 and immunostained for GFP and VGLUT1. The insets show a higher magnification of the main panels to a single neurite. The arrowheads in the inset of e show co-localization of CDKL5 and VGLUT1 at dendritic spines. (g,h) Quantitative analysis of dendritic protrusion density and length of GFP-positive cortex pyramidal neurons. The bars show mean ± s.e.m. n = 15 neurons for each condition (**P < 0.001, t-test). n is derived from three independent experiments. (i) Quantitative analysis of VGLUT1 clusters in GFP-positive cortex pyramidal neurons. The bars show mean ± s.e.m. n = 9 neurons for each condition (**P < 0.001, t-test). n is derived from three independent experiments. Scale bars, 100 μm.

maturation24,25, as a good starting point. Induction of NGL–netrin-G interaction promotes synapse formation26,27. In cells transfected with CDKL5–V5 and the intracellular domain of each of the NGLs, all 3 members were immunoprecipitated by the V5 antibody (Fig. 4a). In particular, CDKL5 binds specifically to a stretch of 5 amino acids located at the amino-terminal side of the intracellular domain (Fig. 4b–d). We also screened other PSD-enriched proteins that were directly or indirectly implicated in RTT-related disorders for CDKL5 interaction, but the results were negative (data not shown).

This prompted us to particularly characterize the association of CDKL5 with NGL-1. A co-clustering assay performed in cells co-transfected with GFP–CDKL5 and Myc–NGL-1 indicated that these proteins form intracellular and surface co-clusters (Fig. 4e–i). In fact, whereas CDKL5 localized in the cytoplasm when transfected alone, co-expression with NGL-1 resulted in CDKL5 translocation to the cell membrane and exact overlapping with NGL-1 (Fig. 4g,h). Further tests in a more physiological context showed that in hippocampal neurons both proteins co-localized precisely at dendritic spines (Fig. 4j). Similarly, Myc–NGL-1 also highly co-localized with endogenous CDKL5 (Fig. 4k–m). Next, the interaction of the proteins was assessed using P4 synaptosome brain extracts. Indeed, NGL-1 was co-immunoprecipitated by the CDKL5 antibody (Fig. 4n), indicating that NGL-1 assembles in the CDKL5 complex in vivo. Notably, both proteins even directly interact as ascertained by a pulldown assay (Fig. 4o).

These data prompted us to speculate that CDKL5 might phosphorylate NGL-1. At first, we analysed whether NGL-1 is phosphorylated in mouse brain by performing two-dimensional (2D) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) from P10 extracts (Fig. 5a). NGL-1-specific staining was resolved in two protein spots. As the addition of the calf intestinal alkaline phosphatase (CIP) robustly reduced the intensity of only spot 2, whereas spot 1 increased its relative signal, we concluded that spot 2 represented the phosphorylated form of NGL-1 (Fig. 5a).

Subsequently, we subjected the entire or parts of the NGL-1 intracellular domain to Phos-tag staining. Interestingly, only the entire and the C2 N-terminal part produced an additional upper band that presents a putatively phosphorylated protein as confirmed by its loss after CIP treatment (Fig. 5b). These results indicate that NGL-1 is present with a mono-phosphorylated form both in vitro and in vivo.

CDKL5 phosphorylates NGL-1 at Ser 631

The C2 N-terminal domains of NGLs have several amino acids conserved between the family members, including Ser 600, Ser 620 and Ser 631. We mutated these serines into non-phosphorylatable alanines
and repeated the Phos-tag assays. Importantly, our results identified NGL-1 Ser 631, but not Ser 600 or Ser 620, to be phosphorylated (Fig. 5c). To obtain direct evidence of the CDKL5 ability to induce NGL-1 protein phosphorylation, the carboxy-terminal half of NGL-1 (amino acids 550–640) was purified and used for in vitro kinase assays. Interestingly, NGL-1 is readily phosphorylated in the presence of CDKL5. In contrast, the mutant S631A was not phosphorylated by CDKL5 (Fig. 5d). The catalytic activity of CDKL5 has been reported to be involved in its autophosphorylation in vitro.\(^{16,18}\) Consistent with this, we observed autophosphorylation when CDKL5 was added (Fig. 5d).

To address whether the NGL-1\(^{1631A}\) mutant could be co-immunoprecipitated by CDKL5, we analysed their interaction. It is noteworthy that GFP–CDKL5 failed to co-immunoprecipitate NGL-1\(^{1631A}\), thus suggesting the relevance of the phosphosylation for reinforcing the interaction (Fig. 5e). To gain further support, we analysed the NGL-1-binding ability of various CDKL5 deletion mutants. Notably, none of the mutants lacking the kinase domain or its activity was able to immunoprecipitate NGL-1 (Supplementary Fig. S4), suggesting that the kinase domain and CDKL5 autophosphorylation are necessary for the stable interaction of CDKL5 with NGL-1.

We next investigated the phosphorylation level of NGL-1 in a fibroblast cell line derived from a female patient who carried an X;7 chromosome translocation that truncated CDKL5 and lacked functional CDKL5 due to inactivation of the normal X chromosome...
Figure 5  CDKL5 mediates phosphorylation in NGL-1 at Ser 631. (a) Total brain extracts were left untreated (left image) or were treated with CIP (right image), subjected to 2D gel electrophoresis and analysed by immunoblotting with anti-NGL-1 antibody. The table reports the results of the densitometric scanning quantification of spots 1 and 2 and the ratio (1/2) of both, in control and CIP-treated samples. (b) Immunoprecipitation assays performed with anti-GFP antibody of HEK293T cells lysates transfected with the indicated plasmids and treated with CIP (+) or left untreated (−). Proteins were resolved in normal SDS-PAGE gels (upper panel) and in phosphate-affinity PAGE gels (lower panel). (c) HEK293T cells were transfected with EGFP–NGL-1 cyt, EGFP–NGL-115631A, EGFP–NGL-115620A or EGFP–NGL-115631A constructs. Immunoprecipitation assays were performed with anti-GFP antibody. Proteins were resolved in a normal SDS gel (upper panel) and a Phos-tag gel (lower panel). (d) In vitro kinase assay performed on HEK293T cell lysates transfected with the indicated plasmids and tested for NGL-1 (left blot) and NGL-115631A (right blot). The mean relative level ± s.e.m. (ref. 8). Importantly, the patient cells, when compared with normal control fibroblasts that express endogenous CDKL5, exhibited reduced levels of the NGL-1 phosphorylated form with respect to the total amount of the protein (Fig. 5f). Thus, these data suggest that the CDKL5–NGL-1 protein interaction is conserved in human cells and that NGL-1 is phosphorylated by CDKL5 also in fibroblasts. Along the same line, an increase of the NGL-1 phosphorylated form was detected when CDKL5 was overexpressed in the patient fibroblasts. This effect was not seen in normal control fibroblasts, suggesting that of NGL-1 and NGL-115631A phosphorylation is illustrated in a histogram (*** P < 0.001, t-test). (e) Total cell lysates of HEK293T cells transfected with the indicated plasmids were subjected to immunoprecipitation assays with monoclonal anti-GFP and anti-Myc antibodies. (f) Control and CDKL5-negative patient fibroblast cells were transfected with EGFP–NGL-1 cyt. Total cell lysates were resolved in a Phos-tag SDS gel and probed with anti-GFP–HRP antibody. Anti-tubulin antibody staining served as a loading control. The relative NGL-1 phosphorylation level is presented as the mean ± s.d. of four independent experiments compared with the total amount of NGL-1 (*** P < 0.001, t-test). (g) Total cell lysates of both control and CDKL5-negative patient fibroblast cells were transfected with the indicated plasmids and resolved in a normal SDS gel or in a Phos-tag SDS gel. GFP protein levels were used to monitor transfection efficiency as indicated in NGL-1/GFP ratios (given in arbitrary units). The relative NGL-1 phosphorylation level is presented as the mean ± SD of three independent experiments compared with the total amount of NGL-1 (*** P < 0.01, t-test). Uncropped images of blots are shown in Supplementary Fig. S8.

NGL-1 binding to PSD95 is sustained by CDKL5 phosphorylation on Ser 631

NGLs have a conserved C-terminal PDZ-binding domain (VQETQI), which specifically binds to PSD95 (ref. 27), a protein that plays a significant role in learning and memory29. NGL-1 spine-inducing capability is promoted by targeting PSD95 to new forming dendritic
Figure 6 CDKL5-dependent phosphorylation of NGL-1 is necessary for NGL-1–PSD95 binding and correct spine morphogenesis. (a) CDKL5 knockdown reduces NGL-1–PSD95 binding in primary cortical neurons (left panels). Lysates of primary cortical neurons (DIV14) infected with either sh-control or sh-CDKL5#1 were immunoprecipitated with antibodies against NGL-1 and immunoblotted with NGL-1 and PSD95 antibodies. CDKL5-dependent phosphorylation of NGL-1 on Ser 631 sustains NGL-1 binding to PSD95 (middle panels). Lysates of HEK293T cells double-transfected with GFP–PSD95 plus Myc–NGL-1 or Myc–NGL-1S631A were immunoprecipitated using an antibody against Myc and immunoblotted with Myc and PSD95 antibodies. Lysates of HEK293T cells double-transfected with GFP–PSD95 plus Myc–NGL-1 or Myc–NGL-1S631E were immunoprecipitated using an antibody against Myc and immunoblotted with Myc and PSD95 antibodies (right panels). Data are presented as means ± s.e.m. ***P < 0.001. (b,c) Effect of overexpression of wild-type NGL-1, NGL-1S631A and NGL-1S631E on dendritic protrusion density and morphology. Primary hippocampal neurons were transfected with the indicated plasmids at DIV7 and seven days later were stained for GFP and Myc. In c, the bars show mean ± s.e.m. n = 12 neurons for each (***, P < 0.001, **P < 0.01, t-test). n is derived from three independent experiments. (d,e) Primary hippocampal neurons were transfected with the indicated plasmids at DIV7 and were stained for GFP and PSD95 seven days after transfection. The arrowheads in d point to dendritic spines. The bars in e show mean ± s.e.m. n = 10 neurons for each (***, P < 0.001, t-test). n is derived from three independent experiments. (f) NGL-1S631E partially rescues the phenotype of CDKL5 knockdown. Representative images of primary hippocampal neurons transfected at DIV7 with the indicated plasmids and labelled for GFP at DIV14. (g) Quantification of dendritic protrusion density and length of sh-control, sh-CDKL5#1 and sh-CDKL5#1 plus GFP–NGL-1S631E-transfected neurons. The bars show mean ± s.e.m. n = 12 neurons for each (***, P < 0.001, **P < 0.01; NS, not significant, t-test). n derives from three independent experiments. Scale bars, 10 μm (b,f); 5 μm (d). Uncropped images of blots are shown in Supplementary Fig. S8.
protrusions\textsuperscript{27}. Given that CDKL5 interacts directly with NGL-1, we examined whether CDKL5 influences the interaction of these proteins. To do this, we performed a co-immunoprecipitation assay in cortical neurons infected at DIV7 with sh-CDKL5\#1. Seven days later, NGL-1 was immunoprecipitated and the amount of PSD95 in the complex was determined. Remarkably, we observed a robust reduction in the level of the NGL-1–PSD95 interaction in CDKL5-knockdown neurons (Fig. 6a). These results indicate that CDKL5 functions in promoting and/or maintaining the NGL-1–PSD95 protein association.

NGL-1 phosphorylated residue Ser 631 is very close to the terminal PDZ-binding domain and therefore located at a convenient site for regulating NGL-1–PSD95 interaction. To investigate this, we transfected cells with either NGL-1 or its S631A phospho-mutant in combination with GFP–PSD95, immunoprecipitated the lysates for Myc–NGL-1 and measured the amount of associated PSD95. Indeed, the phospho-mutant showed a reduced level of association with PSD95 (Fig. 6a). Next, we examined the ability of the mutant to promote synaptogenesis in hippocampal neurons. Notably, dendritic protrusions were reduced in numbers and showed a significantly elongated morphology (Fig. 6b,c). These alterations are both qualitatively and quantitatively similar to the phenotype observed in CDKL5-downregulated neurons. Therefore, it is plausible that the NGL-1\textsuperscript{S631A} mutant acts through a dominant-negative effect on endogenous NGL-1 and its ability to associate with PSD95.

To further strengthen this assumption, the phospho-mimetic NGL-1 mutant S631E was challenged in similar experiments. In particular, this mutant associated better with PSD95 although it was not able to significantly increase spine formation on overexpression (Fig. 6b,c). Despite that, when expressed in CDKL5-knockdown neurons, the mutant was able to restore nearly the correct length and morphology of dendritic spines, but not their numbers (Fig. 6f,g). Conversely, both phospho-mutants retained the ability to induce pre-synaptic terminals similar to the native NGL-1 when expressed by heterologous cells co-cultured with primary neurons\textsuperscript{27} (Supplementary Fig. S5). These findings are consistent with the NGL-1 extracellular domain being the main molecular player of this activity.

Overall, these data indicate that CDKL5 is critical for the maintenance of synaptic contacts, mainly by regulating the NGL-1 phosphorylated state and, thereby, its ability to bind PSD95 and stabilize their association.

**Patient-specific alterations in iPSC-derived neurons**

iPSC technology offers the possibility to model in vitro neurodevelopmental disorders by providing access to human neurons. Therefore, we set out to establish iPSC lines from two female patients diagnosed with atypical RTT caused by CDKL5 pathogenic mutations (R59X, L220P) and whose primary fibroblasts were already characterized\textsuperscript{26,30}. Fibroblasts were infected with OCT4-, SOX2- and KLF4-expressing retroviruses and cultured in the presence of SR and FGF2. After 6–8 weeks, the iPSC-like clones expressed pluripotency markers and showed silencing of the retroviral exogenous genes (Fig. 7a and Supplementary Figs S6a,b and S7a,b). Pluripotent properties were also ascertained by testing the capability to differentiate into cells of the endoderm (SOX17-positive), mesoderm (SM-A-positive) and neuroectoderm (TuJ1-positive) germ layers in vitro and the potential to generate teratomas (Supplementary Fig. S6c,d). Furthermore, correct chromosomal content was ascertained by karyotyping (Supplementary Fig. S7c).

Increasing evidence shows that female iPSCs may or may not retain the inactivated X-chromosome status of the original fibroblasts\textsuperscript{31–33}. Indeed, only iPSCs exclusively expressing the mutant CDKL5 allele will represent a coherent model of disease-affected cells. Thus, we investigated only those clones in which inherent X-chromosome inactivation was revealed by H3K27me3 nuclear staining (Fig. 7a) and selected only cell lines that showed expression of either the mutant or the normal CDKL5 allele for further analysis. This approach allowed the generation of both pathogenic and healthy iPSCs from the same individual, providing stringent control cells for comparative analyses. A list of the cell lines used for each experiment is detailed in Supplementary Table S1.

Undifferentiated mutant and control iPSC clones did not show any evident difference (data not shown). We then induced their differentiation into neurons through the generation of neural-rosette intermediate neural progenitors and their subsequent differentiation for 55–60 days\textsuperscript{34}. In these conditions, neuronal cultures developed a highly intricate neuronal network highly enriched in forebrain-committed glutamatergic neurons (Fig. 7b–h). Interestingly, CDKL5 expression was found to strongly increase following neuronal differentiation (Fig. 8a). In particular, CDKL5 was found enriched in dendritic spines in well-matured neurons (Fig. 8b). These findings establish iPSC-derived neurons as an adequate in vitro model to study CDKL5-dependent biological mechanisms. Absence of CDKL5 did not impair the rate of neuronal differentiation or morphology (Fig. 8c–g). Interestingly, a significantly reduced number of synaptic contacts, indicated by the number of VGLUT1 and PSD95 puncta, was detected in the mutants (Fig. 8h–m). To highlight spine morphology, neurons were infected with a lentivirus constitutively expressing GFP at low multiplicity. This staining permitted visualization of frequent abnormalities in the overall spine structure of mutant neurons (Fig. 8n–r). In particular, many of these altered spines did not present an evident pre-synaptic terminal (Fig. 8p,q). These results are well in line with those obtained for CDKL5-silenced mouse neurons and suggest a conserved role of this protein in neuronal structure homeostasis and activity.

**DISCUSSION**

In this study, we revealed how loss of CDKL5, the molecule responsible for the Hanefeld variant of RTT and early-onset intractable encephalopathy, impacts neuronal function. Our first observations indicate that CDKL5 mainly localizes to dendritic spines where it is juxtaposed to the PSD. Interestingly, CDKL5 was found in the human PSD (ref. 35; www.genescognition.org/HUMAN–PSD), suggesting that its localization at the synaptic PSD is evolutionarily conserved. Our data provide evidence that CDKL5 is necessary for correct genesis and maintenance of dendritic spines and for synapse formation. Indeed, knocking down CDKL5 significantly decreases the density and length of dendritic spines both in vitro and in vivo.

In line with the compromised development of spines, CDKL5-downregulated neurons exhibit abnormal mEPSCs. Thus, our results reveal that CDKL5 is a key-limiting factor in regulating glutamatergic synapse formation and suggest that changes in excitatory synaptic strength might be responsible, at least in part, for the neurodevelopmental symptoms associated with its deficiency.
Figure 7  Assessment of pluripotency-associated markers in iPSCs and forebrain identity of iPSC-derived neurons. (a) Bright-field images of iPSC colonies in the growing phase. iPSCs express pluripotency markers such as OCT4, SOX2, NANOG and TRA-1-60 and exhibit a H3K27-positive inactivated X chromosome. (b–d) Cultured iPSC-derived neurons were immunolabelled with TuJ1 antibody in combination with either VGLUT1, VGAT or TH antibodies. The insets in the right panels of b and c show higher magnifications of a single neurite. (e) Quantification of the mean percentage of either VGLUT1, VGAT or TH TuJ1-positive cells for the neuronal progenies differentiated from four different iPSC lines. The bars show mean ± s.e.m. n = 390 neurons (n is derived from three independent experiments per line). (f) RT–PCR expression analysis of specific brain area genes. iPSC-derived neurons expressed markers of forebrain identity as such Emx1, Tbr1, Ctip2, Cux1, CAMKII and Foxg1. (g) iPSC-derived TuJ1-positive neurons express the cortical layer-5 molecular marker Ctip2. (h) Quantification of the mean percentage of Ctip2 and Tbr1 expression in TuJ1-positive neurons. The bars show mean ± s.e.m. n = 360 neurons (n is derived from three independent experiments per line). Scale bars, 100 μm (a, b, c, d); 50 μm (d, g); 10 μm (a, b, c, d).

We identified CDKL5 as an interactor of NGLs, which are synaptic cell adhesion molecules (CAMs) that exert a pervasive role in synapse homeostasis. Very intriguingly, we have previously found that NTNG1, which encodes the specific NGL-1 partner netrin-G1, was disrupted by a chromosome translocation in a patient with atypical RTT (ref. 23). It is then highly plausible that CDKL5 and NGL-1...
might work, at least in part, in a common pathway, thus providing a molecular framework explaining the similar neuropathological outcome of NTNG1 and CDKL5 mutations.

Our findings prompted us to search for pathogenic mutations in NGL-1 and NGL-2 in patients with atypical RTT. No likely pathogenic changes were detected in these genes, suggesting that they are not a major contributor (data not shown). Although we did not detect NGL-1, -2 mutations, other groups reported deletions including NTNG1 in patients with intellectual disability and seizures, NTNG1 missense mutations in autism, and the association of NTNG1 non-coding single-nucleotide polymorphisms with schizophrenia.

We provide evidence that CDKL5 phosphorylates NGL-1 on a unique serine, which is very close to the PDZ-binding domain that interacts with PSD95. This phosphorylation can also be extracted for VGLUT1/TuJ1 and PSD95/TuJ1 antibody staining. Quantitative analysis reveals a reduced number of both VGLUT1 and PSD95 puncta in the aRTT when compared with wild-type neurons. The bars show mean ± s.e.m. (n is derived from five independent experiments per line; **P < 0.01, t-test.) GFP visualization permits the identification of aberrant spines with a very long and thin appearance in aRTT neurons. Aberrant spines in aRTT neurons lack an evident presynaptic terminal as shown by immunofluorescence microscopy for synaptophysin (SYP). Quantification of the mean length of dendritic spines in wild-type and aRTT neurons. The bars show mean ± s.e.m. (n is derived from five independent experiments per line; **P < 0.01, t-test.) Scale bars, 50 μm (c,f); 20 μm (j); 10 μm (h); 5 μm (n,p).

Figure 8 Patient-specific iPSC-derived neurons exhibit a normal differentiation rate, but aberrant spine structures. (a) The number of CDKL5 transcripts increased in embryoid bodies (EBs) and during neuronal differentiation. (b) CDKL5 immunofluorescence micrograph showing its localization enriched at the synapses of iPSC-derived neurons. (c,d) Low-magnification view of both affected and healthy iPSC-derived neuronal cultures immunodecorated for MAP2 and TuJ1. (e) The neuronal differentiation rate is not altered in aRTT. (n is derived from five independent experiments per line); NS, not significant. (f,g) Representative images of 62-day-old wild-type and aRTT iPSC-derived neurons immunostained for MAP2 and TuJ1. (h) High-magnification images on dendritic tracts of wild-type and aRTT iPSC-derived neurons processed for VGLUT1/TuJ1 and PSD95/TuJ1 antibody staining. (i,m) Quantitative analysis reveals a reduced number of both VGLUT1 and PSD95 puncta in the aRTT when compared with wild-type neurons. The bars show mean ± s.e.m. (n is derived from five independent experiments per line; **P < 0.01, t-test.) (n,o) GFP visualization permits the identification of aberrant spines with a very long and thin appearance in aRTT neurons. (p,q) Aberrant spines in aRTT neurons lack an evident presynaptic terminal as shown by immunofluorescence microscopy for synaptophysin (SYP). (r) Quantification of the mean length of dendritic spines in wild-type and aRTT neurons. The bars show mean ± s.e.m. (n is derived from five independent experiments per line; **P < 0.01, t-test.) Scale bars, 50 μm (c,f); 20 μm (j); 10 μm (h); 5 μm (n,p).
from a phosho-proteomic survey of mouse tissues. So far, little evidence exists showing that the activity of synaptic CAMs is controlled by post-translational mechanisms. The discovery of a regulated phosphorylation process in NGL-1 opens the exciting possibility that the activity of other CAMs can be controlled by their phosphorylation state.

We demonstrated that CDKL5 is able to phosphorylate NGL-1 at Ser 631 both in vitro and in mouse brain. Importantly, the same results were obtained for human cells. In fact, CDKL5-negative patient fibroblasts exhibited reduced NGL-1 phosphorylation when compared with control cells. As NGL-1 phosphorylation was not completely absent, it is plausible to assume that some kinases substitute for CDKL5 in these cells. To overcome the limitations of using skin fibroblasts, we generated iPSC-derived neurons from two female patients with atypical RTT. Interestingly, in these neurons we detected a loss of synaptic contacts together with an increased number of aberrant dendritic spines, indicating that they represent a reliable system for studying CDKL5 function.

We showed that CDKL5-mediated phosphorylation of NGL-1 is necessary to ensure stable binding to PSD95. This mechanism resembles the phosphorylation dynamics of ion channels that mediate changes in the interaction with PSD95 (refs 42, 43). Furthermore, phosphorylation of serine at position 9 outside the PDZ-binding domain in ephrin B ligands is required for the stable interaction with GRIP at the PSD and ensures correct synaptic activity. These results mirror our findings and highlight a new aspect of fine-tuning protein–protein interaction by serine phosphorylation.

Interestingly, overexpression of a NGL-1 non-phosphorylatable mutant leads to significant elongation of dendritic protrusions. This phenotype closely resembles the defects observed in CDKL5-silenced neurons, suggesting that the mutant could act in a dominant-negative manner leading to the disruption of the NGL-1–PSD95 interaction. Thus, these data indicate that synaptic strength and stability can be modified by altering the ratio between phosphorylated and unphosphorylated NGL-1 and, possibly, its homologues.

Our results are consistent with a model in which loss of CDKL5 elicits spine length and synaptic strength by regulating NGL-1–PSD95 interaction. On the other hand, CDKL5 interacts with Rac1 (ref. 21), which participates in stabilizing the actin cytoskeleton. Therefore, it is possible that Rac1 activity is altered by CDKL5 deletion. This is particularly relevant given that actin filament dynamics within the spines control their morphology and stability. Thus, we cannot exclude that some aspects of the alterations described in this study can be ascribed to defects in such mechanisms. In agreement with this assumption, rescue of CDKL5-dependent spine deficits by a phospho-mimetic NGL-1 mutant is incomplete, suggesting that other mechanisms are dysregulated by CDKL5 impairment.

We demonstrate that loss of CDKL5 severely affects spine morphology and reduces functional synaptic contacts, impairing neuronal activity.

We propose a model in which CDKL5-dependent phosphorylation on Ser 631 controls the association of NGL-1 with the postsynaptic molecular hub PSD95. In light of these findings, we provide mechanistic insights into how CDKL5 mutations can impact on neuronal function in atypical forms of RTT and other CDKL5-related neurodevelopmental disorders.
METHODS

Plasmids and constructs. pEGFP–hCDKL5, pEGFP–hCDKL5ΔN, pEGFP–hCDKL5ΔC, pEGFP–hCDKL5ΔNΔC were described previously.20

For generating the NGL-1 cytoplasmic region or its C1 and C2 fragments, we used the pEGFP-C2 vector (Clontech) double-digested with EcoRI and Sall restriction enzymes (New England Biolabs) and PCR products generated using genomic DNA as a template with the following primer pairs: NGL-1 pEGFP EcoRI forward 5'-TAACTCCAAGTAGGAGAGCCACGAG-3' and NGL-1 pEGFP Sall reverse 5'-TGTGCACTGATTGATGCTCTC-3'. NGL-1 C1 pEGFP EcoRI forward 5'-TAACTCCAAGTAGGAGAGCCACGAG-3' and NGL-1 C1 pEGFP Sall reverse 5'-TGTGCACTGATTGATGCTCTC-3'.

The lentiviral knockdown constructs were generated by cloning oligodeoxynucleotides into pLentI.OX.3 (p.L.3) according to the protocol published online by Science gateway (http://www.sciencegateway.org/protocols/lentivirus/cloning.htm). The sh-CDKL5#1 construct targets the region 5'-GAGGACCTATGGGTTGTA-3', the sh-CDKL5#2 construct is directed against the target region 5'-GTGATAGCAGTTCTGGTACA-3'. The sh-control construct is directed against Renilla luciferase and targets the region 5'-GGCCCTTCTACATCTCTAC-3'.

pMyC-hNL1 was a gift from E. Kim (Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea). pEGFP-PSD95 was a gift from L. Muzio (San Raffaele Scientific Institute, Milan, Italy). pMXOCT4, pMXSOX2 and pMXKLF4 were gifts from S. Vortkamp (Max Planck Institute for Molecular Genetics, California, USA). The S631A, S631E and S600A derivatives were generated by site-directed mutagenesis using the QuikChange II site-directed Mutagenesis Kit (Stratagene). pCAGGS-hNL1–iresGFP and pCAGGS–hCDKL5–iresGFP were generated by PCR and cloned into the EcoRI site of pCAGGS–iresGFP. Recombinant hNL1 (amino acids 550–640) and hNL1–100S (amino acids 550–640) were produced from the pYBI–NGL1 and pYBI–NGL1ΔS constructs by PCR into the Ndel and Xhol sites of pTYB1 (New England Biolabs), generating a chitin-binding fusion protein. All PCRs-generated constructs were verified by sequencing.

Cell culture and transfection. HEK293T, COS-7 and fibroblast cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1% penicillin/streptomycin (Sigma Aldrich), 2 mM glutamine (Sigma Aldrich) and 10% fetal bovine serum (Invitrogen) at 37 °C with 5% CO2. Transient DNA transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations or the calcium phosphate precipitation method.

Primary hippocampal and cortical cultures. Primary neuronal cultures were prepared as described previously.20

Mixed-culture assay. The mixed-culture assay was carried as described previously.20

Retrovirus infection, iPSCs derivation and differentiation. Female CDKL5 fibroblasts derived from two patients' specific dermal biopsy explants were provided by L. Muzio (San Raffaele Scientific Institute, Milan, Italy). pMXOCT4, pMXSOX2 and pMXKLF4 were gifts from S. Vortkamp (Max Planck Institute for Molecular Genetics, California, USA). The S631A, S631E and S600A derivatives were generated by site-directed mutagenesis using the QuikChange II site-directed Mutagenesis Kit (Stratagene). pCAGGS-hNL1–iresGFP and pCAGGS–hCDKL5–iresGFP were generated by PCR and cloned into the EcoRI site of pCAGGS–iresGFP. Recombinant hNL1 (amino acids 550–640) and hNL1–100S (amino acids 550–640) were produced from the pYBI–NGL1 and pYBI–NGL1ΔS constructs by PCR into the Ndel and Xhol sites of pTYB1 (New England Biolabs), generating a chitin-binding fusion protein. All PCRs-generated constructs were verified by sequencing.

Immunoprecipitation and western blotting. Immunoprecipitation on cell transiently co-transfected using the calcium phosphate precipitation method or Lipofectamine 2000 (Invitrogen) and western blotting were performed as described previously.20 The following primary antibodies were used: mouse monoclonal anti-GFP (clones 7.1 and 13; 1:1,000, Roche Diagnostics, 1811446000), mouse monoclonal anti-PSD95 (clone K28/43; 1:1,000, University of Nea, 2012 Macmillan Publishers Limited. All rights reserved.

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at 50,000 V h⁻¹ on 7 cm strips. The second dimension was SDS–PAGE 6%, on
reduction with DTE and alkylation with iodoacetamide of the strips. Resolved
proteins were electrotransferred onto Hybond-ECL nitrocellulose membrane with
a miniVE Blot Module (GE Healthcare) for 2 h at 300 mA in transfer buffer (25 mM
tris base, 40 mM glycine, 20% methanol and 0.05% SDS). Membranes were
probed for NGL-1. Detection was by enhanced chemiluminescence (ECL) reaction
(ECL detection kit, GE Healthcare) and proteins were visualized on autoradiography
films (Hyperfilm ECL, GE Healthcare). The lysate (300 μg total protein), without
phosphatase inhibitors, was subjected to a de-phosphorylation reaction using CIP.
Membrane images were acquired with a ProXPRESS 2D Proteomic Imaging System
and quantitative comparisons of spot intensities were carried out using Progenesis
SameSpots (Nonlinear Dynamics).

**In utero electroporation.** In utero electroporation was performed as described previously.⁴¹

**Immunocytochemistry for light microscopy.** Immunocytochemistry on float-
ing 50 μm brain sections, made with a CM 1850 ultraviolet cryostat (Leica), was
performed as described previously.⁴¹ Sections were incubated with mouse anti-GFP
(clone 3E6; 1:2,000, Invitrogen, A11201) and guinea-pig anti-VGLUT1 (1:500,
Synaptic Systems, 135304). For teratoma analysis, up to 2 x 10⁷ iPSCs were injected
subcutaneously into the dorsal flanks of nude mice. After 3 months, teratoma
were explanted, fixed and dissected as described above. Sections were stained with
haematoxylin and eosin for further analysis.

**Dendritic protrusion analysis.** Fluorescence images were acquired using a TCS
SP5 laser scanning confocal (Leica) > 63 oil-immersion lens as z series of 15–25
images taken at 0.3-μm intervals at 1,024 x 1,024 pixels resolution with 1 x zoom.
The confocal microscope acquisition parameters were kept the same for all scans
for each experiment. Each experiment was repeated at least two, mostly three,
times and images were analysed in blind using ImageJ (NIH) software. Dendritic
protrusion density was measured by counting the number of dendritic protrusions on
both primary and secondary dendrites and expressed as the number of dendritic
protrusions per 10 μm dendritic length. The dendritic protrusion length was
measured by manually drawing a vertical line from the protrusion’s tip to the point
where it met the dendritic shaft and the head width was measured by drawing a line
perpendicular to the length. The proportion of spines in each morphology category
was determined for each dendrite. For each experiment, about 300–600 spines from
25–30 dendrites derived from 10–20 neurons were analysed per condition. In the
figure legends, n refers to the number of neurons quantified. Statistical significance
was determined by Student’s t-test.

**Electrophysiology.** Standard patch-clamp whole-cell recordings were taken from
primary hippocampal neurons transfected at DIV7 with sh-control or sh-CDK5 #1
and labelled for GFP at DIV4 as described above. The neurons were continuously
perfused with artificial cerebrospinal fluid of the following composition: 124 mM
NaCl, 2 mM KCl, 3 mM KH₂PO₄, 26 mM NaHCO₃, 1.3 mM MgCl₂, 2.5 mM
CaCl₂ and 10 mM glucose (final pH 7.4). Recording borosilicate glass micropipettes
(outside diameter 1.5 mm, inside 0.86 mm) were pulled to a resistance of
4–10 MΩms when filled with the following medium: 120 mM potassium glutonate,
10 mM KCl, 3 mM MgCl₂, 10 mM HEPES, 2 mM MgATP and 0.2 mM NaGTP, at
pH 7.2 (adjusted with KOH), 280 mM/Sm. mEPSCs were observed in the presence
of artificial cerebrospinal fluid containing 1 mM tetrodotoxin and 10 mM (-)
bicuculline methiodide, using continuous 60 s epochs of mEPSCs recorded at
-60 mV membrane potential with an Multiclamp 700B amplifier (Molecular Devices)
with a low-pass filter of 2 KHz. Data were digitized at 2–5 KHz with a
Digidata 1322A (Molecular Devices) controlled by Clampex 9 software (Molecular
Devices) and analysed off-line with Clampfit 9 software (Molecular Devices), after
processing with a band pass of ~2 Hz to 1 KHz using a single-pole digital filter. Event
selection was visually checked for all data. The statistical analysis was performed
using the paired t-test expressing population mean as the mean ± s.e.m.

**Recombinant protein purification.** hNGL-1 and hNGL-1⁴³ were cloned in
the pTY1 expression construct, expressed in *Escherichia coli* strain ER2566 and
induced with 0.5 mM isoproryl-β-d-thiogalactopyranoside (IPTG) at 30 °C for
5 h. Following induction, extracts were prepared by resuspending the bacteria
in 750 mM NaCl, 20 mM Tris–HCl, at pH 8.0, 1 mM EDTA and 0.1% Triton
X-100 with protease inhibitors (Sigma) and sonicated. Following centrifugation
at 16,000g for 10 min, the lysate was added to chitin-agarose beads (New England
Biolabs). Fusion proteins were cleaved on the column overnight by incubation
with lytic buffer containing 50 mM dithiothreitol. Eluted fractions containing
the bulk of NGL-1 were pooled. Recombinant purified NGL-1 and NGL-1⁴³ were
dialysed for 16 h at 4 °C against the kinase buffer used in the phosphorylation
assay.

**In vitro phosphorylation assays.** HEK293T cells were transfected with 6-
Myc–CDK5 and 6-Myc plasmids. At 48 h post-transfection, transfected cells
were lysed in 50 mM Tris–HCl, at pH 8.0, 500 mM NaCl, 0.1% NP-40, 1 mM
dithiothreitol, phenylmethyl sulphonyl fluoride and protease and phosphatase
inhibitors (Sigma). Equal amounts of protein extracts were pre-cleared for 1 h with
40 μl of 100% mouse IgG–agarose beads at 4 °C, 40 μl of 100% agarose conjugate
anti-c-Myc (Sigma) was added to the extracts and the mixture was incubated for 2 h
at 4 °C. Immunocomplexes were collected by centrifugation, washed 5 times and
incubated with 1.5 μg of recombinant protein in a kinase buffer in the presence of
7 μCi of [γ⁻³²P]ATP and 25 μM of unlabelled ATP and incubated for 30 min at 30 °C.
The reaction was stopped by the addition of Laemmli buffer and the reaction mixture
was directly loaded onto a 15% SDS–PAGE; ³²P-labelled proteins were detected by
autoradiography.

**Pulldown.** Myc–CDK5 was first translated in *in vitro* using the TNT Quick
Coupled Transcription/Translation System (Promega) and then incubated with
tein-immobilized chitin–agarose beads or with hNGL-1–tein-immobilized chitin–agarose
beads for 4 h at 4 °C. Immunocomplexes were collected by centrifugation, washed three times with 750 mM NaCl, 20 mM Tris–HCl, at pH 8.0, 1
mM EDTA and 0.1% Triton X-100 with protease and phosphatase inhibitors
(Sigma), separated on SDS–PAGE and subjected to western blotting with anti-Myc
and anti-chitin-binding-domain antibodies.

**Lentivirus and retrovirus production.** VSVG-coated particles were packaged in
HEK293T cells.

**Synaptosome and PSD preparation.** The synaptosome fraction was prepared from
rat brains as described previously.⁴² PSD fractions were prepared from the
synaptosome fraction as described previously.⁴³

**Mutation search of NGL-1 and NGL-2.** For mutation search the single coding
exons of NGL-1 and NGL-2 together with their immediately flanking non-coding
regions were screened for mutations in a cohort of 51 patients who had been
diagnosed with RTT, infantile spasms or a variant of RTT, in whom no
locking or Phospho-specific antibodies were employed. In other cases immunohistochemistry and immunofluorescent
microscopy were used to verify the specificity of the antibodies. In the
last few years, several groups have developed algorithms to analyse
fluorescence microscopy images. These algorithms have been used to
measure the number of spines on dendritic protrusions and to
quantitate the proportions of spines with different morphologies.

**Figure legends.** In the figure legends, n refers to the number of neurons quantified. Statistical significance
was determined by Student’s t-test.
**Figure S1** CDKL5 protein expression pattern and localization in mouse brain. (a) Multiple-tissue western blot from a postnatal day (P) 21 mouse probed with an antibody against CDKL5. PSD95 was visualized for comparison. Actin was visualized for normalization. (b) Widespread distribution of CDKL5 protein in postnatal day (P) 21 mouse brain regions. Ctx, cortex; Hc, hippocampus; Cb, cerebellum. PSD95 was visualized for comparison. (c,d) Western blot analysis of CDKL5 protein expression levels in mouse brain tissues at different developmental stages. PSD95 was visualized for comparison. GAPDH was visualized for normalization. (e,f) CDKL5 protein level raises in primary hippocampal neurons cultured *in vitro* from DIV 7 to DIV 15. GAPDH was visualized for normalization.
Figure S2 Knock-down of CDKL5 levels using RNAi approach. (a) Total cell lysates of HEK293T cells transfected with the indicated plasmid were subjected to immunoblot analysis using Flag and GFP antibodies. GAPDH was visualized for normalization (***p<0.001, t test). (b) Western blot of endogenous CDKL5 levels in DIV 15 hippocampal neurons infected at DIV 7 with sh-vector, sh-control or sh-CDKL5#1. GAPDH was visualized for normalization (***p<0.001, t test). (c-h) CDKL5 immunostaining in DIV 15 primary hippocampal neurons transfected with sh-control or sh-CDKL5#1. (e,h) merged images. (i-o) Transient expression of the CDKL5 knock-down resistant form (CDKL5R). CDKL5R rescues the phenotype of CDKL5 knock-down neurons. (i) HEK293T cells were transfected with the indicated plasmids, recovered 48 h post-transfection and the expression levels of exogenously expressed GFP-CDKL5 were analyzed by immunoblotting with anti-GFP antibody. GAPDH was visualized for normalization. (j-l) Representative images of primary hippocampal neurons transfected at DIV 7 with the indicated plasmids and labeled for GFP at DIV 14. (m-o) Quantification of dendritic protrusion density (m), length (n) and head width (o) of sh-vector, sh-control and sh-CDKL5#1 transfected neurons. Bar graphs show mean ± s.e.m. n = 12 neurons for sh-vector, n = 16 neurons for sh-control and sh-CDKL5#1 (**p<0.01, *p<0.05, t test). n derived from three independent experiments. (p-q) Primary hippocampal neurons were transfected at DIV 7 with DsRed alone or in combination with GFP-CDKL5K42R and immunolabeled at DIV 14 for GFP and DsRed. q' shows higher magnification of q. Arrowheads in q' show a clear enrichment of the K42R immunostaining in dendritic spines. (r,s) Representative images of single dendrites of DsRed-positive primary hippocampal neurons expressing either, control or GFP-CDKL5K42R. (t,u) Quantification of dendritic protrusion density (t) and length (u) of DsRed or DsRed plus GFP-CDKL5K42R transfected neurons. Bar graphs show mean ± s.e.m. n = 15 neurons for each condition (**p<0.01, t test). n derived from four independent experiments. (v) Schematic representation of dendritic protrusion morphology categories. (w) Quantification of the mean percent of spines in each morphology category in neurons expressing DsRed or DsRed plus GFP-CDKL5K42R. Bar graphs show mean ± s.e.m. n = 15 neurons for each condition (**p<0.01, t test). n derived from four independent experiments. Scale bars: 10 µm (c-h, j-i, p, q), 5 µm (q').
**Figure S3** CDKL5 knock-down does not affect inhibitory synapse number. (a) Primary hippocampal neurons were transfected at DIV 7 with sh-control or sh-CDKL5#1 and immunostained at DIV 14 with anti VGAT and gephyrin antibodies. Bar graph shows mean ± s.e.m. \( n = 15 \) neurons for sh-control and sh-CDKL5#1. \( n \) derived from three independent experiments. (b–d) CDKL5 knock-down in DIV 15 cultured neurons did not reduce the frequency (c) nor the amplitude (d) of mIPSCs. \( n = 5 \) neurons for each condition. \( n \) derived from three independent experiments.
Figure S4 CDKL5 requires the catalytic domain for the interaction with NGL-1. Total cell lysates of HEK293T cells double transfected with Myc-NGL-1 plus GFP-CDKL5 (WT and mutants) were subjected to immunoprecipitation with monoclonal anti-GFP antibody. Immunoprecipitated proteins as well as 10% of the cell extracts were separated by electrophoresis and analyzed by immunoblotting using antibodies indicated on the left side of each panel.
Figure S5 NGL-1 S631A induces pre-synaptic differentiation. HEK293T cells transfected with GFPiresMyc (a,b), GFPiresMyc-NGL-1 (c,d) or GFPiresMyc-NGL-1 S631A (e,f), were co-cultured with hippocampal neurons (DIV 9-12) and stained for GFP and synaptophysin (Syn). Scale bars: 10 µm (a-f).
Figure S6  Generation and characterization of iPSC clones derived from CDXL5 patients. (a) IPS derived clones show complete silencing of the retroviral transgene and activation of the endogenous counterparts. (b) RT-PCR expression analysis of a set of genes associated with pluripotency, which are activated in aRTT iPSCs. (c) Representative images of cells from the three germ layers, endoderm (Sox17+), mesoderm (SMA) and ectoderm (TuJ1) induced by iPSC in vitro differentiation. (d) Histological sections of IPSC-derived teratomas, which display many differentiated areas with tissues of all the three germ layers. Scale bar: 100 µm (c, d).
**Figure S7** Generation and characterization of iPSC clones derived from CDKL5 patients. (a) Sanger sequence analysis of RT-PCR products amplified from iPSC clone derived mRNAs confirming the point mutations in the affected cell lines (aRTT). (b) Total cell lysates of either, HeLa cells, two different R59X CDKL5 mutant iPSC lines or primary hippocampal neurons were separated by gel electrophoresis and analyzed by immunoblotting using CDKL5 antibody. As expected, CDKL5 protein was not detected in the mutant iPSC lines. (c) Karyotype analysis of iPSC lines. All iPSC lines investigated had a normal karyotype in the majority of the cells except for line WT #34 that presented 49 chromosomes in about 20% of the mitotic figures (asterisks).
Figure S8 Full scans.
| Individuals | 46, XX (R50X) | 46, XX (L220P) |
|-------------|--------------|----------------|
| iPSC lines  | R50X #14     | R50X #20       | R50X #30 | WT #21 | WT #26 | L220P #38 | L220P #41 | WT #34 | WT #35 |
| Tri-lineage in vitro differentiation | X | X | X | X | X | X | X | X | X |
| Expression of viral genes | X | X | X | X | X | X | X | X | X |
| Teratoma assays | X | X | X | X | X | X | X | X | X |
| Karyotyping | X | X | X | X | X | X | X | X | X |
| Neuronal differentiation assay | X | X | X | X | X | X | X | X | X |
| Spine morphology | X | X | X | X | X | X | X | X | X |
| PSD95 puncta | X | X | X | X | X | X | X | X | X |
| VGLU1 puncta | X | X | X | X | X | X | X | X | X |

**Table S1** Table showing the iPS cell lines used in each experiment.