Short Communication

Phenotypic characterization of Cdkl5-knockdown neurons establishes elongated cilia as a functional assay for CDKL5 Deficiency Disorder

Alessia Di Nardo a,b,c, Alina Rühmkorf a,b, Patricia Award a, Ashton Brennecke c, Michela Fagiolini a,d, Mustafa Sahin a,b,*

a F.M. Kirby Neurobiology Center, Department of Neurology, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA
b Rosamund Stone Zander Translational Neuroscience Center, Boston Children’s Hospital, Boston, MA 02115, USA
c Genetic and Developmental Disorders Research Unit, Biogen 115 Broadway, Cambridge, MA 02142, USA
*d Present address.

ARTICLE INFO

Keywords:
CDKL5
Neurodevelopmental disorder
Seizure
Cilia
Autism

ABSTRACT

CDKL5 Deficiency Disorder (CDD) is a severe encephalopathy characterized by intractable epilepsy, infantile spasms, and cognitive disabilities. The detrimental CNS manifestations and lack of therapeutic interventions represent unmet needs, necessitating identification of CDD-dependent phenotypes for in vitro disease modeling and therapeutic testing. Here, we optimized a high-content assay to quantify cilia in CDKL5-deficient neurons. Our work shows that Cdkl5-knockdown neurons have elongated cilia and uncovers cilium lengthening in hippocampi of Cdkl5 knockout mice. Collectively, our findings identify cilia length alterations under CDKL5 activity loss in vitro and in vivo and reveal elongated cilia as a robust functional phenotype for CDD.

Although little is known regarding the downstream molecular mechanisms affected by CDKL5 gene loss, previous work has indicated an association between CDKL5 and primary cilia (hereafter cilia): i) the homologue of the human CDKL5 gene in Chlamydomonas, long-flagella (LF) gene LFS, encodes a kinase that regulates ciliary length (Tam et al., 2013); ii) in dividing cells CDKL5 localizes to the centrosome, and its loss is associated with defective centrosome functions (Barbiero et al., 2017); iii) the human CDKL5 kinase localizes to cilia and C. elegans mutants expressing disease-linked mutations showed elongated ciliary length (Canning et al., 2018); iv) the C. elegans CDKL-1 is required for the assembly of the proximal and distal segment of cilia (in press: Park et al., Curr Biology (2021)).

Cilia are evolutionarily conserved, microtubule-based membrane extensions protruding from the surface of cells that coordinate extra-cellular ligand-based signaling and cellular polarity (Lee and Gleeson, 2011). Most ciliopathies are characterized by cilia shortening, resulting in defective ciliary signaling and aberrant neuronal development (Park et al., 2019). Furthermore, altered cilia gene expression has been found as a convergent risk factor for neurological and psychiatric disorders (Migliavacca et al., 2015; Di Nardo et al., 2020a). Cilia lengthening has
CDD have elongated primary cilia and establishes a high-throughput with oxidative stress response and alteration in the Akt/mTOR signaling. mouse brains. Loss of CDKL5 mals were raised and bred in house on a 12 h light/dark cycle with food Hospital Institutional Animal Care and Use Committee (IACUC). Ani drug discovery and therapeutic testing.

Together, our work uncovers that Cdkl5-knockdown neurons have longer cilia and consistent with our model, elongated cilia were also identified in vivo in Cdkl5 KO mouse brains. Loss of Cdkl5 in rat hippocampal neurons was associated with oxidative stress response and alteration in the Akt/mTOR signaling. Together, our work uncovers that in vitro and in vivo rodent models of CDD have elongated primary cilia and establishes a high-throughput cilia length-based platform that could be used in functional assays for drug discovery and therapeutic testing.

All procedures and animal care were approved by Boston Children’s Hospital Institutional Animal Care and Use Committee (IACUC). Animals were raised and bred in house on a 12 h light/dark cycle with food and water ad libitum. To generate experimental animals, heterozygous CDKL5+/− female mice (Wang et al., 2012; JAX#021967) were mated with C57BL/6 J males. Controls were sex- and age-matched littermates. Rat hippocampal neurons were cultured as previously described (Di Nardo et al., 2020a). Lentiviral stocks were prepared as previously described (Di Nardo et al., 2020a, Di Nardo et al., 2020b). Lentiviral stocks were prepared as previously described (Di Nardo et al., 2020a). Control shRNA construct against the luciferase gene (ctrl-sh) was previously prepared (Di Nardo et al., 2009). Cdkl5-sh RNA was from Sigma cat. No.: TRCN0000023097.

Mice were transcardially perfused with saline, followed by phosphate-buffered 4 % paraformaldehyde solution (PFA). Brains were dissected, post-fixed in PFA overnight at 4 °C, cryoprotected in a series of sucrose solutions (10, 20 and 30 %) and frozen in O.C.T. Tissue-Tek (VWR). Coronal slices (40um thick) through the forebrain were sectioned on a cryostat (Leica Microsystems). Brain sections were washed 4 times with Tris Buffered Saline pH 7.4 (TBS), mounted on superfrrost slides and dried overnight. The next day, sections were incubated for 2 h at room temperature in blocking buffer (5 %BSA, 0.1 % Triton X-100, 10 % goat serum). Primary antibodies were incubated in 1 % BSA, 0.1 % Triton X-100, at 4 °C for overnight. The day after, sections were washed in TBS buffer followed by incubation with fluorochrome-coupled secondary antibody. Imaging of the Cdkl5 control and mutant hippocampi was performed by imaging 6–9 random regions in the CA1 of the hippocampus. The average percentage of neurons with cilia (NeuN+/ACIII+ ) was calculated for each of the images. Cilia length measurements were performed by tracing the ACIII stained cilia using the ImageJ software freehand tool. A threshold for cilia count was set such that only ACIII positive objects that measured longer than 1μm were counted as cilia. All the imaging and the quantification were done in a blinded way. Confocal images were acquired with a Nikon Ultraview Vox Spinning Disk Confocal microscope using 63x oil-immersion objective equipped with Hamamatsu camera.

Protein extracts were prepared as previously described (Di Nardo et al., 2020a). The following antibodies were used: Cdkl5 (Sigma, cat. HPA002847), Cdkl5 (Proteintech cat. 12973-1-AP), p-EB2 S222 (Covab-lab, cat. pab0132-P), EB2 (Abcam cat. ab4576), GAPDH (Ambion cat. AM4300), β-actin (Cell Signaling, cat. 3700S), pS6 (Cell Signaling cat. 5364), S6 (Santa Cruz cat. sc-74459), p-Akt Ser473 (Cell Signaling cat. 4060), Akt (Cell Signaling cat. 4691), p-GSK Ser9 (Cell Signaling cat. 5558), GSK (Cell Signaling cat. 2456), HO-1 (Proteintech cat. 10701-1-AP), ACIII (Proteintech cat. 19492-1-AP), Arl13b (Proteintech cat. CL488-17711), NeuN (Millipore cat. MAB377), Cdkl5 (Proteintech cat. HCA), β-Tubulin III (Sigma cat. T7451), β-Tubulin III (Sigma cat. T8660), γ-tubulin (Mil- lipore cat. MAB 1864), GFP (Thermo Fisher Scientific cat. A10262), GLI1 (Abcam cat. ab273018), Smoothened (Invitrogen cat. PA5-113312), active (non-phospho) β-Catenin (Ser45) (Cell Signaling cat. 19807), Dvl2 (Cell Signaling cat. 32245). Western blot quantifications were performed by protein normalization using loading controls. Level of phosphorylated proteins was expressed as the ratio of phosphorylated/total level after loading control normalization. GraphPad PRISM was used for statistical quantifications. Significant different values were considered p < 0.05.

Imaging was performed using the ImageXpress Micro Confocal platform (IXM-C from Molecular Devices) available at the Human Neuroscience Research of the Translational Neuroscience Center (Boston Children’s Hospital). The DAPI channel was used for nuclei identification. When needed, focal planes were adjusted to the best optimal resolution for each channel. Once optimized, Z offsets were kept the same throughout the scans. Imaging of the ciliaHCA was done with a 40x
objective on forty-nine fields of view per well (18% of the well). The nuclei were detected by Hoechst staining at 386 nm emission for 50 ms, the LV-transduced neurons were detected using GFP staining at 485 nm emission for 25 ms, and cilia were detected using ACIII staining at 647 nm emission for 50 ms or using Arl13b staining at 647nm emission for 30 ms using a stack of images at three different focal planes for optimal cilia imaging with a step size of 1 μm/step. Object selection for GFP and ACIII spots identification were filtered using area and shape measurements. Data analysis was done using the MetaExpress Software. After back-ground removal, the cilia were detected using the area measurement as a filter for object identification. A subsequent mask was used to filter the cilia based on the identification of the objects with a minimum length of 1 μm. To identify the transduced neurons, we generated a mask using the GFP staining and used a co-localization module to identify the length of the cilia in the cell bodies of the GFP+ neurons. A threshold for cilia length was set such that only positive objects longer than 1 μm were counted as cilia. Additional cilia length cut-offs were set at 2, 3 and 4 μm.

As a CDD in vitro model, we used rat hippocampal neurons transduced with GFP tagged lentiviral vectors (LV) expressing a short hairpin RNA (shRNA) directed against either the Cdkl5 (Cdkl5-sh) or the...
Tuj1 (n control for protein level normalization. Quantifications are relative to ctrl-sh neurons (unpaired Student’s t test) 

**Fig. 1.** Effects of CDKL5 gene silencing on mTOR activation in cultured neurons. 

Consistent with these findings, we found that Cdkl5-sh knockdown neurons display no change in tubulin expression and post-translational modification. 

**Fig. 3.** Cdkl5 knockdown neurons display no change in tubulin expression and post-translational modification.
(A) Representative western blot of ctrl-sh and Cdkl5-sh lentiviral vectors in a time course experiment at DIV5, 7, 13 and 15. Quantification of γ-Tubulin (n = 3, in (B), Tuj1 (n = 4, in C), Acetylated tubulin (n = 4, in D), and Tyrosinated tubulin (E n = 3). Data are average fold changes of ctrl-sh neurons. GAPDH was used as loading control for protein level normalization. Quantifications are relative to ctrl-sh neurons (unpaired Student’s t test, n.s. not significant, error bars indicate ± SEM).
Fig. 4. Cdkl5 mice have increased cilia length but no changes in components of Wnt and sonic hedgehog signaling pathway.

A. Representative confocal images of the hippocampal CA1 region of WT and Cdkl5 KO mice at P15 stained with the cilia marker ACIII (in red a–h; in greyscale i–l), with the neuronal marker NeuN (in green a–h) and with the nuclei marker Hoechst (in blue a–h). White boxes in a–d represent the regions for zoom in e–l. Scale bar is 10 μm. B. Cilia Length quantification. Data are average cilia length (n = 500 average cilia/mouse * p < 0.05 n = 4 mice/genotype).

C. Representative western blot of protein lysates from brains of control and Cdkl5 KO mice. Quantification of Dvl2 (E n = 5), active β-catenin (non-phosphorylated at S45) (F n = 5), p-GSK (G n = 5), GLI1 (H n = 5), Smo (I n = 5). Loss of Cdkl5 expression and function is shown by reduced Cdkl5 protein level (D n = 5) and EB2 phosphorylation (J n = 5). β-actin was used as loading control for protein level normalization. Phospho-antibodies levels were quantified as the ratio to their respective total antibody after protein normalization to β-actin. Quantifications are relative to ctrl-sh neurons (n = 5 unpaired Student’s t test, ****p < 0.00001, ns = not significant). Error bars indicate ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the Wnt signaling pathway, including the Wnt intermediate Dishevelled (Dvl2), downstream target GSK, and active β-catenin (non-phosphorylated at Ser45) (Fig. 4C, E-G). We did not observe changes in components of the sonic hedgehog pathway, including the transcriptional activator GLI1 and G protein-coupled receptor smoothened (Smo) (Fig. 4C, H-I). Although these data show that Cdkl5-deficient cilia does not affect the expression of these factors, our results do not exclude alterations in their localization or responsiveness to these intracellular signaling mechanisms, as previously reported for other kinases also implicated in cilia-lengthening (Chaya et al., 2014). Future studies are necessary to identify the mechanistic links between Cdkl5 loss, lengthening of cilia, and neuronal dysfunction leading to the neurological symptoms observed in CDD.

Future studies addressing the interplay between cilia assembly and neuronal activity will help elucidate whether defective cilia length might underlie some of the neuronal alterations seen in these epilepsy disorders. High-throughput functional assays represent powerful preclinical approaches to monitor efficacy of drugs screens and gene therapy. The cilia-length based high content assay we have developed represents a valuable platform for unbiased and systematic drug testing in preclinical models of CDD. Finally, given the role of cilia in neuronal function, the establishment of high-content cilia length-based assay in Cdkl5-deficient neurons might also have broader implications in understanding the role of elongated cilia in the neuronal phenotypes associated with CDD.

Declaration of Competing Interest

Mustafa Sahin reports grant support from Novartis, Biogen, Astellas, Aeovian, Bridgebio, and Auta. He has served on Scientific Advisory Boards for Novartis, Roche, Regenbio, and Alkermes.

Acknowledgements

The authors thank the Assistant Director of the Screening Core Facility at the Human Neon Cell Core of the Translational Neuroscience Center (Boston Children’s Hospital) Lee Barrett for conceptual guidance and methodology support for the cilia high-content imaging. The authors thank Drs. Kellen Winden and Nickesha Anderson for constructive discussions on the project. This study was supported by the Loulou Foundation, Orphan Disease Center, administered by the University of Pennsylvania’s Perelman School of Medicine. M.S. is supported by grant funding from the National Institutes of Health (NIH R01NS113591).

References

Abroux, D.N., Koehl, M., Le Moal, M., 2005. Adult neurogenesis: from precursors to network and physiology. Physiol. Rev. 85, 523–569.

Amendola, E., Zhan, Y., Mattucci, C., Castroflorio, E., Calcagno, E., Fuchs, C., Lonetti, G., Foundation, Orphan Disease Center, administered by the University of

Barbiero, I., Valente, D., Chandola, C., Magi, F., Bergo, A., Montonenfo, L., Trambarin, M., Fazzari, M., Sodd, L., Landsberger, N., Rinaldo, C., Kilstrup-

Baltussen, L.L., Negraes, P.D., Silvestre, M., Claxton, S., Moeskops, M., Christodoulou, E., Aeovian, Bridgebio, and Aucta. He has served on Scientific Advisory

Bishop, G.A., Berbari, N.F., Lewis, J., Mykytyn, K., 2007. Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain. J. Comp. Neurol. 505, 571–587.

Bork, K., Park, K., Goncalves, J., Li, C., Howard, C.J., Sharp, T.D., Holt, L.J., Pelletier, L., Bullock, A.N., Leroux, M.R., 2018. CDKL5 family kinases have evolved distinct structural features and ciliary function. Cell Rep. 22, 685–694.

Bovolenta, P., Kovalchuk, Y., Furukawa, T., 2014. Cdk5 is essential for cell type-specific ciliogenesis and the regulation of ciliary transport. EMBO J. 33, 1227–1242.

Briskin, B., Liu, P., Chauliouni, C., Komures, L.G., Hass, P.E., Sandoval, W., Peterson, A.S., 2012. A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain. Nat. Cell. Biol. 14, 61–72.

Di Nardo, A., Kramvis, I., Cho, N., Sadowski, A., Meikle, L., Kwiatkowski, D.J., Sahin, M., 2009. Tuberosus sclerosis complex activity is required to control neuronal stress responses in an mTOR-dependent manner. J. Neurosci. 29, 5926–5937.

Di Nardo, A., Lenoo, I., Vanden, K.D., Ruhmkorff, A., Mod, M.E., Barrett, L., Erkan-

Enver, J., Lehn, M.W., 2012. Ciliopathies: the central role of cilia in a spectrum of pediatric disorders. J. Pediatr. 160, 366–371.

Gerdes, J.M., Davis, E.E., Katsanis, N., 2009. The vertebrate primary cilium in development, homeostasis, and disease. Cell 137, 32–45.

Hector, R.D., Kalscheuer, V.N., Hennig, F., Leonard, H., Downs, J., Clarke, A., Benke, T. A., Armstrong, J., Pineda, M., Bailey, M.E.S., Cobb, S.R., 2017. Cdkl5 variants: improving our understanding of a rare neurologic disorder. Neurol. Genet. 3, e200.

Kim, H.S., Nicas, C., Ruscioni, C., La Montanara, P., Ciceri, D., Berg, A., Bedogni, F., Landsberger, N., 2012. What we know and what we would like to know about Cdkl5 and its involvement in epileptic encephalopathy. Neuropl. 2012, 728367.

Lee, J.E., Gleenon, J.G., 2011. Cilia in the nervous system: linking cilia function and neurodevelopmental disorders. Curr. Opin. Neuro. 24, 98–105.

Migliavacca, E., Golzio, C., Mannik, K., Blumenthal, I., Oh, E.C., Harewood, L., Kosnicki, J.A., Loviglio, M.N., Giannuzzi, G., Hippiolyte, D., Maillard, A.M., Alfai, A. A., Van Haest, M.M., Andrieux, J., Gusella, J.P., Daly, M.J., Beckman, J.S., Jacobsson, S., Talkowski, M.E., Katsanis, N., Seymour, A., 2015. A potential contributory role for ciliary dysfunction in the 16p11.2 600 kb BP4-BP5 pathway. Am. J. Hum. Genet. 96, 784–796.

Munoz, L.M., Morgan, M.E., Pellet, J., Weiland, F., Gregorczyk, M., Brown, F.C., Macartney, T., Toth, R., Trost, M., Rose, J., 2018. Phosphoproteomic screening identifies physiological substrates of the Cdkl5 kinase. EMBO J. 37.

Olton, H.E., Demarent, S.T., Pestana-Knight, E.M., Swanson, L.C., Iqbal, S., Lal, D., Leonard, C., Cross, J.H., Devinsky, O., Benke, T.A., 2019. Cylvka-dependent kinase-like 5 deficiency disorder: clinical case. Clin. Pediatr. Neurol. 19, 25–42.

Omori, Y., Chaya, T., Katoh, K., Kajimura, N., Sato, S., Murakoa, K., Ueno, S., Koyasu, T., Kondo, M., Furukawa, T., 2010. Negative regulation of ciliary length by ciliary male germ cell-associated kinase (Mak) is required for retinal photoreceptor survival. Proc. Natl. Acad. Sci. U. S. A. 107, 22671–22676.

Park, S.M., Jang, H.J., Lee, J.H., 2019. Roles of primary cilia in the developing brain. Front. Cell. Neurosci. 13, 218.

Ruscioni, L., Salvatoni, L., Giudici, L., Bertani, I., Kilstrop-Nielsen, C., Broccoli, V., Landsberger, N., 2008. Cdkl5 expression is modulated during neuronal development and its subcellular distribution is tightly regulated by the C-terminal tail. J. Biol. Chem. 283, 30101–30111.

Srivastava, S., Ramsbottom, S.A., Molinari, E., Alkanderi, S., Filby, A., White, K., Henry, C., Saunter, S., Miles, C.G., Sayar, J.A., 2017. A human patient-derived cellular model of Joubert syndrome reveals cilia defects which can be rescued with targeted therapies. Hum. Mol. Genet. 26, 4657–4667.

Sperka, A., Chen, X., 2018. Neuronal and astrocytic primary cilia in the mature brain. Pharmacol. Res. 137, 114–121.

Tam, L.W., Ranum, P.T., Lefebvre, P.A., 2013. CDKL5 regulates flagellar length and localizes to the base of the flagellum in Chlamydomonas. Mol. Biol. Cell 24, 588–600.

Wang, I.T., Allen, M., Goffin, D., Zhu, X., Fairless, A.H., Brodkin, E.S., Siegel, S.I., Marsh, E.D., Blendy, J.A., Zhou, Z., 2012. Loss of CDKL5 disrupts kinome profile and event-related potentials leading to autistic-like phenotypes in mice. Proc. Natl. Acad. Sci. U. S. A. 109, 21516–21521.

Wang, E.J., Gailey, C.D., Bausigian, D.L., Fu, Z., 2020. Functional alterations in ciliogenesis-associated kinase 1 (CILK1) that result from mutations linked to juvenile myoclonic epilepsy. Cells 9.

Wang, H.T., Zhu, Z.A., Li, Y.Y., Lou, S.S., Yang, G., Feng, X., Xu, W., Huang, Z.L., Cheng, X., Xiong, Z.Q., 2021. CDKL5 deficiency in forebrain glutamatergic neurons results in recurrent spontaneous seizures. Epilepsia 62, 517–528.

Wernier, S., Pimenta-Marques, A., Bettencourt-Dias, M., 2017. Maintaining centrosomes and cilia. J. Cell. Sci. 120, 3789–3800.