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MINI-REVIEW

Molecular and genetic insights into an infantile epileptic encephalopathy – CDKL5 disorder

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BACKGROUND: The discovery that mutations in cyclin-dependent kinase-like 5 (*CDKL5*) gene are associated with infantile epileptic encephalopathy has stimulated world-wide research effort to understand the molecular and genetic basis of CDKL5 disorder. Given the large number of literature published thus far, this review aims to summarize current genetic studies, draw a consensus on proposed molecular functions, and point to gaps of knowledge in CDKL5 research.
METHODS: A systematic review process was conducted using the PubMed search engine focusing on CDKL5 studies in the recent ten years. We analyzed these publications and summarized the findings into four sections: genetic studies, CDKL5 expression patterns, molecular functions, and animal models. We also discussed challenges and future directions in each section.
RESULTS: On the clinical side, CDKL5 disorder is characterized by early onset epileptic seizures, intellectual disability, and stereotypical behaviors. On the research side, a series of molecular and genetic studies in human patients, cell cultures and animal models have established the causality of CDKL5 to the infantile epileptic encephalopathy, and pointed to a key role for CDKL5 in regulating neuronal function in the brain. Mouse models of CDKL5 disorder have also been developed, and notably, manifest behavioral phenotypes, mimicking numerous clinical symptoms of CDKL5 disorder and advancing CDKL5 research to the preclinical stage.
CONCLUSIONS: Given what we have learned thus far, future identification of robust, quantitative, and sensitive outcome measures would be the key in animal model studies, particularly in heterozygous females. In the meantime, molecular and cellular studies of CDKL5 should focus on mechanism-based investigation and aim to uncover druggable targets that offer the potential to rescue or ameliorate CDKL5 disorder-related phenotypes.

Keywords CDKL5 disorder, childhood epilepsy, intellectual disability, mouse model, outcome measure

Cyclin-dependent Kinase Like 5 (CDKL5) (also named *Serine-Threonine Kinase 9, STK9*) was first cloned in 1998 in a positional cloning effort to identify disease genes linked to Xp22 (Montini et al., 1998). In this study, CDKL5 protein was found to carry a putative cyclin-dependent serine-threonine kinase domain in its N terminus. It was not until 2003 when a link between *CDKL5* mutations and disease was described in a case report for two patients with X-linked infantile spasms and mental retardation (Kalscheuer et al., 2003). One year later, two more studies reported that mutations in *CDKL5* are common to the early-onset seizure variant of Rett syndrome (Tao et al., 2004; Weaving et al., 2004).

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Genetic studies

Historically, patients with CDKL5 disorder have been classified as early-onset seizure variant or Hanefeld variant of Rett syndrome, largely because these patients develop clinical symptoms similar to Rett syndrome, such as the stereotypical hand movement, hypotonia, developmental delay, breathing abnormality and intellectual disability (Hanefeld, 1985). However, systematic analysis of clinical symptoms across nearly 100 patients has led to a conclusion that CDKL5 disorder is rather a distinct clinical entity with

early-onset epileptic seizures as the flagship feature (Fehr et al., 2012). Further, it is well-described that Rett syndrome is largely caused by mutations in the X-linked gene, methyl-CpG binding protein 2 (*MECP2*), and is characterized by the regressive loss of learned language and motor skills after a relatively normal early development (Chahrour and Zoghbi, 2007). Thus, the different genetic etiology also supports Rett syndrome and CDKL5 disorder are two distinct disorders.

Since the 2004 genetic discovery, molecular genetic diagnosis has identified nearly 200 patients carrying mutations in *CDKL5* (Fehr et al., 2016). The types of mutations include translocations, deletions, insertions, nonsense and missense mutations, and the majority of *CDKL5* mutations are located within the N-terminal kinase domain, suggesting that the kinase function of CDKL5 is particularly important for brain function (Bahi-Buisson et al., 2008, 2012; Fehr et al., 2016). Moreover, genotype-phenotype correlation study has found mutations in the N-terminal kinase domain are associated with more severe clinical symptoms than mutations in the C terminus (Fehr et al., 2016). Furthermore, it becomes clear that mutations in *CDKL5* exons 19-21 are unlikely pathogenic, because the isoform of CDKL5 that predominantly expresses in the brain does not include exons 20-21 (Hector et al., 2016). Consistently, mutations in these regions have been identified in both patients, healthy siblings or parents, arguing against the clinical relevance of exons 19-21 (Diebold et al., 2014).

Remarkably, a small number of recurrent *CDKL5* mutations have also been identified, but they do not appear to cluster within a specific locus of the gene, except the protein kinase domain (Mastrangelo and Leuzzi, 2012). In addition, clinical phenotypes among patients with recurrent mutations are heterogeneous. For example, patients with the most common recurrent mutation, CDKL5 A40V, show a range of symptoms from severe to mild epileptic encephalopathy (Nemos et al., 2009; Bahi-Buisson et al., 2012). This is likely due to the fact that *CDKL5* is an X-linked gene and the majority of patients are heterozygous females. Due to X-chromosome random inactivation (XCI), patients carrying the same genetic mutation can have different mosaic expression patterns of CDKL5, thus resulting in a spectrum of phenotypes. However, this concept is difficult to prove in human patients as XCI is frequently measured from peripheral blood samples and it is difficult to determine whether the same XCI ratio is also present in the brain. Nevertheless, it is clear that boys carrying mutations in *CDKL5*, though much rare, show more severe epileptic encephalopathy than girls (Fehr et al., 2016), probably because of the expression of *CDKL5* mutation in every cell in males, but in half of the cells in females.

Expression patterns

When CDKL5 was first cloned and sequenced in 1998, Northern blot analysis demonstrated that CDKL5 mRNA is highly expressed in brain tissues, but also detected in liver, kidney and testes, though the size of mRNA appeared to be different in the testes (Montini et al., 1998). Since then, multiple studies have examined mRNA expression patterns of CDKL5 in heart, liver, spleen and lung tissues, but the conclusion remains inconsistent across studies (Lin et al., 2005; Chen et al., 2010). These studies do agree, however, that CDKL5 is highly expressed in the brain. Within the brain, several *in situ* hybridization studies indicate that it is enriched throughout the brain (Weaving et al., 2004; Mari et al., 2005; Chen et al., 2010), while other *in situ* data indicates that it is enriched specifically in the forebrain (Allen Brain Atlas (http://mouse.brain-map.org/experiment/show/740486).

At the protein level, the limitation of specific antibodies against CDKL5 has made a consensus difficult to draw across studies. Using Western blot where a protein band at the predicted molecular size of CDKL5 is missing in samples prepared from *Cdkl5* knockout mice, Wang and colleagues found that CDKL5 protein is highly enriched in forebrain structures, including the cortex, hippocampus, striatum and olfaction bulb, and low in midbrain and hindbrain structures (Wang et al., 2012). At the cellular level, immunostaining of primary neuronal cultures indicates that CDKL5 is expressed in both glutamatergic and GABAergic neurons, but not in glial cells (Rusconi et al., 2008, 2011), whereas a separate study has found a distinct CDKL5 isoform expressed in glia cells (Chen et al., 2010). Notably, a reliable and reproducible antibody that specifically detects CDKL5 in brain sections is currently lacking. Thus, the extent to which CDKL5 protein is expressed and localized *in vivo* in the brain requires further study.

Temporally, the exact time point when CDKL5 protein is expressed *in vivo* in mice remains inconsistent as well. Immunostaining of whole embryos shows broad CDKL5 expression in early embryogenesis (embryonic day 7.5 and 9.5) in one study (Lin et al., 2005), whereas a separate study found CDKL5 is barely detected at embryonic day 16.5 (Rusconi et al., 2008). Western blot analysis is similarly conflicted, showing the onset of CDKL5 expression late embryonically in one study (Chen et al., 2010), but postnatally in other studies (Rusconi et al., 2008; Zhu et al., 2013). However, multiple studies do agree that CDKL5 is detectable in adult animals, supporting a role for CDKL5 after the early stage of development. The inconsistencies related to the time course and distribution of CDKL5 expression are certainly attributed to ineffective antibodies and different experimental methods used in those studies. Thus, to definitively conclude the spatial and temporal expression patterns of CDKL5, a reproducible antibody against CDKL5 that specifically detects CDKL5 protein via immunostaining or immunohistochemistry is imperative at the current stage. Furthermore, the patterns of CDKL5 expression need to be examined in postmortem human tissues as well.

Molecular functions

Despite that the time course and expression patterns of CDKL5 are inconsistent across studies, several laboratories have attempted to characterize the molecular functions of CDKL5 through protein pull-down studies or yeast two-hybrid screens to identify CDKL5 interacting proteins and/or substrates. These studies have identified MeCP2, DNMT1, NGL1, Amphiphysin and Shootin1 as interacting proteins or kinase substrates of CDKL5 (Mari et al., 2005; Kameshita et al., 2008; Ricciardi et al., 2012; Sekiguchi et al., 2013; Nawaz et al., 2016). Mari et al. proposed that MeCP2 functions down-stream of CDKL5 as its kinase substrate, though the exact status of this molecular pathway remains to be further characterized (Mari et al., 2005; Lin et al., 2005). The function of CDKL5 in the phosphorylation of DNMT1 (Kameshita et al., 2008) and regulation of RNA splicing (Ricciardi et al., 2009) awaits further investigation as well for potential cellular phenotypes. Further, the extent to which perturbation of CDKL5-interacting proteins or downstream substrates would lead to functional phenotype in vivo in animal models remains to be determined.

In the meantime, several research groups have examined the function of CDKL5 in cultured primary neurons through shRNA-mediated knockdown of CDKL5 expression. Chen and colleagues found that knocking down CDKL5 expression reduces neuronal dendritic outgrowth and the density of spines (Chen et al., 2010). Importantly, *in utero* electroporation of the same shRNA construct not only reduced neuronal outgrowth *in vivo*, but also delayed neuronal migration. These authors showed evidence that CDKL5 may regulate neuronal outgrowth and migration by forming a complex with Rac1, a known regulator of actin dynamics and neuronal morphology, thereby mediating BDNF-dependent Rac1 signaling (Chen et al., 2010).

However, a separate study has challenged the role of CDKL5 in dendritic outgrowth. Ricciardi and colleagues employed the same knockdown approach with *in utero* electroporation and transfection in cultured primary neurons, and found that dendritic outgrowth is not altered (Ricciardi et al., 2012). Instead, these authors found the spine development is altered by CDKL5 knockdown. Instead of loss of spine density, they found an increase in spine density but at an immature status. Moreover, they described that CDKL5 knockdown reduced both the amplitude and frequency of mEPSCs, pointing to a role for CDKL5 in spine or synapse maturation. Furthermore, they provided immunostaining data showing that CDKL5 is localized exclusively to excitatory synapses and not to inhibitory synapses, indicating that loss of CDKL5 impairs excitatory synapse development and results in the formation of more immature-type dendritic spines. These authors also provided evidence supporting a mechanism by which CDKL5 directly phosphorylates NGL-1, a netrin-G1 receptor that regulates early synapse formation and maturation, and thus promotes an NGL-1-PSD95 interaction in order to stabilize dendritic spines (Ricciardi et al., 2012).

In contrast to NGL-1-PSD95 recruitment model, another study found that CDKL5 binds directly to palmitoylated PSD95, and that it is the CDKL5-PSD95 direct interaction recruiting CDKL5 to excitatory spines (Zhu et al., 2013). Moreover, while the authors of this study used the same method of shRNA-mediated knockdown of CDKL5 and were able to replicate the decrease in excitatory synapse number and reduction in mEPSC frequency identified by Ricciardi and colleagues (Ricciardi et al., 2012), they found a decrease, rather than no change, in spine density and width, and suggested a disease mechanism by which loss of CDKL5 inhibits both spine formation and growth (Zhu et al., 2013). Given previous observation that shRNA-mediated knockdown carries the risk of introducing off-target effects, particularly in experiments related to dendritic morphogenesis and spine formation (Baek et al., 2014), the extent to which these cellular mechanisms and functions are relevant and reproducible *in vivo* in animal models demands further investigation.

Animal models

Despite clinical genetic studies linking mutations in *CDKL5* to infantile epileptic encephalopathy, the causality of *CDKL5* mutations to this clinical entity was not established until the development and characterization of the first knockout mouse model of CDKL5 in 2012 (Wang et al., 2012). In this mouse model, exon 6 of mouse *Cdkl5* was deleted, mimicking a splice site mutation leading to the skipping of human *CDKL5* exon 6 (previously numbered as exon 7) in a CDKL5 patient (Archer 2006; Hector et al., 2016). Because of the exon deletion/skipping, resulting reading frame shift leads to an early truncation of CDKL5 in its N-terminal kinase domain, thereby disrupting CDKL5 kinase activity. Importantly, in this mouse model, truncated or full-length CDKL5 protein are not detected by Western blot using antibodies directed against the N- or C-terminal domains of CDKL5, and *Cdkl5* transcript levels are dramatically reduced, likely due to nonsense-mediated mRNA decay. Thus, this model represents a loss-of-function of CDKL5 and has been named *Cdkl5* knockout (KO) (Wang et al., 2012).

The authors found that *Cdkl5* KO mice are viable and fertile without any gross anatomical abnormalities. Behavioral characterization, however, showed that CDKL5 KO mice mirror multiple symptoms associated with CDKL5 disorder, including impaired motor control on the rotarod, deficits in learning and memory on a fear conditioning assay, and hindlimb clasping, a phenotype believed to be related to deficits in motor control. *Cdkl5* KO animals also demonstrate autistic-like deficits in sociability on a three-chambered social approach assay and in home-cage nesting behavior (Wang et al., 2012). Given the prominence of motor impairment,

intellectual disability, and autism in CDKL5 disorder, these data support the face validity of the *Cdkl5* KO mouse.

Wang and colleagues also found delayed and attenuated auditory-evoked event-related potential (ERP) responses in *Cdkl5* KO mice, suggesting impairments in the strength and timing of cognitive processing. Through an unbiased kinome study, they further found many signaling transduction pathways are disrupted in forebrain regions of *Cdkl5* KO mice (Wang et al., 2012). Interestingly, many of the pathways converge to PTEN-related signaling. Interestingly, multiple signaling pathways reported in this study, including the Akt-mTOR pathway, are altered in other neurodevelopmental disorders, such as autism, Rett syndrome, Fragile X syndrome, and Tuberous Sclerosis, and have been targeted for therapeutic development (Crino 2011). It remains unclear, however, the identified signaling changes are the primary or secondary effects of *Cdkl5* loss-of-function at this point.

Since then, a similar CDKL5 mouse model, deleting exon 4 of *Cdkl5*, was developed and characterized (Amendola et al., 2014). These mice showed deficits in the Akt-S6 and Akt-GSK3b signaling pathways (Amendola et al., 2014; Fuchs et al., 2014, 2015), supporting a convergent deficit in PTEN signaling (Wang et al., 2012). In addition, these mice demonstrate numerous phenotypes consistent with the above mouse model, including hindlimb clasping, impaired working memory, and deficits in visual-evoked potentials (VEPs) (Amendola et al., 2014; Fuchs et al., 2014, 2015). Deficits in neuronal outgrowth and postmitotic cell proliferation are also identified in these mice. Moreover, neuronal tracing of GFP-labeled cells in adult *Cdkl5* KO mice showed reduction in dendritic length of both cortical layer 5 and hippocampal pyramidal neurons (Amendola et al., 2014), and an age-dependent instability of spine formation is also described (Della Sala et al., 2016). These findings are consistent with previous cellular phenotypes identified in cultured neurons to a large extent (Ricciardi et al., 2012), but the discrepancies likely reflect the differences in cell types, developmental stages, and analytical methods used in those studies. Together, these cellular phenotypes offer the potential as cellular outcome measures for compound screening to rescue CDKL5 function and in preclinical studies of CDKL5 animal models.

Surprisingly, despite extensive video-EEG monitoring, neither *Cdkl5* KO mouse models developed spontaneous seizures in both *C57BL/6* and mixed *C57BL/6* with *DBA/2J* genetic backgrounds (Wang et al., 2012; Amendola et al., 2014). Although *Cdkl5* KO mice showed altered EEG patterns following seizure induction (Amendola et al., 2014), the absence of this key phenotype has raised questions concerning the differences between human and mouse and challenged the usefulness of the constitutive KO mouse model. Therefore, additional CDKL5 mouse models, with conditional or mosaic loss of CDKL5 function, that recapitulate the seizure phenotype will be necessary to understand mechanisms underlying the epileptic seizures in CDKL5 patients. Furthermore, given that CDKL5 disorder occurs primarily in heterozygous females with mosaic CDKL5 expression, future molecular, cellular and behavioral studies of animal models of CDKL5 need to focus on heterozygous female mice. Experimentally, it remains as a technical challenge to gain a mechanistic insight into CDKL5 function in heterozygous females because of the lack of reagents and tools to distinguish CDKL5 wild-type-expressing cells from mutant-expressing cells in a mosaic brain. However, the advances in mouse genetics and new developments in labeling cell types should make this study within reach.

Conclusions

In summary, a large array of molecular and genetic studies in human patients, cell cultures and animal models have established the causality of CDKL5 to the infantile epileptic encephalopathy, pointed to a key role for CDKL5 in regulating neuronal function in the brain, and revealed the potential to further characterize CDKL5 function in both neuronal cultures and mouse models. The successfully development and characterization of a mouse model recapitulating a CDKL5 patient mutation, exhibiting clinically relevant behavioral phenotypes, and showing neural circuit deficits and disruptions in multiple signaling pathways (Wang et al., 2012), have advanced CDKL5 research to the preclinical stage. Thus, future identification of robust, quantitative, and sensitive outcome measures would be the key in animal model studies. In the meantime, molecular and cellular studies of CDKL5 should focus on mechanism-based investigation and aim to uncover druggable targets that offer the potential to rescue or ameliorate CDKL5 disorder-related phenotypes, and ultimately bring versatile therapeutic strategies to preclinical and clinical trials.

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Compliance with ethics guidelines

A.Z., S.H. and Z.Z. declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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