Role of calcium/calmodulin-dependent kinase 2 in neurodevelopmental disorders

Martina Proietti Onori\textsuperscript{a,b}, Geeske M. van Woerden\textsuperscript{a,b,*}

\textsuperscript{a} Department of Neuroscience, Erasmus MC, Rotterdam, 3015 GD, the Netherlands
\textsuperscript{b} The ENCORE Expertise Center for Neurodevelopmental Disorders, Erasmus MC, Rotterdam, 3015 GD, the Netherlands

\textbf{ARTICLE INFO}

Keywords:
CAMK2
Intellectual disability
de novo variants
Plasticity

\textbf{ABSTRACT}

Neurodevelopmental disorders are a complex and heterogeneous group of neurological disorders characterized by their early-onset and estimated to affect more than 3\% of children worldwide. The rapid advancement of sequencing technologies in the past years allowed the identification of hundreds of variants in several different genes causing neurodevelopmental disorders. Between those, new variants in the Calcium/calmodulin dependent protein kinase II (CAMK2) genes were recently linked to intellectual disability. Despite many years of research on CAMK2, this proves for the first time that this well-known and highly conserved molecule plays an important role in the human brain. In this review, we give an overview of the identified CAMK2 variants, and we speculate on potential mechanisms through which dysfunctions in CAMK2 result in neurodevelopmental disorders. Additionally, we discuss how the identification of CAMK2 variants might result in new exciting discoveries regarding the function of CAMK2 in the human brain.

\section{Introduction}

Neurodevelopmental disorders (NDDs) are early onset disorders that result from abnormal brain development, caused by a known (genetic or environmental) cause, or with a yet unidentified etiology (Sherr, 2016). They encompass a broad clinical spectrum, including autism spectrum disorder, attention deficit disorder and intellectual disability (ID), with often multiple clinical symptoms (co-morbidity) seen together. ID is one of the most common early onset NDDs with a prevalence in the general population of 1–3\%, characterized by a general limitation in intellectual functioning (IQ < 70) (Maulik et al., 2011; McKenzie et al., 2016). Its severity is highly variable, which is reflected by a wide genetic heterogeneity (Mefford et al., 2012; Mir and Kuchay, 2019). After the first pilot study in 2010 (Vissers et al., 2010), trio-based exome sequencing is now one of the primary methods to identify new de novo genetic variants as possible causes for sporadic ID. Whereas initially 13–35 \% of the most severe ID cases were shown to be caused by de novo variants in known ID genes (De Ligt et al., 2012; Rauch et al., 2012), this number rapidly increased to 60 \%, following additional Whole Genome Sequencing (WGS) studies (Gilissen et al., 2014). Currently, more than 700 genes (including X-linked, autosomal dominant and recessive genes) are linked to ID and ID-associated disorders (Mir and Kuchay, 2019; Vissers et al., 2016).

Despite the clinical diversity seen in NDDs, the underlying molecular pathways often overlap (Plummer et al., 2016). For example, one large group of genes identified as risk factors for several NDDs, including ID, encode postsynaptic density (PSD) proteins (Kaizuka and Takumi, 2018; Verpelli et al., 2013; Zoghbi and Bear, 2012). The PSD is part of the postsynaptic membrane, characterized by thousands of proteins densely packed together, including neurotransmitter receptors, channels, scaffolding and cytoskeleton proteins, cell adhesion molecules and signaling enzymes (Cheng et al., 2006; Trinidad et al., 2008; Ziff, 1997). The molecular composition of the PSD and its flexibility underlie the expression of neuronal synaptic plasticity (Meyer et al., 2014; Zeng et al., 2018). Hence it is not surprising that mutations in components of the PSD, for example in genes encoding neuraligins (NLGNs), SH3 and multiple ankyrin repeat domains (SHANKs), Glutamate Ionotropic Receptor NMDA Type Subunit 2B (GRIN2B) or Synaptic Ras GTPase Activating Protein 1 (SYNGAP1), have been associated with human neurological disorders (Bayès et al., 2011; Grant, 2012; Kaizuka and Takumi, 2018; Laumonnier et al., 2007; Zoghbi and Bear, 2012). These findings reveal the impact of synaptic changes on the genesis of neurodevelopmental pathologies, including ID. Surprisingly, one of the most abundant synaptic proteins, Calcium/calmodulin dependent protein

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kinase II (CAMK2), was never directly linked to neurological disorders, until a few years ago. In this review, we summarize the recent discoveries that link CAMK2 to ID and describe the role of CAMK2 in neurodevelopmental disorders, highlighting the importance of CAMK2 for human cognitive and adaptive functions.

2. CAMK2

2.1. Structure and regulation

CAMK2 is encoded by four distinct genes, known as CAMK2A, CAMK2B, CAMK2D and CAMK2G (Sloutsky et al., 2020; Tombes et al., 2003). Each CAMK2 subunit is composed of an N-terminal catalytic domain, a C-terminal hub domain and an autoregulatory domain in the middle. While these domains are highly homologous between the different paralogs, most of the variability lies in the linker region (also called the variable domain) connecting the autoregulatory domain to the hub domain (Bennett and Kennedy, 1987; Bulleit et al., 1988; Hanley et al., 1987; Lin et al., 1987). Much of the variability seen in the linker region results from alternative mRNA splicing. Indeed, already in the late 80 s - beginning 90 s, different CAMK2 transcripts originating from alternative splicing were identified in the rodent brain (Bennett and Kennedy, 1987; Brocke et al., 1995; Bulleit et al., 1988; Mayer et al., 1993; Miller et al., 1988; Schwerzer et al., 1993). Recently, more than 70 different CAMK2 transcripts expressed in the human hippocampus were found, also resulting from extensive alternative mRNA splicing mainly in the linker domain of the different paralogs (Sloutsky et al., 2020). These variations in the linker domain are regulated in a temporal manner (e.g. the embryonic CAMK2B isoform lacks the F-actin binding domain (Brocke et al., 1995) and spatial manner (e.g. the CAMK2A isoform containing a Nuclear Localization signal (NLS) is expressed mainly in midbrain and hindbrain, but far less in forebrain (Cook et al., 2018)). Additionally, different splice variants functionally impact CAMK2 by conferring specific properties to different isoforms, such as affinity for calcium/calmodulin (Sloutsky et al., 2020) and the balance between inhibitory and activating autophosphorylation (Bhattacharyya et al., 2020), but also the subcellular localization (Ma et al., 2014; Srinivasan et al., 1994; Takeuchi et al., 1999 and for review Sloutsky and Stratton, 2020). Single CAMK2 subunits form a unique homo- or heteromeric structure (holoenzyme) through interaction of the C-terminal hub domains of 12–14 subunits, which constitute the central core in a double ring shape; the N-terminal catalytic domains extend towards the outside in a “hub-and-spoke” fashion (Braun and Schulman, 1995; Chao et al., 2011; Kanaseki et al., 1991; Kołodziej et al., 2000; Morris and Török, 2001).

In the basal state, the autoinhibitory segment of each CAMK2 subunit is bound to the kinase domain, keeping the enzyme catalytically inactive (reviewed in Stratton et al., 2013). When the level of intracellular calcium rises, it binds to calmodulin upon which the calcium/calmodulin complex can bind the C-terminal end of the autoregulatory domain disrupting the position of the autoinhibitory segment (Lisman et al., 2002). As a consequence, a critical phosphorylation site now gets exposed, Thr286 for CAMK2A (287 for the other paralogs) and can be phosphorylated by an activated neighboring subunit in the holoenzyme (Hansson et al., 1994, 1989; Kuret and Schulman, 1985; Lai et al., 1986; Molloy and Kennedy, 1991). Autophosphorylation at this site increases the affinity of CAMK2A for calcium/calmodulin by 1000-fold resulting in the trapping of the calcium/calmodulin complex to the enzyme (Meyer et al., 1992). Even when calcium drops in the cell, the calcium/calmodulin complex remains trapped for several seconds, ensuring full catalytic activity of the kinase. After calcium/calmodulin dissociates from the kinase, the presence of a phosphate group on Thr286/287 ensures that the kinase remains partially active (autonomous state) until dephosphorylation (Lou et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986; Shields et al., 1985). Additionally, other sites within the calmodulin-binding domain now become available for phosphorylation (Threonines 305/306 or 306/307 for the other paralogs). Phosphorylation at these sites prevents calcium/calmodulin from re-binding to the kinase, causing insensitivity to subsequent increase in calcium level (Colbran and Soderling, 1990; Hanson and Schulman, 1992; Mukherji and Soderling, 1994; Patton et al., 1990). This regulatory mechanism is thought to have important implications for neuronal function, specifically in mediating relevant aspects of synaptic functions related to learning and plasticity (Coultrap and Bayer, 2012; Hudmon and Schulman, 2002). Besides these crucial well-studied residues on CAMK2, several other residues at different locations in CAMK2 have been shown to be of importance for the autoregulatory processes (Yang and Schulman, 1999).

2.2. Role of CAMK2 in plasticity, learning and memory

CAMK2 was originally purified from rat brain homogenates, with the initial characterization of the major brain paralogs alpha and beta (Bennett et al., 1983; Kennedy et al., 1983; Kennedy and Greengard, 1981; Lin et al., 1987). However, presence of CAMK2 is not exclusive to mammals but is conserved across the animal kingdom. Some species such as the fruit fly (Drosophila) and the nematode (C. elegans) have only one single CAMK2 ortholog, which is implicated in several behaviors (Tombes et al., 2003). In Drosophila, CAMK2 is enriched in the mushroom body memory center (Takamatsu et al., 2003). Inhibition of CAMK2 in this species is shown to cause synaptic defects as well as memory deficits in the courtship-conditioning behavior (Griffith et al., 1994, 1993). In C. elegans, the only orthologue of CAMK2, UNC-43, is shown to cause multiple defects in locomotion and in spontaneous activity when mutated (Reiner et al., 1999). Additionally, loss of UNC-43 results in absence of the AMPA-type glutamate receptor GLR-1 at synaptic sites, suggesting that UNC-43 regulates the density of central glutamatergic synapses in vivo (Rongo and Kaplan, 1999). In vertebrates, multiple copies of Camk2 are present, which likely emerged from gene duplication of the common ancestral gene (Tombes et al., 2003). The high abundance of CAMK2 in the brain, especially in dendritic spines, led to the assumption that this kinase might play a substantial role in neuronal activity and synaptic transmission also in mammals (Griffith, 2004). Over the years, we learnt from experimental in vitro and in vivo rodent studies that CAMK2A mediates synaptic plasticity by regulating some of the main components of the PSD through sequential steps upon influx of calcium. These mechanisms involve: 1) the rapid translocation of CAMK2A from the cytosol to the PSD (Shen and Meyer, 1999) where it binds the GluN2B subunit of the NMDA receptor (Leonard et al., 1999; Strack et al., 2000, 1997; Strack and Colbran, 1998), which leads to prolonged activation of the holoenzyme even in absence of calcium (Bayer et al., 2001; Pradeep et al., 2009); 2) the enhancement of the conductance of the channel AMPA receptor, responsible for basal synaptic transmission (Barria et al., 1997; Derkah et al., 2002) and 3) insertion of new AMPA receptors in the membrane (Hayashi et al., 2000). All of these steps and the molecules and channels involved, are crucial for regulating synaptic strength (Lisman et al., 2012). Of the 4 CAMK2 proteins present in the brain of vertebrates, CAMK2A is the most abundantly expressed in the hippocampus, the brain region known to be important in the acquisition of explicit memory. Therefore, maybe not surprisingly, the first knock-out mouse model generated in the field of learning and memory, was the Camk2a knockout mouse (Silva et al., 1992a,b). This CAMK2A-null mutant shows reduced hippocampal NMDA-dependent long-term potentiation (LTP) and impaired spatial learning in the Morris water maze, with normal gross brain morphology, proving the requirement of CAMK2A for hippocampal plasticity and learning (Elgersma et al., 2002; Silva et al., 1992a,b).

A more detailed understanding of the role of CAMK2A in these processes (its enzymatic as well as structural requirements) came from additional mouse models generated over time. The importance of tight regulation of the enzymatic activity of CAMK2A in LTP and learning was demonstrated using different knock-in and transgenic mouse models
As well, both at the presynapse (Hojjati et al., 2007) and at the post-synapse, where CAMK2 accounts for 2-6% of total protein and represents an important interaction partner for many scaffolding proteins (see below and reviewed by Hell, 2014). However, it should be noted that despite its long-considered important role in the organization of the PSD, simultaneous absence of CAMK2A and CAMK2B does not seem to affect the biochemical composition of the PSD (Kool et al., 2019).

CAMK2B is the second most highly expressed CAMK2 gene in the mammalian brain, sharing high homology with CAMK2A. Despite this high homology, differential effects on synaptic strength and dendritic arborization were shown, due to the CAMK2B-specific F-actin binding domain (Fink et al., 2003; Thiggarajan et al., 2002). Additionally, in contrast to CAMK2A, CAMK2B is expressed already prenatally, and shown to play a role in neurodevelopment, as prenatal deletion of CAMK2B disrupts neuronal migration (Küry et al., 2017; Nicole et al., 2018). Further evidence supporting an important and unique role for CAMK2B in learning and plasticity comes from CAMK2B knockout mice, which similar to CAMK2A knockout mice, exhibit impaired hippocampal LTP as well as hippocampus-dependent learning deficits (Borgesius et al., 2011). However, in contrast to CAMK2A, CAMK2B was shown to play a unique structural role in the hippocampus, regulating the location of CAMK2A through its F-actin binding domain (Borgesius et al., 2011; Shen et al., 1998; Shen and Meyer, 1999). Indeed, a knock-in mouse model carrying a point mutation in the calcium/calmodulin binding site (CAMK2Bp.Ala303Arg) which prevents enzymatic activation but preserves the actin-binding properties of CAMK2B, showed normal CAMK2A targeting to the synapse as well as normal hippocampal LTP and spatial learning (Borgesius et al., 2011). Besides these unique functions of CAMK2A and CAMK2B, evidence exists for functional redundancy. Whereas the enzymatic function of CAMK2A is crucial for hippocampal LTP, residual LTP is seen in the CAMK2A knockout (Borgesius et al., 2011; Elgersma et al., 2002), which is completely lost upon additional deletion of CAMK2B in the adult brain (Kool et al., 2019). Additionally, whereas CAMK2A mutant mice and CAMK2B mutant mice are viable, deletion or loss of enzymatic activity of CAMK2A and CAMK2B simultaneously results in premature death (Kool et al., 2019). This highlights the requirement of CAMK2 for some critical functions, which remain to be uncovered.

CAMK2G and CAMK2D are expressed at much lower levels in the brain compared to CAMK2A and B, and their precise roles in learning and plasticity largely remain unknown. Some of the splice variants of CAMK2G and CAMK2D contain an NLS (Brocke et al., 1995; Mayer et al., 1993; Tobimatsu et al., 1988) and these variants are shown to play an important role in excitation-transcription coupling (Ma et al., 2014; Shioda et al., 2015; Takeuchi et al., 2002). Absence of CAMK2G (using a CAMK2G knockout mouse) was shown to cause deficits in hippocampal learning and late-phase LTP, proving for the first time a unique function for CAMK2G in these processes (Cohen et al., 2018). Knockdown of hippocampal CAMK2D using antisense oligonucleotides indicated a role for this paralog in the formation of long-lasting memories (Zalcman et al., 2018).

Despite the vast amount of literature on the role of CAMK2 in different animal species, much less is known regarding its role in cognitive functions in humans. The first direct demonstration that CAMK2 plays a critical role in learning and memory also in the human brain was provided only a few years ago with the identification of patients suffering from ID carrying mutations in the CAMK2 genes (Akita et al., 2018; Chia et al., 2018; Küry et al., 2017; Proietti Onori et al., 2018; Rizzi et al., 2020).

### 3. The involvement of CAMK2 in neurodevelopmental disorders

In the past years, alterations of CAMK2 expression and/or activity were found in animal models of a broad range of neurological disorders and pathophysiological processes including: ischemia, drug addiction, depression, schizophrenia, epilepsy and rare neurodevelopmental disorders such as Angelman Syndrome, as extensively reviewed in (Coultrap et al., 2011; Robison, 2014; Takemoto-Kimura et al., 2017). However, a direct link between CAMK2 (mutations or deletions) and human ID remained to be found. This is surprising, since the Z score for missense variants (defined as a signed Z score of the chi-squared deviation of observation from expectation, where a positive Z score reflects less variants observed than expected (Samocha et al., 2014)), is 4.68 for CAMK2A, 4.07 for CAMK2B, 3.8 for CAMK2G and 3.11 for CAMK2D (Zek et al., 2016), suggesting that variations in the CAMK2 genes are not well tolerated. The first evidence of a contribution of CAMK2A to ID was shown in 2014 in a genetic study in which a large chromosomal deletion encompassing the CAMK2A gene was identified in patients with Treacher Collins syndrome with intellectual disability (Vincent et al., 2014). However, no functional studies were performed to directly link the ID to the CAMK2A gene deletion.

#### 3.1. CAMK2A and CAMK2B

The first CAMK2 missense variants were discovered in whole exome sequencing studies, where large groups of patients with unexplained ID or autism were sequenced (De Ligt et al., 2012; Iossifov et al., 2014). One of the variants found was a de novo missense variant in CAMK2A (p. Glu183Val) thereby providing, for the first time, a direct connection between CAMK2A and autism (Iossifov et al., 2014). The pathogenicity and functional effect of this mutant was proven by the group of Roger Colbran (Stephenson et al., 2017). Subsequently, more studies were published with larger cohorts of patients with a neurodevelopmental phenotype carrying variants in the different CAMK2 genes (summarized in Table 1), thereby expanding the CAMK2-related disorder (Akita et al., 2018; Chia et al., 2018; Küry et al., 2017; Proietti Onori et al., 2018; Rizzi et al., 2020). Currently there are 15 variants published in CAMK2A, 9 variants in CAMK2B, and 1 variant in CAMK2G (Fig. 1). CAMK2D is highly expressed in the heart, where its crucial role is already well established (as reviewed by Beckendorf et al., 2018), but ID causing variants have not yet been identified in literature.

In the first CAMK2 cohort study published in 2017, 19 different heterozygous variants in CAMK2A and CAMK2B were identified in 24 unrelated individuals suffering from ID (Küry et al., 2017). The majority of the identified variants were confirmed to be de novo and consisted mainly of missense variants besides a few nonsense as well as canonical splice site variants (Fig. 1). Since these variants are localized in regions that are highly conserved across species, bioinformatics tools can predict their pathogenicity, but not the direction of pathogenicity (e.g. gain-of-function (GoF), dominant negative or loss-of-function (LoF)). For some variants, predictions could be made based on existing data thanks to previous biochemical studies where multiple residues in the CAMK2A gene were mutated in order to understand their role in CAMK2 function (Yang and Schulman, 1999). However, pathogenicity for most of the variants remained to be tested.

Küry and colleagues found that the majority of the variants caused GoF, some LoF and some caused a dominant effect (Küry et al., 2017). Overall, it appeared that missense variants that affect the autoregulatory domain correlate with more severe ID on a clinical level. A subsequent publication of additional CAMK2 variants identified in patients with ID and epilepsy, further strengthened this finding (Akita et al., 2018). However, the cohort is still too small to draw reliable conclusions on genotype-phenotype correlations.

It might be considered surprising that of all the variants discovered...
so far, very few cause haploinsufficiency. Based on the data presented in the gnomAD database (https://gnomad.broadinstitute.org), the probability that haploinsufficiency is not tolerated (pLI) is high for CAMK2A (pLI = 1) and only slightly lower for CAMK2B (pLI = 0.74) (Karczewski et al., 2020). This suggests that haploinsufficiency is expected to be damaging. But of all the cases described until now, only 1 premature stop was found for both CAMK2A and CAMK2B (Küry et al., 2017). If haploinsufficiency would indeed not be tolerated, this number would be much higher in the ID patient population. Additionally, of the missense mutations found to cause a loss-of-function (LoF) based on the phosphotransferase activity, most are shown to affect the function of the holoenzyme in a dominant negative manner (Stephenson et al., 2017; Küry et al., 2017). This indicates that although they appear as LoF, these mutations are not recessive (haploinsufficient) mutations. To date, one true recessive variant in CAMK2A (His477 Tyr) was published (Chia et al., 2018). Both parents are unaffected carriers, whereas two siblings, homozygous for this variant, show a range of neurological symptoms such as severe ID and epilepsy (Chia et al., 2018). This mutation is located in the hub domain (Fig. 1) and causes partial disruption of self-oligomerization (Chia et al., 2018). Since the parents are carriers of this variant, these results suggest that LoF variants, which cause haploinsufficiency and do not affect the holoenzyme in a dominant negative manner, could potentially be tolerated. However, to date the number of individuals carrying variants in CAMK2 is still too low to draw conclusions. Future identifications will hopefully shed more light on the role of haploinsufficient variants in NDD.

3.2. CAMK2G

Surprisingly, CAMK2A and CAMK2B, the most abundant paralogs in the brain, were not the first of the CAMK2 family to be linked to human neurological disorders. Before the publication of the Iossifov study in 2014, a large-scale genomic study including children with severe ID identified a de novo candidate variant in CAMK2G, p.Arg292Pro (De Ligt et al., 2012). Even though CAMK2G is less abundant in the adult brain compared to CAMK2A and CAMK2B, CAMK2G represents the major brain paralog in the developing system together with CAMK2D (Bayer et al., 1999; Proietti Onori et al., 2018). Interestingly, CAMK2G, together with NMDA, PKA and metabotropic glutamate receptors was described as an important gene for human memory function, by correlating the genetic variability of this cluster of genes together with episodic memory performance (de Quervain and Papassotiriopoulos, 2006d). CAMK2G has also been reported to be the target of a miRNA, miR-219, which is involved in neurological and psychiatric disorders (Kocerha et al., 2009; Lukowi, 2007; Pan et al., 2014; Zhang et al., 2015). However, whether this mutation was indeed pathogenic and causing the NDD, remained to be shown.

Two research groups studied the pathogenic effect of the CAMK2Gp.Arg292Pro variant, for which later a second unrelated individual was identified (Cohen et al., 2018; Proietti Onori et al., 2018). Interestingly, whereas both groups found that the variant is indeed pathogenic, the mechanism behind the pathogenicity remains not well understood. One of the roles proposed for CAMK2G, more specifically for the brain specific CAMK2G isoform containing an NLS, is to act as a shuttle for calcium/calmodulin bringing this complex into the nucleus (Ma et al., 2014). The CAMK2Gp.Arg292Pro variant was shown to result in the loss of the shuttling capabilities of CAMK2G to the nucleus in both studies. However, whereas one group concludes that it is this lack of shuttling that might be the cause of the neurodevelopmental disorder (Cohen et al., 2018), the other group additionally showed that the variant causes CAMK2G to be constitutively active, and that in their assays the nuclear localization does not play a role in the pathogenicity, but that processes in the cytosol are likely also affected (Proietti Onori et al., 2018). These studies show that the ID-related mutation might exert its pathogenic function through multiple mechanisms, namely through a cytosolic gain-of-function effect, rendering the kinase constitutive active, and/or through a loss of function towards its nuclear shuttling function and control of gene expression. This illustrates the need for a broad set of functional studies to assess the pathogenicity of CAMK2 variants, and suggests that multiple mechanisms might contribute to the neurodevelopmental disorder seen in the children.

4. Mechanisms of pathogenesis

The core clinical symptoms in the previously described CAMK2 studies are neurological, with varying degrees of ID. This is not surprising, given the LTP and learning deficits observed in CAMK2 knockout mouse models (Achterberg et al., 2014; Bachstetter et al., 2014; Borgesius et al., 2011; Kool et al., 2019; Silva et al., 1992a,b; Van Woerden et al., 2009). However, variants in CAMK2A and CAMK2B result also in other, non-neurological phenotypes, such abnormalities of the digestive system (Küry et al., 2017). This might not be unexpected for patients carrying variants in CAMK2B considering that CAMK2B is also expressed in the skeletal muscle, intestines and endocrine system (Brocke et al., 1995; Tobimatsu and Fujisawa, 1989; Tombes et al., 2003). The presence of supposedly non-neurological symptoms might also argue for parallel and independent pathways being disrupted by dysregulation of CAMK2.
4.1. CAMK2 interacting partners in the PSD and their involvement in ID

When considering the ID phenotype seen in CAMK2-related disorders, the underlying mechanism likely involves differentially affected substrates or binding partners of CAMK2 in the synaptic compartments. Despite differences in molecular composition of the PSD between different neuronal types and brain regions (Bayes et al., 2011, 2012; Grant, 2019; Zhu et al., 2018a,b), some key elements such as PSD-95, Shank, Homer and CAMK2 constitute the main scaffold around which the postsynaptic compartment assembles (Chen et al., 2005; Cheng et al., 2006; Hell, 2014; Sheng and Hoogenraad, 2007). Other major components of the PSD are neurotransmitter receptors (NMDA, AMPA) and trans-synaptic adhesion molecules (N-cadherin, Neuroligins, Eph receptors) at the plasma membrane, and signaling proteins in the cytosol such as SYNGAP, Kalirin-7, Arc and B-catenin (Sheng and Hoogenraad, 2007) (Fig. 2). CAMK2 sits at a central position in the PSD, serving both as a structural molecule and a signaling molecule with several interacting partners (Hell, 2014). Binding of CAMK2 to the NMDA receptor as well as regulation of the activity and insertion of AMPA receptors into the post-synaptic membrane are crucial for controlling synaptic strength (Barria et al., 1997; Barria and Malinow, 2005; Hayashi et al., 2000; Lisman et al., 2012; Strack and Colbran, 1998; Zhou et al., 2007). Other examples of CAMK2-substrate interaction in the PSD are i) interaction between CAMK2A and SHANK3 (Baucom et al., 2015), which is essential in regulating neuronal L-type calcium channel signaling to the nucleus (Ferré et al., 2020), ii) the CAMK2-mediated phosphorylation of SYNGAP which results in activation of Ras followed by AMPAR insertion and LTP (Araki et al., 2015), iii) phosphorylation of Stargazin, resulting in binding of Stargazin to PSD-95 and thereby indirectly stabilizing AMPAR at synapses (Opazo et al., 2010) iv) phosphorylation of Kalirin-7 which promotes F-actin polymerization (Hell, 2014; Xie et al., 2007) and v) binding and phosphorylation of Tiam1, a Rac-GEF shown to promote...
stable actin-polymerization during LTP (Saneyoshi et al., 2019). Therefore, several signaling pathways might be affected upon CAMK2 dysfunction in ID, either through its enzymatic or structural role. For example, the CAMK2Ap.Glu183Val mutant presents disrupted interactions with SHANK3, as well as with the GluN2B subunit of the NMDA, the voltage gated calcium channel β2a subunit and the mGlu5 metabotropic glutamate receptor (Stephenson et al., 2017).

Mutations in many components of the PSD have been associated with neurodevelopmental disorders, including ID (Bayés et al., 2011; Kaizuka and Takumi, 2018). When focusing on some of the well-established interacting partners and known substrates of CAMK2, ID causing mutations were identified in GRIA1 (De Ligt et al., 2012), SYNGAP1 (Berryer et al., 2013; Gamache et al., 2020; Hamdan et al., 2011a, 2011b, 2009), IQSEC1 and IQSEC2 (Alexander-Bloch et al., 2016; Ansar et al., 2019; Levy et al., 2019; Rogers et al., 2019; Shoubridge et al., 2010; Tran Mau-Them et al., 2014), CACNG2 (Hamdan et al., 2011b), KALRN (Makrythanasis et al., 2016), SHANK3 (Cochoy et al., 2015; Hamdan et al., 2011b; Schmeisser and Verpelli, 2016; Zhu et al., 2018b) and GRIN2A and GRIN2B (De Ligt et al., 2012; Enende et al., 2010; Freunsch et al., 2013; Hu et al., 2016; Lemke et al., 2014) (Fig. 2). These findings indicate that multiple synaptic pathways potentially underlie the phenotypes observed in CAMK2-related disorders. Additionally, they illustrate the possibility of common pathways involved in NDDs, providing possible targets for further mechanistic studies and therapeutic intervention for multiple disorders.

4.2. CAMK2 and epilepsy

A subset of patients with CAMK2 mutations also suffer from epilepsy (Akita et al., 2018; Chia et al., 2018; Köry et al., 2017; Rizzi et al., 2020). This is not unexpected because previous studies have linked CAMK2 to epilepsy. However, there is no clear consensus on the precise role that CAMK2 plays in epilepsy. For example, in the CAMK2Ap.Thr305Val/Thr306Ala mouse mutant, where CAMK2A is constitutively active, seizures could be induced upon handling (Elgersma et al., 2002), suggesting that increased CAMK2 activity reduces the threshold for epilepsy. However, acoustic induction of epilepsy is also enhanced in Angelman Syndrome (AS) mice (Jiang et al., 1998), which have decreased CAMK2A activity due to increased Thr305/Thr306 phosphorylation (Weeber et al., 2003). Moreover, crossing AS mice with the CAMK2Ap.Thr305Val/Thr306Ala mice to reduce the inhibitory phosphorylation largely rescued the epilepsy phenotype (Van Woerden et al., 2007). More evidence that loss of CAMK2 or loss of its enzymatic function could be involved in epilepsy comes from several epilepsy rodent models, including kindled rats, pilocarpine and kainic-acid models of epilepsy where a reduction of CAMK2 activity was observed (Churn et al., 2000a; Goldenring et al., 1986; Wu et al., 1990; Yamagata et al., 2006). However, it should be noted that finding reduction of T286 phosphorylation in these models does not necessarily imply a causative link with the expression of seizures. Loss of CAMK2A using CAMK2A knockout mouse models also was found to reduce the threshold for developing seizures, showing limbic epileptiform activity if stimulated with normally subconvulsive brain stimuli (Butler et al., 1995). In line with these findings, reducing CAMK2 expression or activity in vitro results in the generation of epileptic-like activity (Ashpole et al., 2012; Churn et al., 2000b). Taken together, most studies suggest that reduced CAMK2A activity or expression plays a role in epileptogenesis, but the exact mechanism through which this is obtained remains to be fully

Fig. 2. CAMK2 interacting partners and substrates in the PSD involved in ID.
Schematic representation of a selection of the most abundant proteins that constitute the PSD. Mainly proteins that are known to directly interact with CAMK2 or that play a role in the CAMK2 signaling pathway are represented. CAMK2 is indicated as a holoenzyme and shown in transparency within the PSD to highlight its possible localizations and interactions, depending on the state of the synapse. Proteins indicated in bold and italic are linked to ID (see text for references). Abbreviations: SynGAP, synaptic Ras/Rap-GTPase-activating protein 1; AMPAR, AMPA-type glutamate receptor; Arc, activity-regulated cytoskeleton-associated protein; IQSEC, IQ motif- and SEC7 domain-containing protein; GKAP, guanylate kinase-associated protein; mGluR, metabotropic glutamate receptor; NMDAR, NMDA-type glutamate receptor; EphB-RTK, Ephrin type-B receptor-receptor tyrosine kinase; PSD-95, Postsynaptic density protein 95; Tiam-1, T-lymphoma invasion and metastasis-inducing protein 1; SHANK, SH3 and multiple ankyrin repeat domain protein.
understood.

So far, only one study tried to explore the possible mechanisms underlying the seizures seen in an individual with ID carrying the hyperactive CAMK2Ap.Pro212Gln variant (Akita et al., 2018). Since it was shown before that CAMK2 activity can enhance the surface expression levels of Kv4.2 channel (Varga et al., 2004) and children carrying de novo missense variants in KCN2D encoding for the Kv4.2 show seizure activity (Lee et al., 2014), the authors assessed the I\textsubscript{h} current (dependent on the Kv4.2 channel) in murine hippocampal neurons, upon overexpression the CAMK2Ap.Pro212Gln variant (Akita et al., 2018). Their results suggested that increased I\textsubscript{h} currents caused by hyperactive CAMK2A might indeed be underlying the epileptic phenotypes (Akita et al., 2018; Lee et al., 2014). However, given the complex interaction between proteins belonging to the PSD and the involvement of many proteins and channels in some forms of epilepsy (reviewed in Keith and El-Hussein, 2008; Torres et al., 2017), additional mechanisms behind the epilepsy phenotype caused by CAMK2 variants are likely to be involved. In future research, with more variants being discovered, it will be valuable to explore the contribution of GoF and LoF mutations respectively to the epilepsy phenotype.

4.3. CAMK2 heterogeneity in human disorders

Of the four paralogs of CAMK2, only CAMK2A is strictly brain specific. The widespread expression of the other CAMK2 paralogs in various tissues in the body suggests functional implications for CAMK2 that go beyond the neurological aspect. This, in combination with the recent systematic analysis of human hippocampal tissue where up to 70 different CAMK2 family transcripts that originate from alternative splicing were detected (Sloutsky et al., 2020; Sloutsky and Stratton, 2020), could potentially explain the wide variety of symptoms observed in CAMK2-related disorders. Each of these variants might have a different spatiotemporal expression pattern and/or interacting partner or substrate, supporting multiple and specific physiological functions for each isoform. Additionally, it is formally possible that although a variant is found in a shared domain, this variant might affect differently the function of a certain splice variant more than others of the same CAMK2 paralog. Future research is needed to determine the expression patterns as well as interactors of these different CAMK2 variants.

The use of mouse models to study the role of the different CAMK2 proteins in an organism, revealed that each paralog has specific enzymatic and/or structural functions. The differences in requirement of each of these functions in cellular processes can be another explanation for the variety in symptoms, as depending on the nature of the variants found in each individual (LoF or GoF), the impact on CAMK2 functions can differ significantly. The identification of additional individuals with NDDs, carrying variants in one of the CAMK2 genes will hopefully allow future genotype/phenotype correlations.

Furthermore, a recent study using 3D electron microscopy revealed that CAMK2 holoenzymes are not fixed structures but are very dynamic and highly flexible. The widespread expression of the other CAMK2 paralogs in various tissues in the body suggests functional implications for CAMK2 that go beyond the neurological aspect. This, in combination with the recent systematic analysis of human hippocampal tissue where up to 70 different CAMK2 family transcripts that originate from alternative splicing were detected (Sloutsky et al., 2020; Sloutsky and Stratton, 2020), could potentially explain the wide variety of symptoms observed in CAMK2-related disorders. Each of these variants might have a different spatiotemporal expression pattern and/or interacting partner or substrate, supporting multiple and specific physiological functions for each isoform. Additionally, it is formally possible that although a variant is found in a shared domain, this variant might affect differently the function of a certain splice variant more than others of the same CAMK2 paralog. Future research is needed to determine the expression patterns as well as interactors of these different CAMK2 variants.

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5. A new chapter in CAMK2 research

After more than 25 years from the generation of the very first mouse model for learning and memory, which showed the contribution of CAMK2A in synaptic plasticity and learning, a new line of investigation is starting to emerge. The discovery of individuals suffering from a neurodevelopmental disorder in which ID is the main characteristic, carrying variants in the CAMK2 genes switched the attention to the role that CAMK2 plays in the development and regulation of learning capabilities as well as synaptic plasticity in the human brain. Despite an extensive study of the basic functionality of CAMK2 in the mouse brain, the new challenge will be to understand how disruption of mechanisms regulated by CAMK2 in the human brain can lead to neurodevelopmental disorders. The investigation of the pathogenic effects caused by each variant identified in CAMK2 genes demonstrates that there can be multiple ways in which disruption of CAMK2 could potentially lead to a disorder. Combining the generation of novel mouse models and new techniques that will implement the use of human induced pluripotent stem cell (iPSCs) will allow to study the mechanisms underlying CAMK2-related neurodevelopmental disorders. The use of an in vitro disease model with robust physiological relevance like iPSC derived neurons will eventually constitute an important preclinical platform through which we can assess the validity of mechanistic hypotheses, acquired also from mouse models, as well as to screen the efficacy of specific drugs.

Author statement

Martina Proietti Onori: Writing – Original draft, Writing – Review & Editing; Geeske M. van Woerden: Writing – Original draft, Writing – Review & Editing. Funding acquisition.

Funding

This research was supported by the NWO-VIDI(016.Vidi.188.014 to G.M.v.W.)

Declaration of Competing Interest

The authors declare no conflict of interest

Acknowledgements

We would like to thank Dr. Danielle C. M. Veenma for reading the manuscript and giving valuable input.

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