Review

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Convergence on CaMK4: A Key Modulator of Autism-Associated Signaling Pathways in Neurons

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ABSTRACT

Although the precise underlying cause(s) of autism spectrum disorder remain unclear, more than 1000 rare genetic variations are associated with the condition. For many people living with profound autism, this genetic heterogeneity has impeded the identification of common biological targets for therapy development for core and comorbid traits that include significant impairments in social communication and repetitive and restricted behaviors. A substantial number of genes associated with autism encode proteins involved in signal transduction and synaptic transmission that are critical for brain development and function. *CAMK4* is an emerging risk gene for autism spectrum disorder that encodes the CaMK4 (calcium/calmodulin-dependent protein kinase 4) enzyme. CaMK4 is a key component of a Ca^{2+}-activated signaling pathway that regulates neurodevelopment and synaptic plasticity. In this review, we discuss 3 genetic variants of *CAMK4* found in individuals with hyperkinetic movement disorder and comorbid neurological symptoms including autism spectrum disorder that are likely pathogenic with monogenic effect. We also comment on 4 other genetic variations in *CAMK4* that show associations with autism spectrum disorder, as well as 12 examples of autism-associated variations in other genes that impact CaMK4 signaling pathways. Finally, we highlight 3 environmental risk factors that impact CaMK4 signaling based on studies of preclinical models of autism and/or clinical cohorts. Overall, we review molecular, genetic, physiological, and environmental evidence that suggest that defects in the CaMK4 signaling pathway may play an important role in a common autism pathogenesis network across numerous patient groups, and we propose CaMK4 as a potential therapeutic target.

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Autism spectrum disorder is a lifelong neurological condition that affects approximately 1% of the global population. It is defined by a group of core symptoms characterized by deficits in social communication and interaction, restricted interests, and repetitive behaviors (1,2). Approximately one-third of the autistic population have profound autism and experience major difficulties accessing health services, education, independent living, and engaging with the community (3). In addition to core traits, affected individuals also experience a higher burden of psychiatric and medical comorbidities than the general population that frequently aggravate the core symptoms and reduce their quality of life (4). Common comorbidities include gastrointestinal complications, epilepsy, and sleep disorders (5–7).

Although the etiology of autism spectrum disorder is not fully understood, genetic factors play a major role (8). Recent advances in genomic technologies, such as high-throughput sequencing, genome-wide association studies, and whole-exome sequencing, have facilitated the discovery of a multitude of heritable and spontaneous genetic alterations associated with autism spectrum disorder. These range from rare, highly penetrant variations in single genes to common variations in both coding and noncoding regions across the genome (9). Copy number variations, de novo variants, and the aggregate effects of common single nucleotide polymorphisms across multiple genes have each been implicated in the genetic architecture of the condition. Environmental factors also play a role in shaping the developmental trajectory and severity of autism spectrum disorder including parental age, prenatal exposure to environmental agents, and maternal health (10–16). The impact of these environmental factors varies with individual genetic predispositions, giving rise to a spectrum of phenotypes.

Here, for the first time, we draw on evidence that implicates the CaMK4 (calcium/calmodulin-dependent protein kinase 4) signaling pathway as a potential unifying mechanism connecting the contribution of 19 genetic variations (including 7 that impact *CAMK4* directly) and 3 environmental factors to autism spectrum disorder.

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CaMK4 IS A KEY COMPONENT OF AUTISM-ASSOCIATED SIGNALING PATHWAYS IN NEURONS

Ca^{2+} signaling regulates a variety of pathways critical for neuronal communication, synaptic plasticity, and information processing in the brain (17). Many neuronal processes regulated by Ca^{2+} are mediated by calmodulin, a universal Ca^{2+}-sensing protein that decodes information carried by Ca^{2+} signals by binding and modifying the function of a diverse range of effector proteins (18). In humans, 3 separate genes (*CALM1*, *CALM2*, and *CALM3*) located on chromosomes 14, 2, and 19, respectively, produce identical calmodulin proteins (19). All 3 *CALM* genes are abundantly expressed in both the central and enteric nervous systems but display differential expression in peripheral tissues (20). This gene redundancy underscores the importance of calmodulin in maintaining neurological function. At least 7 rare genetic variations in *CALM1–3* are associated with a range of neurological disorders including autism, intellectual disability, and attention-deficit/hyperactivity disorder (Table 1) (19).

CaMK4 is a serine/threonine protein kinase and a core constituent of a Ca^{2+}-calmodulin activated signaling pathway that plays a crucial role in neuronal function. For example, CaMK4 impacts gene expression regulation, synaptic plasticity, neuronal differentiation, mitochondrial energy generation, and neuronal survival (Figure 1) (21–25). CaMK4 is activated by neurotransmitters and voltage-gated Ca^{2+} channels that increase intracellular Ca^{2+} levels and cause accumulation of the Ca^{2+}-calmodulin complex, which allosterically activates CaMK4.

MECHANISMS OF CaMK4 REGULATION

The regulation of CaMK4 activity involves a complex interplay between allosteric activation by calmodulin and post-translational modifications such as phosphorylation and O-linked-*N*-acetylglucosamine (O-GlcNAc) glycosylation on CaMK4 regulatory sites. The domain structure of CaMK4 comprises an N-terminal kinase domain and a C-terminal regulatory region that contain overlapping autoinhibitory and calmodulin-binding sequences (26,27) (Figure 2). CaMK4 is regulated by a multistep process. Firstly, Ca^{2+}-calmodulin allosterically activates CaMK4 by preventing the autoinhibitory sequence from blocking the catalytic site in the kinase domain (26). The autoinhibitory sequence is a critical regulatory feature that ensures that CaMK4 signaling remains inactive in the absence of a Ca^{2+} stimulus. Secondly, Ca^{2+}-calmodulin binding enables phosphorylation of a threonine located at position 200 (Thr200) within the activation loop of CaMK4 by activating upstream kinases (i.e., CaMKK1 and CaMKK2 [CaMK kinase 1 and 2]) (27–29). This phosphorylation of Thr200 is a critical step because it enables CaMK4 to remain activated after the Ca^{2+} stimulus has diminished. Thirdly, CaMK4 is returned to its inactive state by dephosphorylation of Thr200 by PP2A (protein phosphatase 2A) (30).

GENETIC VARIATIONS IN CaMK4 ARE ASSOCIATED WITH AUTISM SPECTRUM DISORDER

Whole-exome sequencing, candidate gene association, and copy number variation studies have uncovered numerous genetic variations in *CAMK4* in populations diagnosed with autism spectrum disorder (Table 2).

Table 1. Genetic Variations Relevant to the CaMK4 Signaling Pathway Identified in Patients With Autism Spectrum Disorder and Related Neurodevelopmental Disorders

Gene	Variant	Relationship to CaMK4	Diagnosis	Reference
CALM1	p. Asp312Val and Phe142Leu	Allosteric activator of CaMK4	Autism spectrum disorder, intellectual disability, epilepsy	(19)
CALM2	p. Glu46Lys	Allosteric activator of CaMK4	Autism spectrum disorder, intellectual disability, epilepsy	(19)
CALM3	p. Asp96His and Phe142Leu	Allosteric activator of CaMK4	Autism spectrum disorder, developmental delay	(19)
CACNA1C	p. Gly406Arg	Cell surface activator of CaMK4	Timothy syndrome, a multisystem condition characterized by a range of complications including cardiac arrythmias, syndactyly, and autism spectrum disorder	(42)
PPP2CA	p. Cys196Arg	Negative regulator of CaMK4 PP2A inactivates CaMK4 by reversing Thr200 phosphorylation.	Intellectual disability, hypotonia, seizures, and autism spectrum disorder	(51)
OGT	p. Cys921Tyr	Negative regulator of CaMK4 OGT prevents CaMK4 activation by blocking Thr200 phosphorylation.	X-linked intellectual disability and autism spectrum disorder	(57)
HDAC4	2q37.3 deletion	Epigenetic regulator and direct substrate of CaMK4	Brachydactyly syndrome	(78)
FMR1	p. Ile304Asn	*FMR1* gene expression is regulated by CaMK4.	Fragile X syndrome	(109)
MeCP2	p. Arg306Cys	Epigenetic regulator and direct substrate of CaMK4	Rett syndrome	(110)

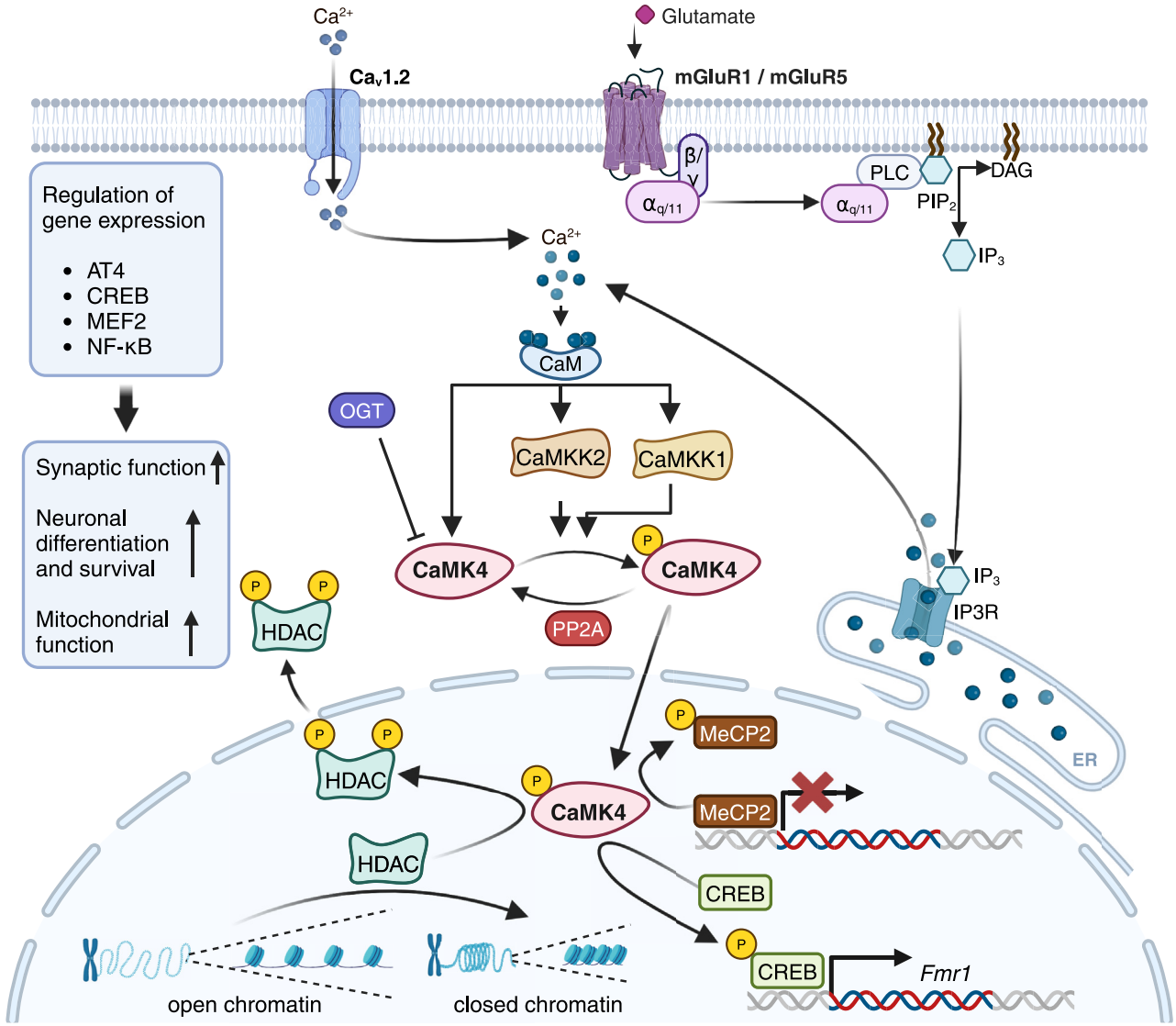

Figure 1. CaMK4 signaling pathway in neurons. CaMK4 is activated by the voltage-gated Ca^{2+} channel ($Ca_V1.2$) and the metabotropic glutamate receptors mGluR1 and mGluR5, all of which cause increases in intracellular Ca^{2+} and accumulation of Ca^{2+}-calmodulin complex. Activated CaMK4 directly phosphorylates 3 known downstream effectors (CREB, HDAC4, MeCP2) through which it regulates the expression of a range of genes that support brain function. CaMK4 is inactivated by OGT and deactivated by PP2A, which ensures signal fidelity and prevents overactivation of CaMK4 signaling. DAG, diacyl glycerol; ER, endoplasmic reticulum; NF-κB, nuclear factor-κB; PLC, phospholipase C.

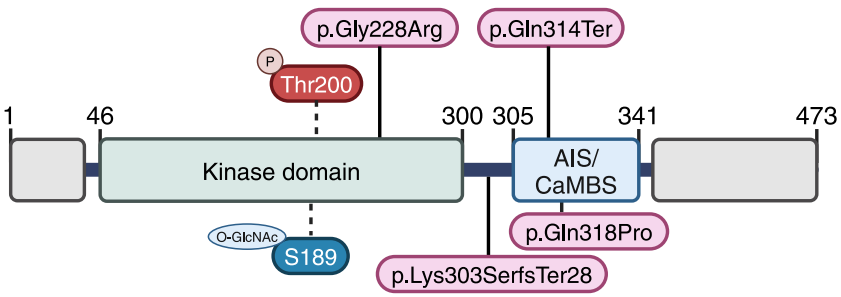

Figure 2. Domain structure of CaMK4 and mechanisms that regulate CaMK4 activity. Linear schematic of the domain structure of human CaMK4 illustrating the position of the kinase domain, the autoinhibitory sequence (AIS), the calmodulin-binding sequence (CaMBS), and the regulatory phosphorylation (Thr200) and O-GlcNAcylation (Ser189) sites, as well as genetic variations (Gly228Arg, Lys303SerfsTer28, Gln314Ter, Gln318Pro) associated with autism spectrum disorder.

Table 2. Summary of *CAMK4* Variants Associated With Autism Spectrum Disorder and Related Neurodevelopmental Conditions

Sex	Variant	Variant	Inheritance	Mechanism of Action	Diagnosis	Reference
M	Frameshift	p. Lys303SerfsTer28	De novo	Gain of function	Hyperkinetic movement disorder, severe generalized dystonia, intellectual disability, autism spectrum disorder	(31)
F	Nonsense	p. Gln314Ter	De novo	Suspected gain of function based on a truncated CaMK4 construct	Dystonia, generalized chorea, autism spectrum disorder	(34)
M	Missense	p. Gln318Pro	De novo	Unknown	Hyperkinetic movement disorder, generalized epilepsy, intellectual disability, autism spectrum disorder	(33)
M	Missense	p. Gly228Arg	De novo	Unknown	Autism spectrum disorder	(35)
M	Splice site	Circa 1101$G > A$	Unknown	Increased messenger RNA expression of a truncated *CAMK4* isoform	Autism spectrum disorder, fragile X syndrome	(36)
M	CNV duplication	5:g. (110,260,870–110,730,612) duplication	Unknown	Copy number duplication	Autism spectrum disorder, intellectual disability	(40)
M	CNV duplication	5:g. (91,562,441–111,573,636) duplication	De novo	Copy number duplication	Autism spectrum disorder, intellectual disability, mild microcephaly	(41)

CNV, copy number variation; F, female; M, male.

To date, 3 rare de novo variants of *CAMK4* have been reported in individuals exhibiting a range of neurodevelopmental abnormalities. A dominant heterozygous variant (OMIM: 114080.0001) located in an essential splice site in *CAMK4* was identified by whole-exome sequencing analysis of an individual with hyperkinetic disorder, severe generalized dystonia, intellectual disability, and comorbid autism spectrum disorder–like features (31). This variant causes an out-of-frame deletion that results in a premature stop codon within the last exon, which gives rise to a truncated CaMK4 protein (Lys303SerfsTer28) that lacks the autoinhibitory and calmodulin-binding sequences (Figure 2). The Lys303SerfsTer28 variant causes constitutive activation of CaMK4 signaling because fibroblasts obtained from the affected individual showed elevated CREB (cyclic adenosine monophosphate response element binding protein) Ser133 phosphorylation (a marker of increased CaMK4 activity) compared with fibroblasts from their neurotypical relatives. Strikingly, the Lys303SerfsTer28 variant is absent from population-reference databases of human genetic variation, demonstrating that this region of *CAMK4* is subject to strong purifying selection that prevents the accumulation of deleterious, function-altering genetic variations in the population (32). Collectively, these findings indicate that the Lys303SerfsTer28 variant of CaMK4 is likely pathogenic with monogenic effect and manifests the clinical phenotype through a gain-of-function mechanism.

Two additional heterozygous variants that encode amino acid substitutions (Gln314Ter and Gln318Pro) in CaMK4 have been identified in individuals with clinical phenotypes similar to the Lys303SerfsTer28 variant (33,34). These variants map to the autoinhibitory sequence of CaMK4 (Figure 2). Therefore, the location of these substitutions suggests that they also result in gain of function of CaMK4 signaling and are likely pathogenic. The Gln314Ter variant introduces a premature stop codon that results in a truncated form of the CaMK4 protein. There is evidence to suggest that the Gln314Ter variant results in gain of function. Specifically, a genetically engineered CaMK4 truncation construct that terminates at position 317 was found to show constitutive activity in a cell-free in vitro assay (28).

In addition to genetic variations clustered around the autoinhibitory sequence of CaMK4, a whole-exome sequencing study of more than 2500 families, each with a child diagnosed with profound nonverbal autism spectrum disorder, identified a genetic variation in *CAMK4* that results in a Gly228Arg substitution in the kinase domain of CaMK4 (Figure 2) (35). Although the functional consequences of the Gly228Arg substitution have not yet been determined, there are no reports of variations at this position in human genetic databases, which demonstrates that this site is highly conserved and likely functionally important. Based on the crystal structure of CaMK4 (Protein Data Bank Identification: 2W4O), Gly228 is positioned in close proximity to the activation loop. Therefore, the Gly228Arg substitution could impact CaMK4 activity by altering the conformation of the activation loop and through subsequent interactions with activating upstream kinases such as CaMKK1 and CaMKK2.

In terms of common variations, a candidate gene association study of a patient cohort with diagnoses of early childhood autism, Asperger's syndrome, or atypical autism found a significant association with a missense polymorphism in *CAMK4* (rs25925; 1.9×10^{-1} minor allele frequency), where the minor allele was associated with increased messenger RNA (mRNA) expression (>2-fold) of a truncated isoform of *CAMK4* (36).

However, it has not yet been established whether the minor allele results in increased expression of truncated CaMK4 protein and contributes to the clinical phenotype observed in the patient cohort.

Copy number variations are the most common form of genetic variation in the genome and are important contributors to the pathogenesis of autism spectrum disorder (37). These variations can be inherited or occur by de novo events, and they often alter the dosage of specific genes by increasing or decreasing the number of copies, thereby disrupting the finely tuned balance of gene expression required for normal brain development and function (38,39). A recent genetic study of copy number variation in 2 large cohorts identified an association between autism spectrum disorder and a duplication of approximately 470,000 base pairs in the genome locus 5q22.1 harboring the *CAMK4* gene (40). A larger duplication of 20.1 Mb pairs of the genome locus spanning 5q14.3-5q22.1 has also been detected in 2 individuals with intellectual disability and autism spectrum disorder but not in their neurotypical parents (41). While these studies hint at a potential role for *CAMK4* in driving the observed phenotype in these patients, this remains to be determined and may involve other genes located in this region of the genome.

Overall, these findings indicate that functionally disruptive genetic variations in human *CAMK4*, particularly those that encode gain-of-function substitutions in CaMK4 that result in overactive signaling, are strong candidates as contributors to autism spectrum disorder.

UPSTREAM REGULATORS OF CaMK4 AND POTENTIAL ROLES IN AUTISM ETIOLOGY

Dysfunction in 3 cell surface activators of CaMK4 signaling ($Ca_V1.2$, mGluR1 [metabotropic glutamate receptor 1], and mGluR5), which are encoded by the genes *CACNA1C*, *GRM1*, and *GRM5*, respectively, are associated with autism spectrum disorder (Table 1). For example, a rare gain-of-function variation in $Ca_V1.2$ causes Timothy syndrome (OMIM: 114205.0001), a multisystem condition characterized by a range of complications including cardiac arrythmias, syndactyly, and autism spectrum disorder (42). Several studies have reported elevated levels of mGluR1 and mGluR5 protein in various brain regions in individuals with autism spectrum disorder compared with neurotypical controls (43–45). Furthermore, pharmacological inhibition of mGluR1 and mGluR5 receptors has been shown to rescue autism-like phenotypes in several mouse models of autism spectrum disorder (46–49).

As mentioned, PP2A deactivates CaMK4 by dephosphorylating Thr200. PP2A associates with CaMK4 by binding to the autoinhibitory sequence. Loss of PP2A/CaMK4 association prevents Thr200 dephosphorylation and causes hyperactivation of CaMK4, which demonstrates that this regulatory step is crucial for regulating CaMK4 signaling (30,50). This deactivation step is necessary for healthy brain development and function because overactive CaMK4 signaling is associated with autism spectrum disorder and related neurodevelopmental disorders (31). Consistent with these data, a reciprocal loss-of-function variation in the catalytic subunit of PP2A is associated with intellectual disability and autism spectrum disorder (Table 1) (51).

CaMK4 activity is also regulated by O-GlcNAcylation, which is a posttranslational modification and form of glycosylation that involves the reversible attachment of the modified sugar β-*N*-acetylglucosamine (O-GlcNAc) to hydroxyl groups of specific serine and threonine residues in proteins (52,53). O-GlcNAc glycosylation is particularly abundant in the brain and modulates a myriad of cellular processes including neuronal signal transduction and gene expression (54–56). O-GlcNAcylation of CaMK4 on Ser189 prevents activation of CaMK4 by blocking phosphorylation of Thr200 (52). A missense variation that gives rise to a Cys921Tyr substitution in O-GlcNAc transferase, the only enzyme known to catalyze O-GlcNAc modification, causes a catalytic deficiency associated with X-linked intellectual disability and autism spectrum disorder (Table 1) (57). The central role of O-linked GlcNAcylation in neurotransmission and cellular signaling in the nervous system as well as a specific genetic variation in this enzyme contributing to autism diagnoses offer additional evidence for considering converging CaMK4 signaling pathways in the etiology of autism.

Given that the 3 regulatory steps relevant to controlling CaMK4 activity are modulated by genes that are associated with autism spectrum disorder when altered, we propose that focusing on CaMK4 dysfunction in clinical populations may identify a broad but targetable therapeutic approach for improving outcomes for affected individuals and families, in particular for cases of profound autism in which individuals experience multiple debilitating comorbid traits.

CaMK4 DOWNSTREAM EFFECTORS CREB, FMRP, AND HDAC4 ARE ASSOCIATED WITH AUTISM SPECTRUM DISORDER

In addition to genetic variations in *CAMK4* and in regulatory proteins that modify the activity of CaMK4 enzyme (i.e., $Ca_V1.2$, mGluRs, PP2A, and OGT [O-GlcNAc transferase]), there are potential autism-associated impacts of modifying molecular targets downstream of CaMK4. It has been reported that genetic variations that impact effector proteins of CaMK4 such as CREB and HDAC4 (histone deacetylase 4) also contribute to autism pathology. Furthermore, *FRM1*, a target gene of CREB, is strongly associated with autism.

At the subcellular level, inactive CaMK4 is primarily cytoplasmic, whereas activated CaMK4 predominantly resides in the nucleus but is also present in the cytoplasm, suggesting that CaMK4 can shuttle between these cellular compartments (58,59). Direct nuclear effectors of CaMK4 include the transcription factor CREB, as well as the epigenetic regulator HDAC4 (23,24,60–64). CaMK4 modulates the activity of these effector targets by directly phosphorylating key regulatory residues that control effector target subcellular localization or ability to interact with other components of the transcriptional machinery. Through these effectors, CaMK4 coordinates gene expression programs that are essential for neurodevelopment and healthy brain function.

CREB is highly expressed in the cerebral cortex, cerebellum, and hippocampus and orchestrates transcription of multiple genes involved in pathways that support learning and memory

function (65). Many target genes regulated by CREB encode proteins essential for neuronal function, such as *FMR1*, which encodes FMRP (fragile X messenger ribonucleoprotein), which is very strongly implicated in autism spectrum disorder and is in the top ranking category (1S score) of autism-associated genes in the Simons Foundation Autism Research Initiative gene database (66–68). Genetic variations that result in a loss or reduction of FMRP expression result in fragile X syndrome (e.g., OMIM: 309550.0001), which is the most widely recognized cause of autism spectrum disorder (Table 1) (69). CREB-dependent transcription is activated by multisite phosphorylation, which in turn is mediated by several protein kinases including CaMK4 (23,61,70). Phosphorylation of CREB at Ser133 (located in the kinase-inducible domain of CREB) by CaMK4 promotes transcription of CREB target genes by facilitating the recruitment of two transcriptional coactivators, CREB-binding protein and its paralog p300 (71). Ser133 phosphorylation is critical for maintaining neuronal plasticity and cognition because mice that express a forebrain-specific, nonphosphorylatable CREB missense substitution (Ser133Ala) show deficits in spatial cognitive flexibility, basal synaptic transmission, and long-term potentiation (72). Notably, impairments in cognitive flexibility and executive function are significantly associated with autism diagnoses (73,74).

HDAC4 is highly expressed in the brain, where it regulates a transcriptional program in neurons that is essential for synaptic plasticity and information processing (75). Within the nucleus, HDAC4 acts as a transcriptional repressor, dampening the expression of genes involved in regulating synaptic structure and function. The transcriptional activity of HDAC4 is tightly controlled by a mechanism that involves shuttling of HDAC4 from the nucleus to the cytoplasm to spatially segregate HDAC4 from its target genes (76). CaMK4 relieves HDAC4 transcriptional repression by promoting its export from the nucleus and retention in the cytoplasm, which results in increased gene expression (64). Like CaMK4, emerging lines of evidence implicate HDAC4 dysregulation in autism spectrum disorder (Table 1). For example, increased *HDAC4* mRNA expression has been reported in the prefrontal cortex of brains from individuals with autism compared with neurotypical controls (77). In addition, a rare de novo genetic variation that results in truncation of HDAC4 is associated with brachydactyly syndrome (OMIM: 605314.0001), a syndromic form of autism (Table 1) (78). A separate study found that this truncated form of HDAC4 is confined to the nucleus, which induces a state of constitutive transcriptional repression of HDAC4 target genes. This outcome was in contrast to the subcellular shuttling of wild-type HDAC4 and resulted in impaired neurotransmission and spatial memory deficits in mice (75). These studies demonstrate the importance of nuclear-cytoplasmic shuttling of HDAC4 for maintaining optimal neuronal function. Given the core role of CaMK4 in this regulatory mechanism, it is plausible that genetic variations that impair CaMK4 signaling could also hinder HDAC4 shuttling, potentially leading to similar clinical phenotypes. These findings support the idea that the CaMK4 signaling pathway is a crucial mechanism that connects multiple rare genetic variations that have already been established to cause autism. In doing so, it provides a signaling road map for autism spectrum disorder.

CaMK4, RETT SYNDROME, AND DEFECTS IN HOMEOSTATIC PLASTICITY

The CaMK4 signaling pathway plays a pivotal role in maintaining homoeostatic neural plasticity, which is a key regulatory mechanism that actively balances excitatory and inhibitory signals within neurons to prevent the emergence of hyperactivity or hypoactivity (79). Activation of CaMK4 in cortical neurons diminishes synaptic strength and spontaneous firing rates, whereas inhibition of CaMK4 heightens both, suggesting that CaMK4 activation produces a negative feedback signal to regulate neuronal firing rates (79).

Defects in homeostatic plasticity of synapses that lead to unrestrained neuronal activity are implicated in autism spectrum disorder and other related neurodevelopmental conditions (80–82). For example, loss-of-function variations in the gene that encodes the transcriptional repressor MeCP2 cause Rett syndrome, an X-linked neurodevelopmental disorder in which patients frequently show symptoms that overlap with autism spectrum disorder (Table 1) (83). Mice that lack *Mecp2* show impaired synaptic plasticity and imbalances in synaptic excitation and inhibition within neocortical circuits (84). Interestingly, CaMK4 phosphorylation of Thr308 on the MeCP2 protein has been reported, but this has yet to be confirmed in vivo. Thr308 is located within the MeCP2 transcription repressor domain, and phosphorylation of Thr308 impairs the ability of MeCP2 to repress transcriptional activity, resulting in increased gene expression (85). Knock-in mice expressing either the Rett syndrome–associated Arg306Cys substitution (OMIM: 300005.0016) or a Thr308Ala substitution that prevents phosphorylation of MeCP2 display Rett syndrome–like phenotypic traits (85,86). These significant findings demonstrate that phosphorylation of MeCP2, which is potentially mediated by CaMK4, plays an essential regulatory role in neurodevelopment and brain function.

VALPROATE, CANNABINOIDS, AND HYPOTHYROIDISM MODIFY CaMK4 AND ARE ENVIRONMENTAL RISK FACTORS FOR AUTISM SPECTRUM DISORDER

Although autism spectrum disorder is highly heritable, environmental risk factors are recognized as important contributors to its development, and several of these factors have links to CaMK4 (Table 3). Advanced parental age is a well-documented risk factor, which may be due to increased frequency of germline de novo genetic variations (87–90). Maternal exposure to medications, environmental toxins, and illicit drugs that impact neurodevelopment, as well as untreated medical conditions during pregnancy such as hypothyroidism, have also been linked to autism spectrum disorder (10–14,16).

Multiple epidemiological studies have demonstrated a significant association between the prenatal use of the antiepileptic and mood-stabilizing medication, valproate, and an increased risk of autism spectrum disorder in offspring (11,12,16). This association holds even after accounting for factors such as parental epilepsy and psychiatric conditions. Consistent with these studies, maternal exposure to valproate during pregnancy in nonhuman primates has been shown to impair neurogenesis and induce autism-like behaviors in offspring (91). Treatment with valproate has been reported to

Table 3. Evidence for Environment-Gene Interactions Influencing CaMK4 in Epidemiological Studies and Preclinical Models of Autism

Environmental Factor	Epidemiology	Preclinical	References
Valproate	Significant association between prenatal use of valproate and an increased risk of autism spectrum disorder in offspring.	Valproate exposure during pregnancy in nonhuman primates impaired neurogenesis and induced autism-like behaviors in offspring. Treatment of human forebrain organoids with valproate (an established preclinical model of autism) increases expression of CaMK4.	(11,12,16,91,92)
Cannabinoids	Maternal cannabis use during pregnancy is associated with increased incidence of autism spectrum disorder in offspring.	Treatment with the synthetic cannabinoid agonist JWH-081 disrupts hippocampal-dependent memory and learning in mice through a mechanism involving CaMK4-CREB signaling via CB_1 receptors.	(13,94,95)
Hypothyroidism	Maternal hypothyroidism during pregnancy is associated with an increased risk of autism spectrum disorder in offspring.	Elevated levels of CaMK4 expression in two rat models of hypothyroidism.	(14,96–100)

increase the expression of *CAMK4* and other risk genes for autism spectrum disorder and disrupt synaptic transmission in a human forebrain organoid model of brain development (92).

A large retrospective cohort study reported that maternal cannabis use during pregnancy was associated with increased incidence of autism spectrum disorder in offspring compared with unexposed children (13). Exogenous cannabinoids primarily act via cannabinoid CB_1 and CB_2 receptors, with the former being abundantly expressed in the brain (93). In relation to CaMK4, treatment with the synthetic cannabinoid agonist JWH-081 disrupted hippocampal-dependent memory and learning in mice through a mechanism involving CaMK4-CREB signaling (94). Another study demonstrated that increased levels of the endocannabinoid anandamide in the brain adversely affected CaMK4 signaling in the hippocampus, leading to deficits in memory and learning in mice (95). In both studies, the effects of cannabinoids on CaMK4 signaling were observed only in wild-type mice but not in mice that lacked CB_1 receptors, demonstrating an important contribution of CB_1 receptors in the modulation of CaMK4-associated cognitive functions. While the precise mechanisms remain poorly understood, cannabinoid-induced disruptions in CaMK4 signaling may play a role in the increased risk of autism diagnoses in offspring following cannabinoid use.

Several studies indicate that maternal hypothyroidism during pregnancy is associated with an increased risk of autism spectrum disorder in offspring (14, 96–98). Elevated levels of *Camk4* mRNA and CaMK4 protein expression have been observed in neocortical neurons from pups born from 2 distinct rat models of late maternal hypothyroidism, suggesting a potential role for CaMK4 (99). Supporting this idea, a separate study that examined changes in gene expression in the cerebral cortex of pups from the same rat models revealed that a large proportion of the differentially expressed genes were downstream targets of CaMK4-CREB signaling (100).

These intersecting impacts of valproate, cannabinoids, and hypothyroidism on CaMK4 activity, mRNA expression, and protein abundance reveal a functional biological signature relevant to the etiology of autism and associated comorbid traits. Importantly, this information could assist to enhance the design of targeted therapies for individuals with autism who experience significant issues. These findings highlight the possibility that diverse environmental factors may contribute to the development of autism spectrum disorder through a common mechanism that involves increased CaMK4 signaling.

LIMITATIONS OF THE REVIEW

Although there are a number of compelling connections between CaMK4 dysregulation and autism spectrum disorder, direct evidence that links the two is still limited. To close this knowledge gap, new tools are needed to directly investigate the role of *CAMK4* variants in autism. This includes generating induced pluripotent stem cell and knock-in mouse models carrying patient-derived *CAMK4* variants. These tools are crucial for advancing the field and providing a clearer understanding of how CaMK4 signaling contributes to autism spectrum disorder and related neurological conditions.

FUTURE DIRECTIONS

Despite recent advances in understanding genetic, neurobiological, and environmental factors that contribute to autism spectrum disorder, current therapeutic options to manage core symptoms are limited for individuals with profound autism (101). The complex genetic diversity and wide range of clinical traits associated with autism spectrum disorder pose challenges for developing pharmacological approaches for specific therapeutic targets and identifying biomarkers crucial for guiding the development of therapies. However, personalized medicine based on targeting common pathways that connect multiple genes associated with autism spectrum disorder offers a promising approach for tailoring interventions to the unique molecular and genetic profiles of individuals with significant impairments. In this regard, the CaMK4 signaling pathway is a potential candidate for drug targeting. Here, we demonstrated, using specific examples identified in preclinical and clinical studies, that genetic variations that result in dysregulation of CaMK4 can contribute to key aspects of autism spectrum disorder including defects in signal transduction, homeostatic plasticity, and environmental risk factors.

How can the information summarized in this review be applied to help patients and their families? Given that the

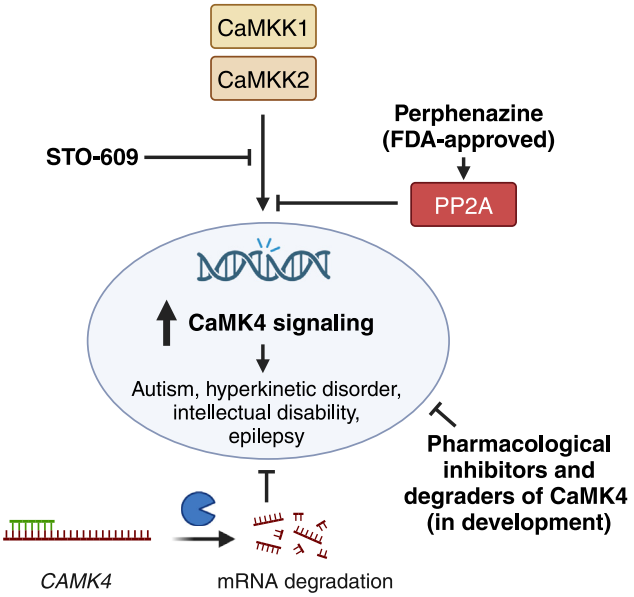

Figure 3. Potential therapeutic strategies to treat profound autism and related neurological disorders associated with overactive CaMK4 signaling. Schematic illustrating potential strategies to target the CaMK4 signaling pathway to treat profound autism and related neurological disorders. STO-609 is a pharmacological inhibitor of both CaMKK1 and CaMKK2. Perphenazine is a pharmacological activator of PP2A. FDA, Food and Drug Administration; mRNA, messenger RNA.

current evidence overwhelmingly points to overactive CaMK4 signaling as a key underlying mechanism, one potential approach is to develop antisense oligonucleotide therapies to selectively knockdown *CAMK4* expression in the brain (102) (Figure 3). A major advantage of this approach is that it can be tailored to individual cases. A similar therapeutic strategy has been used successfully to treat patients carrying pathogenic gain-of-function variants in *SOD1*, which causes an adult-onset form of amyotrophic lateral sclerosis (103,104). Similarly, developing small-molecule inhibitors and ligand-directed degraders that target CaMK4 represents another potential strategy. Protein kinases are highly amenable to drug development given the plethora of approved drugs directed at this enzyme class (105). In fact, there are several potential therapeutics targeting protein kinases currently undergoing preclinical and clinical development for brain-related disorders (106). Although no potent and selective inhibitors of CaMK4 are currently available, it may be possible to pharmacologically manipulate CaMK4 indirectly by targeting the proteins that regulate it (Figure 3). For example, PP2A deactivates CaMK4 by dephosphorylating Thr200 (30,50). Significantly, the Food and Drug Administration–approved antipsychotic drug perphenazine is an activator of PP2A and may have clinical potential in counteracting some of the neurological effects of CaMK4 overactivation in the brain (107,108). Another possible strategy involves targeting the upstream kinases that activate CaMK4. As a proof of concept, overactive CaMK4 signaling caused by the gain-of-function Lys303SerfsTer28 variant has been shown to be reversed by STO-609, a pharmacological inhibitor that blocks CaMKK1 and CaMKK2 from activating CaMK4 (31).

CONCLUSIONS

Further research is needed to fully validate the CaMK4 signaling pathway as a therapeutic target and to translate these insights into clinically effective treatments for relevant patient cohorts. Tapping into the therapeutic potential of the CaMK4 signaling pathway holds promise for improving outcomes and quality of life for individuals living with profound autism spectrum disorder.

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