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Ph.D. Thesis

First insights on the signaling pathways related to CDKL5 regulation and on its possible involvement in synaptic plasticity

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“There’s plenty of room at the bottom”

Richard P. Feynman

Contents

ABSTRACT

Rett syndrome (RTT) is an X-linked form of mental retardation that occurs sporadically once every 10,000-15,000 female births. After a period of normal development (6-18 months), the patients show a rapid regression of acquired speech and motor skills and the development of several symptoms including mental retardation, seizures, intermittent hyperventilation and stereotypic hand movements. This condition is mainly stable and signs of progressive neurodegeneration are absent. Almost 80% of Rett cases are associated with mutations in the *MECP2* (methyl CpG binding protein 2) gene. MeCP2 is a nuclear protein that binds methylated DNA and recruits histone deacetylases and co-repressor complexes to suppress transcription. It belongs to the MBD family of proteins involved in the epigenetic regulation of gene-expression.

Recently, mutations in the X-linked gene cyclin-dependent kinase-like 5 (*CDKL5*) have been found in patients characterized by a subset of Rett clinical phenotypes and generally suffering of infantile spasms and severe mental retardation. The product of *CDKL5* is a serine/threonine kinase that belongs to the CMGC family; the exact functions exerted by this kinase and its regulatory mechanisms remain mainly unknown.

CDKL5 is present in the nucleus and in the cytosol of neurons and its expression shows a continued increase during development; accordingly, CDKL5 is a critical regulator of neuronal morphogenesis, neurite growth and dendritic arborization. In the cytosol, CDKL5 phosphorylates NGL-1 (Netrin-G1 Ligand 1), a regulator of early synapse formation and maturation. In the nucleus, CDKL5 binds and phosphorylates in vitro MeCP2. Furthermore, in the nucleus CDKL5 colocalizes with nuclear speckles and is probably involved in the regulation of mRNA splicing.

Recently our group has demonstrated that the expression levels and the subcellular distribution of CDKL5 are modified by neuronal activation. In particular, a glutamate bath induces in cultured hippocampal neurons the rapid exit of the kinase from the nucleus and its proteasome-dependent degradation. The significance of this response remains to be elucidated. Furthermore, BDNF induces in rat cortical cultures, a rapid phosphorylation of CDKL5.

The main aim of this work was to study how neuronal depolarization or activation by BDNF affects Cdkl5 regulation, in terms of gene transcription, post translation modifications of its final protein product and the involved signaling pathway(s). We found that, both in primary murine neuronal cultures and cortical slices, depolarization affects the expression of the gene,

both at the transcriptional and post-transcriptional levels, together with its phosphorylation state. The response is affected by the maturation stage of the treated neurons and the involved signaling pathways have been characterized. We speculate that the observed regulation of Cdkl5 during neuronal depolarization could be related to a role of the kinase in neuronal activation. Electrophysiological approaches will be required to confirm the involvement of CDKL5 in the regulation of neuronal activity; furthermore, the identification of novel interactors of Cdkl5 should help in understanding its physiological functions in the central nervous system and the pathological consequences of a malfunctioning CDKL5.

INTRODUCTION

Rett Syndrome

Rett syndrome (RTT) is a severe progressive neurodevelopmental disorder mainly affecting female patients during early childhood. The prevalence is estimated to be approximately one in 10.000/15.000 females [1]. Pediatric patients with RTT develop normally up to 6–18 months of age when the development unexpectedly slows down and the patients show general growth retardation, weight loss and muscle hypotonia. Deceleration of head growth, a sign linked to the neurological involvement, is very precocious and leads to microcephaly within the second year of life. Simultaneously, stagnation and progressive loss of the cognitive skills that patients had previously gained (speech, purposeful hand use) occur. Patients start presenting autistic features (self-abusive behavior, irritability, loss of visual contact, loss of speech and social skills, indifference to the environment) and characteristic repetitive hand stereotypies (typically wringing/washing hand-movements, but also flapping and mouthing). Other signs, proving an involvement of the central nervous system (CNS), are the loss of motor coordination, the deterioration of autonomic functions, breathing irregularities (hyperventilation followed by breath-holding and apnea) and sleep disorders [2,3]. Seizures are common in Rett syndrome (50-80% of cases [4]), often starting at 2-3 years of age and becoming most common with age through puberty. The occurrence of seizures is described as a factor associated with the worst clinical phenotype. No correlation between seizure-onset and head growth has been established [4]. Seizures range from easily controlled to medically refractory epilepsy, but the most common presentation is given by partial complex and tonic-clonic seizures [5]. Between 5 to 10 years of age it is possible to see an improvement in the autistic features and in the communication skills of the patients, although they continue to have major physical problems as osteopenia, scoliosis, and rigidity. Also emotional disorders like night crying, irritability, low mood and anxiety become less evident [6,7,8]. Adolescent girls are characterized by a severe worsening of the motor abilities consisting of rigidity, curvature of the spine and spasticity associated with deformity. Some autonomic problems can also become more acute (cold blue feet, constipation, tachy-bradycardia). During adolescence, cognition and social skills in general do not decline and a decrease of hand stereotypies may be observed [3,9]. Summarizing, the clinical outcome can be subdivided in 4 stages (**Table 1**).

Tab.1 Typical RTT syndrome clinical outcome

Period	Clinical features
STAGE 1 "**Developmental stagnation**" 6-18 months of age	decreasing head growth reduced eye contact reduced interest in environment delayed motor development
STAGE 2 "**Developmental regression**" 1-3 years of age	loss of acquired hand and speech skills stereotypies and motor difficulties regression of social communication autistic-like symptoms breathing irregularities
STAGE 3 "**Stationary state - Seizures**" 3-10 years of age	onset of seizures motor difficulties improvement in behavior and communication
STAGE 4 "**Motor worsening**" Adolescence and adulthood	reduced mobility stop walking scoliosis spasticity and deformity cognition and social interactions do not decline

Historical overview of RTT and diagnostic criteria

Andreas Rett in 1966 published the first description of a girl with the syndrome that later got his name [10]; worldwide recognition of RTT was obtained in 1983 when Hagberg and colleagues published a report describing 35 cases [11].

In 1985, Dr. Hagberg developed consensus criteria exclusively for females [12], which were afterwards modified (1988) to include also males [13]. Subsequently, after the identification

of the gene involved in most cases of typical RTT [14], those criteria were further modified to achieve a major diagnostic selectivity (International Consensus Meeting, European Paediatric Neurology Society, Baden 2001). Consequently Hagberg proposed *necessary* and *supportive* diagnostic criteria [15,16] for typical RTT syndrome, which were further revised in 2010 by the RettSearch Consortium, an international group of clinicians (**Tab.2**) [17]. The criteria proposed by Hagberg and revised in 2010 underline the concept of "regression" ("*loss*" of purposeful hand skills, "*loss*" of acquired speech, "*regression followed by recovery or stabilizatio*n"). The importance of regression for the diagnosis of RTT was described since 1986, when Goutieres and Aicardi wrote: "*The absence of normal initial development, followed by secondary deterioration and of loss of previously acquired voluntary hand grasp is especially important, as it is one of the essential traits of Rett Syndrome*" [18].

In 1985, Dr. Hanefeld described a girl with infantile spasms who also developed many characteristics of RTT syndrome. Soon after the "Hanefeld variant" of RTT (infantile spasms, early-onset seizures and RTT-like features) was reported, the discovery of a large number of other clinical variations led the International Medical Community to draw up an increasing list of "atypical" manifestations of RTT, leading to the identification of clinically independent entities. Consequently, the development of consensus criteria for variant forms of RTT became important [19]. More recently, RettSearch Consortium completed the review of the main criteria for the diagnosis of atypical RTT [17] (**Tab. 2**), including those *core features* that are essential to clarify the diagnosis of RTT or its variants [20]. The presence of regression in atypical RTT syndrome was emphasized, although the timing of appearance is different in comparison with typical RTT. In typical RTT syndrome the regression is generally present at the age of 6 months, while in atypical RTT syndrome it could be delayed. Although the majority of patients carrying mutations in *CDKL5* (cyclin-dependent kinase-like 5) are female, there are rare occurrences in males suffering from infantile spasms, early-onset epilepsy and severe mental retardation [3].

To summarize, several clinically defined variant forms of RTT have been recognized, but three distinct forms are the most frequent: a) the preserved speech variant, or "Zappella-RTT" (hand stereotypies, regression, autism), b) the congenital variant (hypotonia and mental retardation from the very first months of life), c) the early-onset seizure variant (Hanefeld variant, which starts with generalized convulsions or spasms at 10 days – 1.5 months).

Tab.2 RTT Diagnostic Criteria (adapted from Neul et al., 2010 [17])

RTT Diagnostic Criteria 2010
Main Criteria 1. Partial or complete loss of acquired purposeful hand skills 2. Partial or complete loss of acquired spoken language 3. Gait abnormalities: impaired (dyspraxic) or absence of ability. 4. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms
Required for typical or classic RTT 1. A period of regression followed by recovery or stabilization 2. All main criteria and all exclusion criteria 3. Supportive criteria are not required, although often present in typical RTT
Required for atypical or variant RTT 1. A period of regression followed by recovery or stabilization 2. At least 2 out of the 4 main criteria 3. 5 out of 11 supportive criteria
Exclusion Criteria for typical RTT *1*. Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems 2. Grossly abnormal psychomotor development in first 6 months of life
Supportive Criteria for atypical RTT 1. Breathing disturbances when awake 2. Bruxism when awake 3. Impaired sleep pattern 4. Abnormal muscle tone 5. Peripheral vasomotor disturbances 6. Scoliosis/kyphosis 7. Growth retardation 8. Small cold hands and feet 9. Inappropriate laughing/screaming spells 10. Diminished response to pain 11. Intense eye communication - “eye pointing”

Typical RTT syndrome is related to *MECP2*-gene mutations

Given that the majority of patients with RTT are females, it had been speculated for many years that genetic defects in the X chromosome might be involved and that an X-linked dominant mode of inheritance could explain the male lethality in this condition. RTT syndrome was initially described as a "*postnatal phenotypic manifestation of a prenatal developmental alteration*" [21,22] and linkage studies were not possible, since more than 99% of RTT cases are sporadic. However, in 1998 a family with a maternal inheritance was identified, permitting exclusion mapping studies and leading to the identification of the Xq28 locus (1-5) as a candidate region for the involved gene. Using a systematic gene screening approach, Amir and colleagues identified the first mutations in the *MECP2*-gene (methyl CpG binding protein 2) in 5 sporadic cases. It is now clear that not all RTT patients carry mutations in *MECP2*; indeed, by considering typical RTT patients, *MECP2* mutations can be identified in almost 90-95% of cases. The number of *MECP2*-negative patients increase to up to 30-50% when considering patients with atypical presentations of RTT.

MeCP2 structure and functions

The *MECP2* gene is located at q28 of the human X chromosome and four exons code for the two different isoforms of the protein derived from alternative splicing of exons 1 and 2. Furthermore, four different transcripts, differentially expressed in nervous and non-nervous tissues, can be alternatively generated because of a large 3'-untranslated region containing multiple polyadenylation sites.

The *MECP2* gene codes for a nuclear protein, MeCP2, that constitutes the founding member of the Methyl-CpG binding protein family. Indeed, MeCP2 was isolated in 1992 as a protein capable of binding selectively to methylated DNA containing just one single symmetrically methylated CpG [23]. MeCP2 is composed of four functional domains (**Fig.1**): the methyl-binding domain (MBD), the transcriptional repression domain (TRD), a nuclear localization signal (NLS) and the WW domain binding region [123]. The MBD, located in the N-terminus, is sufficient to direct specific binding to methylated DNA whereas the TRD, located in the central core of the protein, recruits transcriptional corepressors such as c-Ski, N-CoR and mSin3A, which interact with class I histone deacetylases, therefore regulating chromatin conformation and gene-transcription. The WW domain (also known as WWP domain) of the MeCP2 interactors is a short domain with two signature tryptophan residues, that provides a platform for the assembly of multiprotein networks involved in molecular processes, such as

transcription and RNA processing [24].

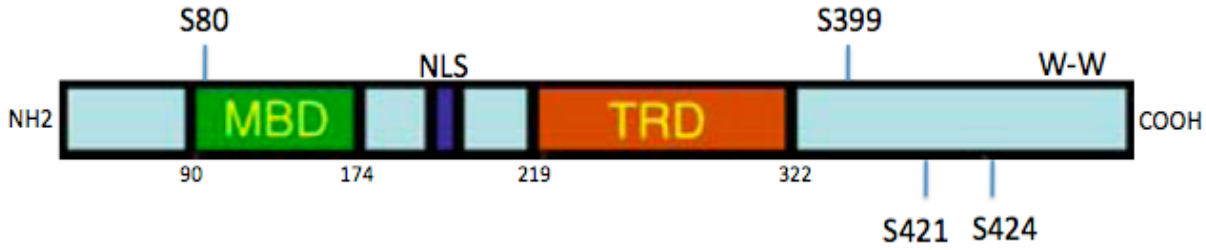


Figure 1. **A schematic representation of MeCP2** illustrating the positions of the methyl-CpG-binding domain (MBD), the nuclear localization signal (NLS) and the transcriptional repression domain (TRD). Phosphorylation sites relative to serine 80, serine 399, serine 421 and serine 424 are also reported (adapted from [38])

The expression of MeCP2 is ubiquitous in peripheral tissues and in CNS, with highest expression levels in neurons. During embryogenesis, the expression levels of MeCP2 in neurons increase [25] and correlate with the maturation of the central nervous system; indeed, in mouse, Mecp2 appears first in ontological older tissues (spinal cord and brainstem) and then in cortex, where the expression in deeper cortical layers precedes that in the superficial layers [26]. The modulation of MeCP2 expression is complete only when cells reach maturity and, in humans (but not in mice), expression increases also during the post-natal period (until the age of 10 years).

The functions of MeCP2 in the nucleus are mainly directed to the regulation of gene expression in the context of epigenetic mechanisms linked to the presence of methylated DNA sequences.

Considering the well-known role of DNA methylation in transcriptional silencing, several laboratories have used candidate approaches and genome-wide expression profile studies to identify direct target genes of MeCP2. These studies have contributed to the notion that MeCP2 might function as transcriptional repressor of brain derived neurotrophic factor (BDNF) gene [27-29]. However, this first suggestion has not always been confirmed by following reports and so far no bona fide MeCP2-target genes are univocally recognized. Also the first classification of MeCP2 as a transcriptional repressor is reductive considering a recent work showing the ability of MeCP2 to activate the transcription of some genes through the association with CREB1 [30,31]. Furthermore, it has been demonstrated that MeCP2 can function as an architectural chromatin protein, functioning directly without corepressor or

enzymatic activities [39]. Indeed, depending on the molar ratio of MeCP2 to nucleosomes, MeCP2 assembles novel secondary and tertiary chromatin structures. Importantly, recent data finely measuring the abundance of MeCP2 in neurons have led to hypothesize that in brain MeCP2 could be considered a *global regulator* of chromatin architecture, coating all chromosomes by tracking methylated DNA. In accordance with a structural role of MeCP2, its deficiency leads to global changes in chromatin structure, such as an increase in histone acetylation and H1 levels. Indeed, MeCP2 can substitute histone H1 in methylated chromatin [32,33].

Besides a role of MeCP2 in regulating gene expression and chromatin structure, a role in regulating protein synthesis has also been hypothesized. Indeed, a recent publication has also shown that both *Mecp2*-null hemizygous males and heterozygous females are characterized by a reduction in the AKT/mTOR signaling (a crucial pathway involved in controlling neuronal cell soma, dendrite arborization, synaptic function, structure and plasticity) and a significant impairment in protein synthesis [39]. It is worthwhile to recall that aberrant neuronal protein synthesis is considered a probable cause of the clinical features of autism spectrum disorders; thus these data might suggest a novel, direct or indirect function of MeCP2 that could be relevant for understanding the pathogenesis of RTT.

A second mechanism by which MeCP2 could be implicated in the regulation of protein synthesis is represented by its role in mRNA-splicing processes. Indeed, in the mammalian brain, MeCP2 directly interacts with the splicing factor *Prpf3* (*pre-mRNA processing factor 3*), forming a novel *brain-derived* MeCP2 *complex* proposed to regulate mRNA splicing. This function of MeCP2 could be related with RTT pathophysiology, since many *MECP2* RTT truncations disrupt the MeCP2-Prpf3 complex, probably leading to the aberrant mRNA splicing evident in a mouse model for RTT [40].

A new approach for the comprehension of MeCP2 functions is given by the study of its site-specific phosphorylation. Indeed, it has been demonstrated that MeCP2 functions in the nucleus are regulated by specific events of phosphorylation that affect its activity in response to extracellular cues. In particular, in rodent brain, serine 80 (S80) and S399 are the two major phosphorylation sites of MeCP2 under resting conditions, whereas S424 and S421 show specific depolarization-dependent phosphorylation [37, 38]. It has been suggested that S80 phosphorylation increases the affinity of MeCP2 for euchromatin whereas the modification of S421 was found *in vitro* to lead to a decrease in the binding of MeCP2 to specific methylated promoters. We still need to reveal the consequences of these post-translational modifications for gene expression. Furthermore, very recent *ChIP-seq analyses* of the phosho-S421 isoform of MeCP2 suggest that *in vivo* this form is globally bound to methylated DNA, therefore

questioning once again the previous results. Furthermore, the kinases and the phosphatases involved in the post-translational modifications of MeCP2 remain mainly uncharacterized even though the involvement of CaMKIV in the phosphorylation of S421-MeCP2 and of HIPK2 in S80 phopshorylation have been suggested. CDKL5, the kinase mutated in some patients with a RTT variant (see below), is also capable of phosphorylating MeCP2 *in vitro* on yet non-identified sites [41]. This observation underlines the importance of MeCP2-phosphorylation in the clinical presentation of RTT [243]. Accordingly, some symptoms observed in RTT patients, such as the weight gain and the decreased locomotor activity, are also present in a *knock-in* mouse model carrying a substitution of S80 with the non-phosphorylatable alanine (S80A), whereas a S421/424A model is characterized by hyperactivity [37].

MECP2 mutations

So far, hundreds of different mutations in *MECP2* have been described, including missense, nonsense, frameshift mutations, wide deletions of whole exons and complex rearrangements. Eight missense and nonsense mutations (in the MBD and in the TRD) account for approximately 70% of all mutations. These so called "*hot spot mutations"* are C to T transition mutations, probably caused by unrepaired deamination of methylated cytosine bases in the paternal germline. Small C-terminal deletions account for approximately 10% of mutations: indeed, a section with repetitive sequence elements between nucleotides 1050 and 1200, encoding the C-terminus of MeCP2, is a *hot spot* for this kind of deletions and for larger rearrangements present in around the 16% of the remaining patients. Alterations in the expression levels of MeCP2 could also be related to neurologic disfunctions, like the *MECP2* duplication syndrome (hypotonia, mental retardation, poor speech development, progressive spasticity, seizures), 100% penetrant in males [124,125]. Mouse models have confirmed that RTT-like features can be caused both by loss of expression, underexpression and overexpression of Mecp2 [42,43]; this is important considering that the MeCP2 levels are sensitive also to the presence of some point mutations [44].

The establishment of a link between genotype and phenotype in RTT patients is not easy because of the X-chromosome inactivation that renders females mosaics for the mutation [45]. However, several genotype-phenotype correlation studies have been reported and some data are starting to emerge. Some clinical differences between patients with truncating versus missense mutations have been described [46]. Generally, point mutations involving the MBD

or the NLS, affecting the possibility of MeCP2 to bind methylated DNA, and truncating mutations in the N-terminus of the protein lead to a more severe phonotype [79]. On the contrary, point mutations affecting the TRD (e.g. R306C, associated only with language disabilities [77]) or late-truncating mutations, where the functions of the protein are partially conserved, are usually associated with a less severe phenotype [78]. In a large cohort of typical RTT syndrome, patients with the early truncating mutation R168X showed very poor abilities to walk, to use the hands and to speak in comparison with the patients carrying the R294X truncation [77]. In addition, different mutations in the MBD, probably affecting DNA binding to different degrees, are related to more severe (e.g. T158M, a mutation found in particularly ataxic and rigid patients) or more favourable (e.g. R133C [48,76]) prognosis.

Atypical RTT syndrome is frequently caused by mutation in genes other than *MECP2*

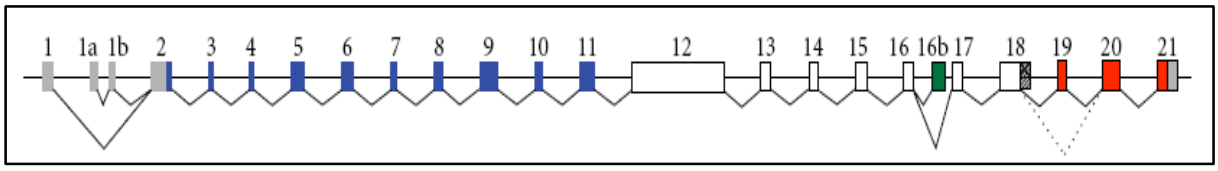
Mutations in loci other than *MECP2* have been found in some atypical RTT patients. In particular, the genes identified so far are related to two different and well characterized RTT variants: the *early-onset seizure variant* and the *congenital form*. The first gene to be identified was *CDKL5*, located on the X-chromosome and coding for the serine-threonine kinase *cyclin-dependent kinase-like 5*. Mutations in *CDKL5* were for the first time described in 2003 [47] in two unrelated epileptic patients, at the age of respectively two and three months, with identical phenotypes: early-onset severe infantile spasms, global developmental arrest, hypsarrhythmia and severe mental retardation. In 2004, Weaving et al. demonstrated for the first time the presence of *CDKL5*-mutations in a girl with a RTT-overlapping phenotype, in her sister with autistic disorder and in her brother with profound intellectual disability and seizures who died at the age 16 years. *MECP2* mutations were absent in these patients and three candidate regions were identified by microsatellite mapping (Xp22.31-pter; Xp22.12-p22.11 and Xq21.33); the authors sequenced two genes, the *ARX* and *CDKL5* genes, located within the Xp22 region, leading to the establishment of the involvement of *CDKL5* in RTT [48]. In 2005, Scala et al. [80], by analyzing two *MECP2*-negative patients affected by the Hanefeld variant of RTT and by considering the clinical overlap between the Hanefeld variant and West syndrome, studied the same two genes *CDKL5* and *ARX*; *CDKL5* frameshift deletions were found in both patients, confirming the involvement of this gene in RTT. It is important to mention that *CDKL5* mutations have never been found in patients with typical Rett syndrome, thus linking this gene to a very specific clinical picture.

The second gene involved in atypical RTT syndrome was identified by Ariani et al. [49] in 2008; the authors reported the presence of *FOXG1*-gene truncation mutations in two patients affected by the *congenital variant* of Rett syndrome. The *FOXG1* gene is located on chromosome 14 and codes for a brain-specific transcription repressor, forkhead box protein G1.

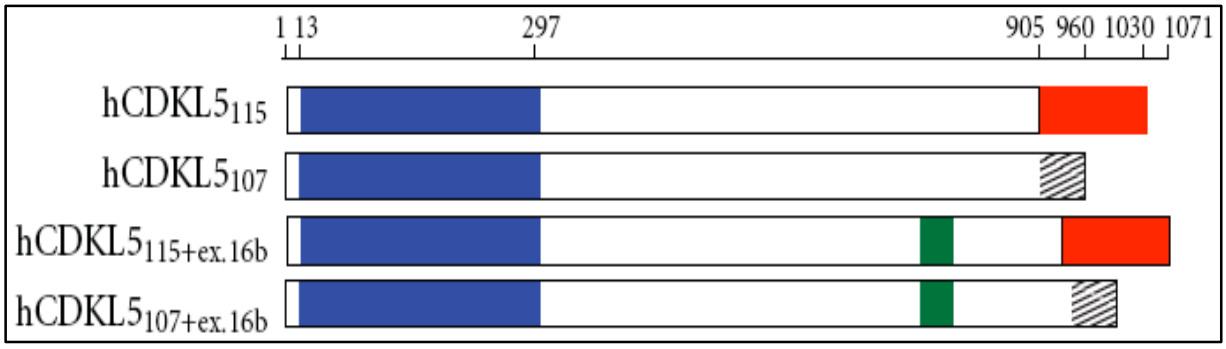
CDKL5 structure

The human *CDKL5* gene (Xp22 [50,51]) is composed of 24 exons of which the first three (exons 1, 1a and 1b) are untranslated [52]. Due to alternative splicing of exons 19-21 and exon 16b, four different hCDKL5 isoforms might exist, differing within the C-terminal region. The first isoform to be identified was the 1030 a.a. protein (CDKL5-115; 115 kDa) that is specific for primates and is mainly expressed in testis. A second isoform is a 960 a.a. protein (CDKL5-107; 107 kDa), conserved in human and mouse, that seems to be the predominant one in brain. The presence of exon 16b would generate the CDKL5-115+ex.16b and/or CDKL5-107+ex.16b (**Fig.2**). At the functional level, the only difference that has been observed between these isoforms is their stability: in fact, CDKL5-107 appears to be more stable than the longer human CDKL5-115 isoform [53]. Considering the first non-translated exons (1, 1a and 1b), other two *CDKL5*-mRNA splice variants with distinct 5'UTRs have been found: isoform I, containing exon 1, is present in a wide range of tissues, whereas isoform II, containing exons 1a and 1b, is present only in testis and fetal brain [51,52,53].

A)



B)



C)

Fig.2 The genomic structure of *CDKL5* and its splice variants.

A) The human *CDKL5* gene can be subdivided in 6 regions: I) the non translated exons 1, 1a and 1b (gray), II) the exons encoding the catalytic domain (blue), III) the exons encoding the common C-terminal region (white), IV) exons 19-21 specific for CDKL5-115 (red), V) exon 16b (green), VI) intron 18 retained in CDKL5-107 (hatched).

B) Human CDKL5 protein isoforms differing in the C-terminal region (the color code corresponds to that of panel A).

C) The murine CDKL5 isoforms. a) mCDKL5-105 harbors a distinct C-terminal region encoded by a mouse-specific exon 19 (orange); b) mCDKL5-107 isoform, with the retention of intron 18. Adapted from [52]

In expression studies in human and mouse tissues, the *CDKL5*/*Cdkl5* mRNA was detected in a wide range of peripheral districts such as testis, lung, spleen, placenta, uterus and prostate, but highest levels were found in the brain [50,52,53].

Cdkl5-mRNA levels in adult mouse brain are particularly high in the forebrain. Interestingly, higher expression levels are detected in the most superficial cortical layers, involved in the intercortical connectivity. In particular, there are some cortical areas in which the mRNA-expression is more evident (the frontal cortex, the motor cortex and the cingulate gyrus), suggesting a region specific role of *CDKL5* and the putative involvement of the kinase in mental diseases related to the physiology of these areas [52]. High expression levels detected

in the entorhinal cortex and in the hippocampus might suggest an involvement of *CDKL5* in high cognitive functions like learning, memory, and the development of individuality. Between different neuronal sub-populations, there are also differences in the expression of *Cdkl5*: the glutamatergic and the gabaergic neurons (the main populations in the striatum) are the two cellular types expressing highest CDKL5 levels in brain; in particular, *Cdkl5* is expressed in both glutamatergic and GABAergic primary mouse cultured neurons, but is certainly better detectable in the latter cell type [52,54]. Specific future experiments are required to explain the role and the regulatory mechanisms of *Cdkl5* expression in different brain regions, including the thalamic geniculate nuclei and the cerebellum where the transcripts are also detected even if at later stages.

Studies of CDKL5-protein expression applied to adult rat extracts mainly confirmed the mRNA-expression studies [56]. At the cellular level, the kinase is highly expressed in virtually all NeuN-positive neurons while very low levels are present in the glia [56]. In mouse brain, the kinase is widely distributed in hippocampus, cortex, thalamus, and striatum, mainly in neurons [52,54].

The expression profile of *Cdkl5* is finely regulated during development: indeed, the kinase is only minimally detectable at embryonic stages, it is induced during late pre-natal and early post-natal stages and its levels increase till reaching a plateau at P14 [54].

The *CDKL5* gene codes for a serine-threonine kinase, named CDKL5 (Cyclin-Dependent Kinase–Like 5), belonging to the CMGC family of kinases which includes the family of cyclin-dependent kinases (CDK), the mitogen-activated protein kinases (MAP kinases or *extracellular-signal-regulated kinases* (ERKs)), the glycogen synthase kinases (GSK) and the CDK-like kinases.

The catalytic domain of CDKL5 is homologous to that of the other CDKL-family members, while the presence of a long C-terminus of more than 600 amino acids is unique for CDKL5.

The catalytic domain of CDKL5 (**Fig. 3**), which is located in the N-terminal portion of the protein (13-297 a.a.), presents three kinase signatures homologous to other serine–threonine kinases:

1) the ATP-binding motif, located between amino acids 13-43 a.a., with a glycine-rich stretch close to a lysine residue (K42) involved in ATP binding [60];

2) the serine/threonine protein kinase active site, between amino acids 127-144, with a conserved aspartic acid (D135) important for the catalytic activity [60];

3) the "activation-loop" with a DFG domain and a TEY motif (DFGFARNLSEGNNANYTEY; 153-171 a.a.). It is important to mention that the universally conserved DFG motif forms polar contacts with other regions of the kinase, stabilizing its active conformation either directly or through the coordination of magnesium atoms; the TEY motif, mainly studied in the activation loop of ERK kinases is dually phosphorylated during activation by the MEK-MAPKK (MAPK/ERK kinase - MAPK kinase) [62]. It has been shown that CDKL5, as some other members of the CMGC group, is capable of autophosphorylating its TEY motif [55,61].

The long C-terminal tail of CDKL5 (from a.a. 298) that does not share homology with other human proteins acts as a negative regulator of the catalytic domain and is also involved in the regulation of the stability of CDKL5 [55]. Three putative signals, regulating the intra-cellular localization of CDKL5, are present in the tail: two *nuclear localization signals* (NLS) and one *nuclear export signal* (NES), as shown in Fig.3. Lastly, three PxxP sites in the C-terminal tail (within 525 a.a. and 781 a.a.) constitute a putative binding site for Src homology 3-domain proteins [55].

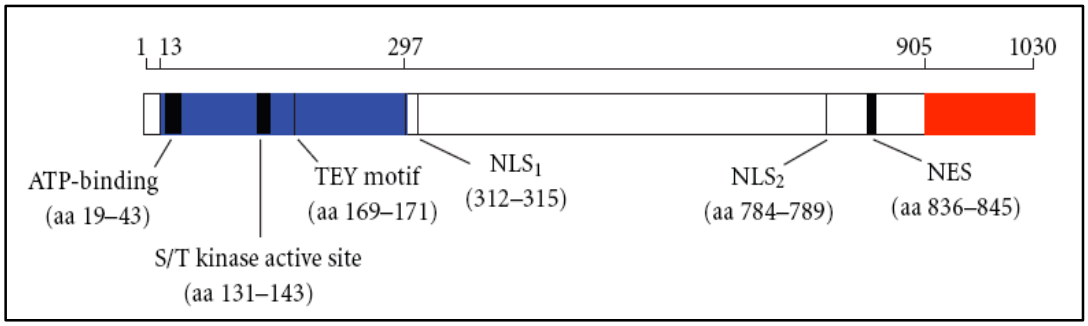


Fig.3 Schematic representation of CDKL5-115 with the functional domains and signatures indicated. In blue the catalytic domain with the ATP-binding site and the TEY motif. In white and red the COOH-terminal tail. NLS: nuclear localization signal; NES: nuclear export signal (adapted from [52]).

CDKL5 mutations

Almost 90 different *CDKL5* patients have been described so far, harboring a wide range of clinical conditions. Data from genetic screenings for *CDKL5* in cohorts of patients with atypical RTT syndrome show a wide range of pathogenic mutations including missense and nonsense mutations, deletions, insertions, aberrant splicing and frameshifts (**Fig.4**).

Among almost 75 identified mutations so far, only 5 can be considered as "*hot* spots"

(indicated with an asterisk in Fig.4) [52].

Missense mutations localize mainly in the catalytic domain, confirming the relevance of the kinase activity of CDKL5 for proper neuronal functions, and lead generally to loss of funtions. Two missense mutations in the catalytic domain (C152F and R175S) have been suggested to interfere with, respectively, phosphotransfer and either kinase activation or substrate specificity [61]. On the contrary, truncating mutations occur anywhere in the gene.

The relevance of the rather uncharacterized C-terminal part of CDKL5 is suggested by the fact that many pathogenic alterations involve this region. The C-terminal tail is, as already mentioned, probably involved in regulating the catalytic activity and the subcellular localization [52].

Regarding the subcellular localization of CDKL5, the protein is present in both the cytoplasm and nucleus of expressing cells. Importantly mis-localization of the kinase is evident in both the pathogenic derivatives, L879X and R781X, causing the truncation of the very last portion of the C-terminus and confining CDKL5 to the cell nucleus.

Some studies have reported pathogenic duplications of X chromosome regions including *CDKL5* [52,70] suggesting that also CDKL5 levels must be finely tuned within the brain. This is in accordance with the fact that the expression of the kinase is finely regulated in developing and mature neurons.

So far, no clear genotype-phenotype correlation of CDKL5 mutations have been established. Some reports, not always confirmed, suggested that mutations in the C-terminal tail originate milder clinical pictures than those caused by mutations in the catalytic domain [52]. The phenotypic variability of the patients is probably due to the different penetrance of mutations [71] and to skewed X-Chromosome inactivation. Weaving et al. [48] reported two genetically identical *CDKL5*-mutated twin girls with a significant discordant phenotype (the first proband showed a phenotype overlapping RTT, while her sister showed autistic disorder and mild-to-moderate intellectual disability); in this particular case, the reason of these different phenotypes could be attributed primarily to environmental and/or epigenetic factors.

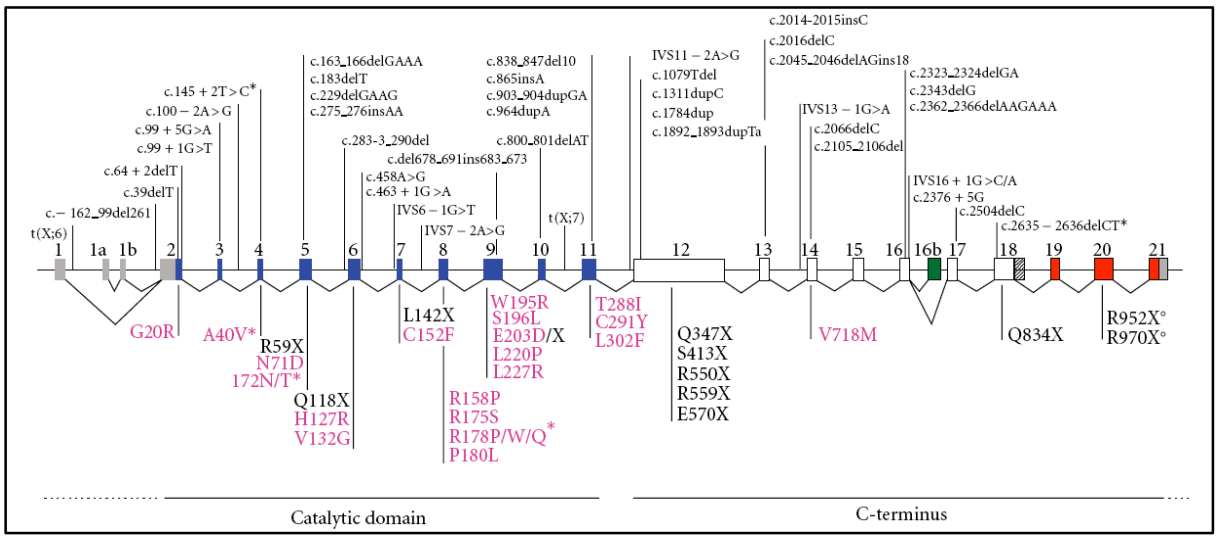


Fig.4 All mutations in *CDKL5* reported to date. Mutations shown above the *CDKL5* gene are deletion and frame shift mutations as well as splice variants indicated with cDNA nomenclature. Missense and nonsense mutations (fuchsia and black, resp.) are represented with amino acid nomenclature below the *CDKL5* gene. ∗: recurrent mutations; □: uncertain pathogenicity. Adapted from [52].

CDKL5 functions

Our present knowledge of CDKL5 functions is still rather limited, but first evidences suggest the involvement of the kinase in processes related to the regulation of gene expression and neuronal morphogenesis. CDKL5 is present in neurons both in the nucleus and in the cytosol, where it interacts with specific proteins. The study of these interactions provide a platform to understand the functions of CDKL5, the signaling pathways in which it could be involved and, finally, the relationship between *CDKL5* mutations and the pathogenesis of Rett syndrome. In the nuclear compartment of primary hippocampal neurons (E18, DIV10-12), endogenous CDKL5 shows a diffuse staining with brilliant nuclear dots that do not overlap with heterochromatic DAPI-positive DNA [63], while the cytosolic CDKL5-immunostaining is finely dotted in both the soma and along the dendrites. Similarly, in adult (P21) mouse brain the punctate pattern of CDKL5-immunoreactivity is evident in cell bodies, along dendrites and in synaptic spines [64]. In the nucleus, clinical and biochemical data suggest that CDKL5 belongs to the same molecular pathway as MeCP2, since CDKL5 binds, both *in vitro* and *in vivo*, and phosphorylates MeCP2 *in vitro* [59]. Two other nuclear CDKL5-interactors have also been found, both involved in the regulation of gene expression. The first was DNMT1 (*DNA (cytosine-5)-methyltransferase 1*), an enzyme that recognizes and

methylates hemimethylated CpG after DNA replication. Indeed, it was found that a truncation derivative of CDKL5, containing only the catalytic domain, binds and phosphorylates in vitro DNMT1 in the N-terminal region in the presence of DNA [65]; further studies are required to confirm the interaction *in vivo* and with a full length kinase. The second nuclear CDKL5-interacting protein is the SR-family splicing factor, SC35, which co-localizes with CDKL5 in the neuronal *nuclear speckles* both *in vitro* and *in vivo* and also co-immunoprecipitates with the kinase [66]. Regarding the putative functions of CDKL5 in the nucleus, we can conclude that this kinase could represent an important regulator of gene expression via different molecular interactions, but so far no *bona fide* CDKL5-target genes are univocally recognized.

In the cytoplasmic compartment, CDKL5 was recently found to participate in the regulation of the excitatory dendritic spine development and dendritic morphogenesis. The first regulatory mechanism involves the molecular interaction between CDKL5 and NGL-1 (*Netrin-G1 Ligand 1*), a transmembrane protein localized in the Post Synaptic Density compartment (PSD) [64]. NGL-1 specifically interacts with Netrin-G1, a lipid-anchored protein related to the netrin family of axon guidance molecules, promoting the early synapse formation and subsequent maturation [67]. Among PSD-enriched proteins, the three components of the NGL-family (NGL-1, NGL-2 and NGL-3) were identified as CDKL5-interacting partners. CDKL5, which co-localizes both *in vitro* and *in vivo* with PSD95 and with excitatory synapse markers (Shank, NR2 and GLUT1), binds and phosphorylates NGL-1 *in vitro* and this phosphorylation strengthens the interaction between NGL-1 and PSD95 [64]. On the other hand, CDKL5 participates in neuronal morphogenesis regulation through the interaction with Rac1 (*Ras-related C3 botulinum toxin substrate 1*), a critical regulator of actin remodeling. Accordingly, this small signaling G protein, which is a member of the Rho family, appears to regulate cell growth and cytoskeletal reorganization [68]. BDNF stimulation enhances the interaction between CDKL5 and Rac1, leading to the effects of CDKL5 on neuronal morphogenesis (see below). Furthermore, CDKL5 was found to co-localize with F-actin in the peripheral domain of growth cones in cultured neurons at DIV2, suggesting that CDKL5 might be involved in regulating the actin cytoskeleton also in this compartment [56].

Chen *et al*. [56] recently studied the effects of CDKL5 silencing in rat hippocampal primary cultures and reported a decrease in total length of both dendrites and axons (DIV3 neurons silenced at DIV0), and a marked reduction in dendritic arborization (DIV8 neurons silenced at DIV5). The same data were confirmed *in vivo* (P4 rats) when CDKL5 was silenced in neuronal progenitors through *in utero* electroporation at E15. In these experiments, also the

migration of neurons in the cortex showed defects. Migration was not blocked but delayed, since neurons devoid of CDKL5 were able to migrate into cortical layers during the first post-natal days, extending apical dendrites toward the pial surface by P14. The impairment in dendritic arborization was not a secondary consequence of the migratory defect because similar deficits in arborization were seen using a less effective shRNA construct that had little effect on neuronal migration.

In a recent paper, Ricciardi *et al*. demonstrated the importance of CDKL5 in the development of dendritic spines. Knocking down CDKL5 in mouse primary neuronal cultures increases the dendritic protrusion density and alters their morphology: the protrusions are thinner and show a filopodia-like configuration. These morphological alterations are associated with a reduction in the number of excitatory synapses and synaptophysin puncta. The electrical counterpart is represented by a significant decrease of mEPSCs, suggesting an involvement of CDKL5 in the regulation of neuronal activity. Confocal images of coronal slices of P11 mouse brain after CDKL5-silencing by *in-utero* electroporation at E13.5 confirms the data *in vitro*: cortical pyramidal neurons showed an increase in the protrusion density along with abnormal morphology. In the same neurons also the density of VGLUT-1 puncta, identifying excitatory pre-synaptic buttons, was reduced.

Altogether these data suggest that, since the regulation of dendritic and spine morphogenesis is one of the essential target points of synaptic plasticity, CDKL5, showing a crucial role in these processes, could be considered as an interesting new plasticity-related molecule.

Rett syndrome is characterized by a number of synaptic deficits [69] and the impairment in synaptic plasticity could explain, although partially, some clinical features such as motor disabilities, the speech absence or the severe cognitive dysfunctions. Interestingly the expression of CDKL5 increases during development, correlating with neuronal maturation and the formation of new complex synaptic networks in cortical and sub-cortical areas. Other molecular processes linked to neuronal activation during synaptic plasticity (transcription and post-transcriptional events) could also be regulated by CDKL5 via specific interactions with transcriptional regulators and epigenetic factors (MeCP2, DNMT1), or with splicing factors (SC35).

Lastly, to complete this hypothetic overlap between CDKL5-functions and plasticity-related processes, we can mention the response of the kinase to specific neuronal stimuli. In a recent publication [63], the sensitivity of CDKL5 to glutamate, the most abundant excitatory neurotransmitter, was tested in hippocampal cultured neurons in terms of expression and sub-cellular localization of the kinase. A rapid exit of CDKL5 from the nucleus, followed by a massive proteasome-dependent degradation was observed after glutamate treatment. The

meaning of this response remains to be elucidated, but probably the expression levels and the sub-cellular localization of the kinase could be related with different roles played by different molecular interactions. Interestingly, the kinase-dead K42R-CDKL5 is much less abundant in the nucleus than in the cytosol [61], suggesting a link between the kinase activity and its sub-cellular localization. Other mechanisms, related to unknown signals during development, can regulate this nucleocytoplasmic distribution since the abundance of CDKL5 in the nucleus increases during development and reaches, in several brain districts of adult mice, almost 50% of total CDKL5 levels [54,63]. In the future it would be very interesting to understand better the response of CDKL5 to different stimuli and the signaling pathways involved in regulating its expression and sub-cellular localization in different developmental stages.

RESULTS

CDKL5 expression during early neuronal activation

As already mentioned, recent biochemical data suggest that CDKL5 is involved in proper neuronal functions and in the regulation of dendritic and spine morphogenesis [56,64], but very little is known about the molecular pathways regulating its activities in developing and mature neurons. It is already known that CDKL5 in neurons is sensitive to specific neuronal stimuli, such as glutamate, that regulates the sub-cellular localization and degradation of the kinase [63]. Therefore, we decided to investigate the response of CDKL5, in terms of transcription, translation, post-translational modifications, and sub-cellular localization to neuronal depolarization induced by KCl treatment. We started evaluating the endogenous Cdkl5-expression levels in hippocampal primary cultures prepared from E18 mouse embryos before and after KCl treatment. Neurons were cultured for 3, 7 and 12 days *in vitro* (DIV), and were exposed for 5 min (minutes) to 50 mM KCl or, as control, to KRH containing 5 mM KCl. Neurons were directly collected in Laemmli buffer and Cdkl5-expression was assayed by Western blotting (8% SDS-PAGE) using the signal of neuron-specific Class III β-tubulin (Tuj1) as internal standard. Moreover, the efficacy of the KCl treatment in activating neurons was assessed by the increase in Erk-1/2 phosphorylation using a phospho-specific antibody. Interestingly, we found that in KCl treated neurons, Cdkl5-expression was increased significantly up to 60% (**Fig.5A, B**), while other proteins that are known to play essential roles in the early phase of neuronal activation (c-Fos, Erk-1 and Erk-2) did not change their expression levels during the first 5 min of stimulation (**Fig.5A, C, D**). In accordance with literature, we found an increase in c-fos expression only after 40 min of KCl treatment (data not shown).

In agreement with a recently published article [54], we found that endogenous Cdkl5 levels gradually increase with maturation *in vitro* from DIV3 to DIV12. Furthermore, KCl treatment caused an increase in Cdkl5 protein levels also when DIV3, 7 and 12 hippocampal neurons were treated for 5 min. Moreover, similar results were obtained when cortical primary neuronal cultures obtained from E18 mouse embryos were treated with KCl (data not shown) altogether indicating that the KCl induced increase in Cdkl5 levels does not depend on maturation stage and neuronal type. To investigate the mechanisms involved in the KCl-dependent activation of the *Cdkl5*-gene, we analyzed the synthesis of the kinase at the

transcriptional and translational levels after 5 min stimulation with KCl. For the transcriptional response, total RNA was extracted from treated and non-treated hippocampal primary cultures (DIV7) and *Cdkl5*-mRNA levels, along with those of c-fos-mRNA, were analyzed by quantitative RT-PCR (qPCR). Both mRNA levels, *Cdkl5* and *c-fos*, were significantly increased during early depolarization (+34% and +59% respectively, **Fig.5E**).

Since an increase in mRNA content reflects a change in gene transcription and/or a modification of mRNA stability, we used actinomycin-D to block RNA pol II activity allowing us to analyze whether the increase in *Cdkl5* mRNA levels might be due to a KCl induced alteration of the mRNA half life. Transcription was inhibited by treating neurons with 20 µg/ml of actinomycin-D for 30 min whereafter depolarization was obtained by a 5 min KCl treatment. No increase in *Cdkl5*-mRNA and c-fos-mRNA levels were observed, suggesting that the KCl-dependent activation of *Cdkl5* is mediated by transcriptional activation (data not shown).

A prolonged stability of the kinase might also participate in the observed up-regulation; therefore, cultured neurons were treated with cycloheximide (an inhibitor of translation elongation) for 30 min before applying KCl; no increase in the level of Cdkl5 expression was found, suggesting that protein synthesis plays a dominant role in the observed response (**Fig. 5F**).

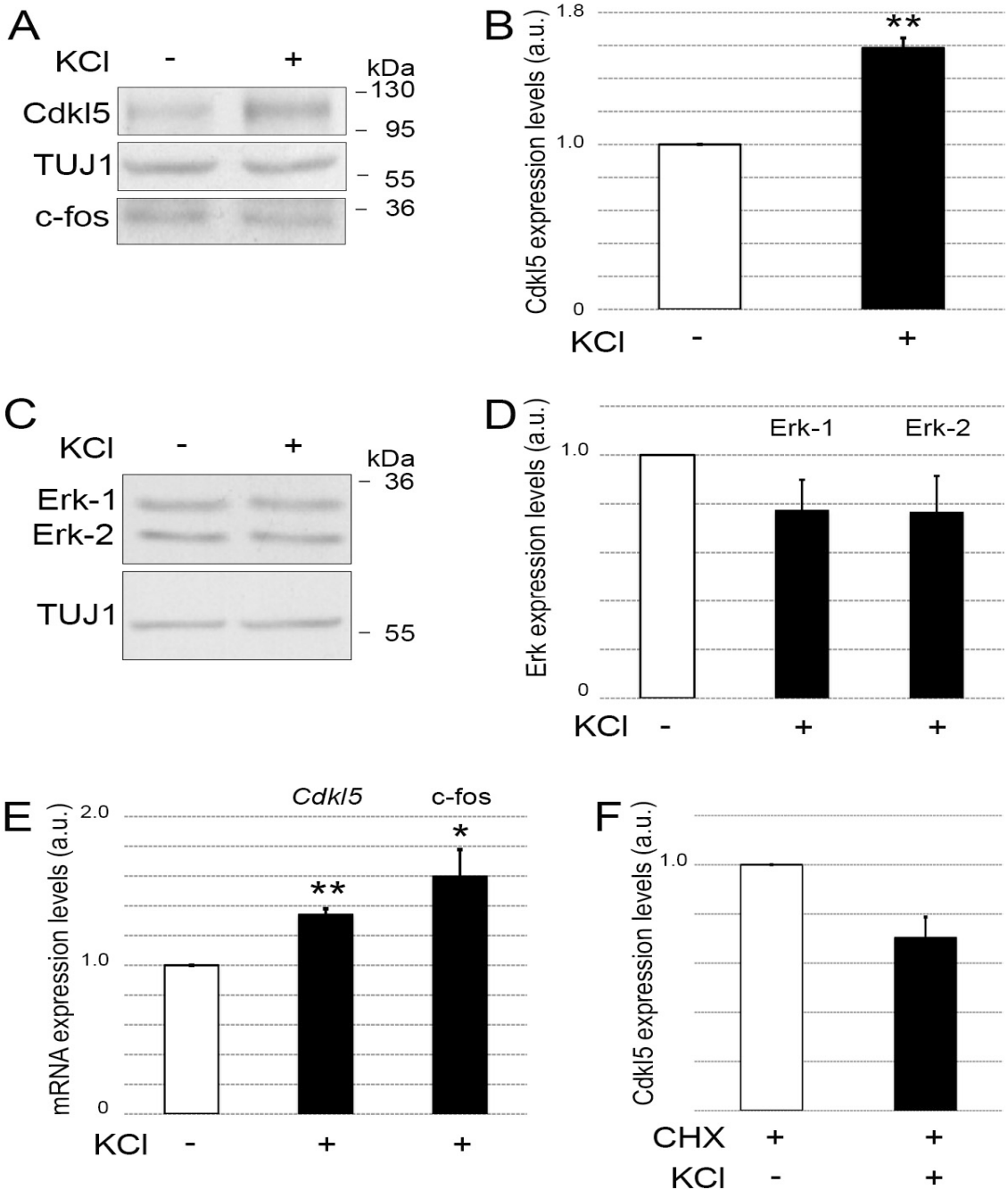


Fig. 5 Cdkl5 expression is induced during early neuronal depolarization in hippocampal neurons *in vitro*. Hippocampal neurons were isolated from E18 mouse embryos and treated with 50 mM KCl or, as control, KRH for 5 min at DIV7. All values are expressed as the average of at least 3 different experiments ± standard error of the mean (SEM). The significance was evaluated by Student's *t* test and statistical significance was established as $p < 0.05$ (*) or $p < 0.01$ (**).

A) Western blot analysis of Cdkl5 and c-fos expression in E18-DIV7 hippocampal neurons treated with KCl as indicated. Beta-tubulin III (TUJ1) was used as internal standard. **B)** Statistical analysis (t-test, N>3) after densitometric quantification (QuantityOne Biorad software). **C, D)** Western blot analysis of Erk-1/2 expression in treated neurons and t-test statistical analysis. **E)** *Cdkl5* and *c-fos* mRNA levels determined by RT-qPCR (N=3). Total RNA was collected from E18-DIV7 hippocampal neurons treated for 5 min with 50 mM KCl and subjected to RT-qPCR analysis. *c-fos* mRNA levels were used as indicator of neuronal activation, and the

expression of *Gapdh* as internal standard. The statistical analysis was performed by using the "ΔΔCt method" to compare relative expression. **F)** Graphic illustration showing Cdkl5 protein levels in E18-DIV7 hippocampal neurons treated for 30 min with 40 μM cycloheximide (CHX) and then exposed to 50 mM KCl for 5 min. No increase in the level of Cdkl5 protein levels was found. Statistical analysis (t test, N=3) after densitometric analysis of WB (QuantityOne Biorad software).

To validate these results with a different model of neuronal activation, KCl was applied to cortical slices. Frontal cortical slices of adult mice (P35-P38) were prepared and maintained alive in a solution saturated with 95% oxygen and 5% carbonic anhydride. Depolarization was obtained with a perfusion buffer (1 ml/min) containing 30 mM KCl for 5 min [110-112]. Cdkl5 levels in depolarized slices were determined by Western blotting and compared with those in paired slices perfused for the same time using a low-KCl solution (3 mM). Interestingly, an increase in Cdkl5 levels similar to that observed in cultured primary neurons could be observed also in this *in vivo* system (+60%, **Fig.6A, 6B**). As before, Erk-1 and Erk-2 expression levels (**Fig. 6C, 6D**) were not significantly changed.

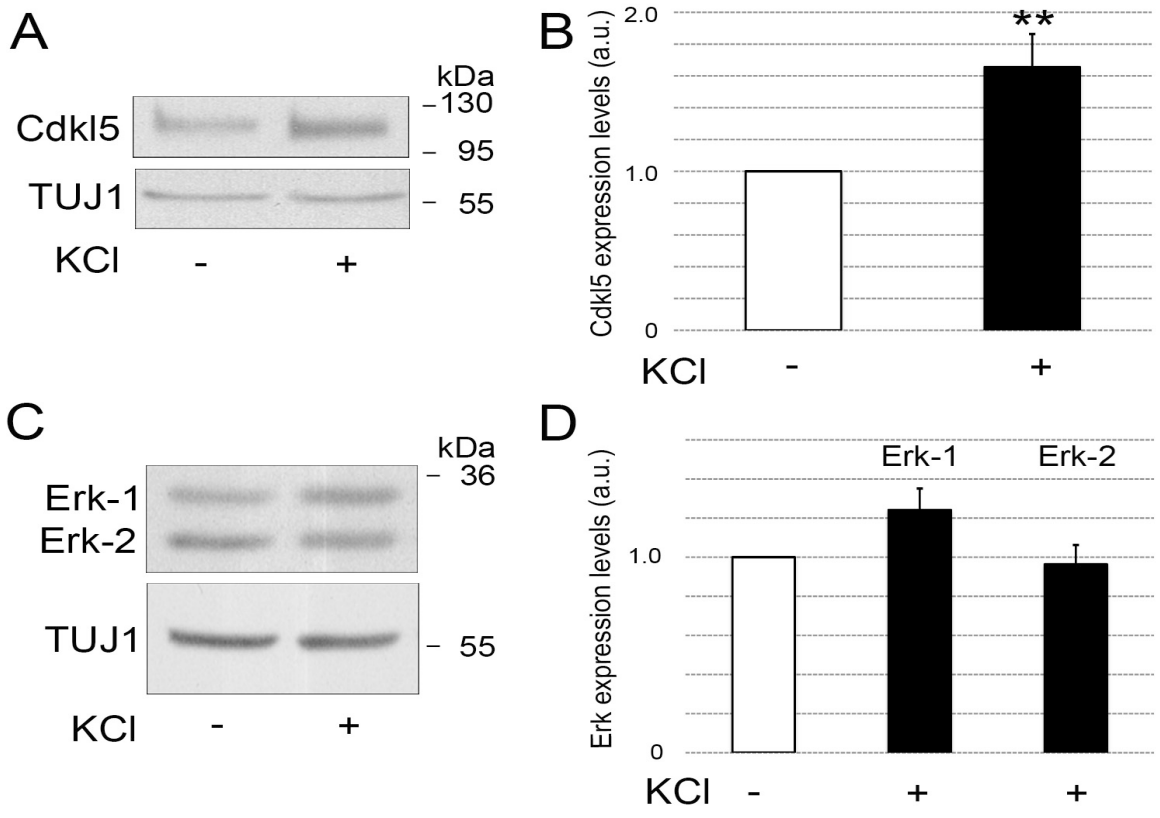


Fig.6 Cdkl5 expression is induced during early neuronal depolarization in cortical neurons in an *ex-vivo* model. A, C) Western blot analysis showing Cdkl5 and Erk-1/2 protein levels in P35-P38 mouse cortical slices treated or not with 30 mM KCl for 5 min. The cortical slices were maintained in a solution with 95% oxygen and

5% CO_2 for 30 min at 32°C and at 25°C for other 30 min before perfusion with a gassed solution containing 30 mM KCl for 5 min. The control slices were perfused using a low-KCl (3 mM) gassed solution. Beta-tubulin III (TUJ1) was used as internal standard. **B, D)** Graphic illustration of Cdkl5 and Erk-1/Erk-2 protein expression levels in treated and untreated slices (N=3; standard error expressed as SEM; the significance of results was evaluated by Student's *t* test and statistical significance was established as $p < 0.01$ (**)).

Signaling pathway(s) involved in the regulation of CDKL5 expression

In order to characterize the receptors and signaling pathway(s) activated by KCl and leading to the observed increase in Cdkl5-expression, we evaluated the activation of the excitatory synapses [64] by inhibiting specific molecular targets essential for synaptic transmission. The entry of calcium in neurons after glutamatergic transmission activates downstream pathways involving PI-3K, PKA, PKC, αCaMKII and MAPKs (mitogen-activated protein kinases) (**Fig. 7**), which we choose as targets for our experiments.

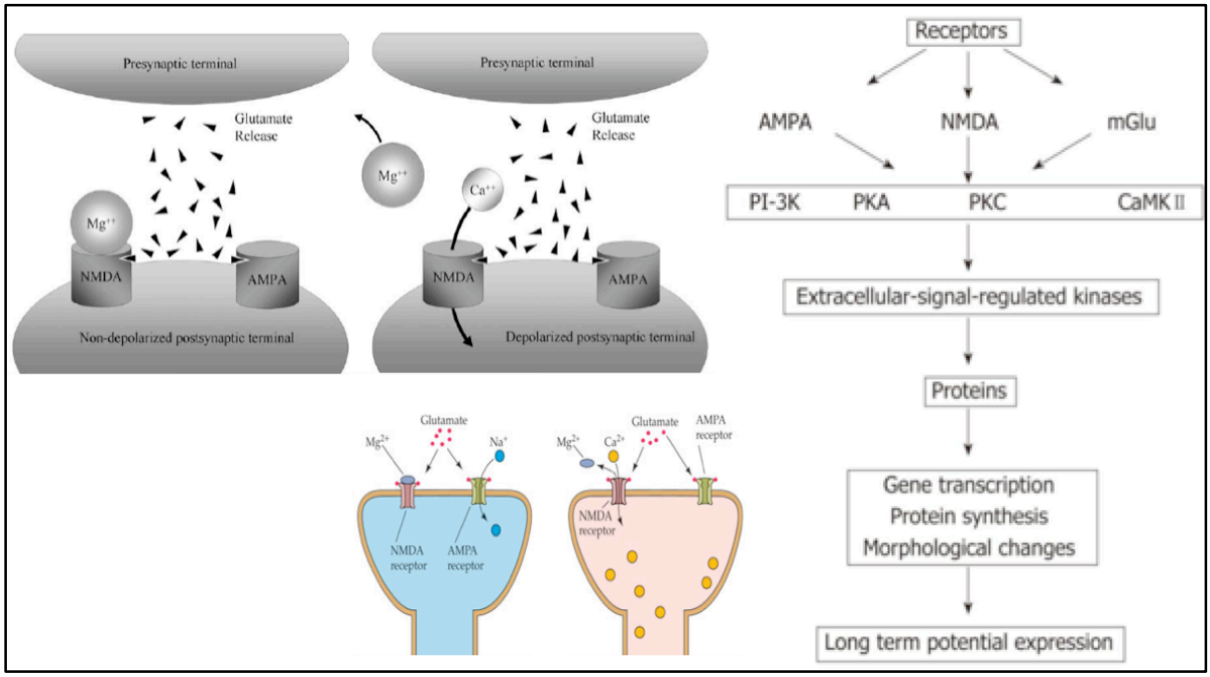


Figure 7. **Glutamatergic synapse: proposed sequences leading to changes in long term potentiation (LTP)**. Glutamate released from the pre-synaptic membrane binds both AMPA and NMDA receptors, but the NMDA-Rs remain functionally blocked by magnesium ions. AMPA-Rs conduct mostly sodium ions thereby depolarizing the post-synaptic membrane. With sufficient post-synaptic depolarization, magnesium ions are released from the NMDA-Rs and calcium ions enter the cell triggering the activation of molecules related to the LTP signaling pathways: PI-3K, PKA, PKC, αCaMKII and ERKs/MAPKs.

Abbreviations: NMDA, *N-methyl D-aspartate;* mGlu*: Metabotropic glutamate receptors*; AMPA, *α-amino-3-*

hydroxy-5-methyl-4-isoxazolepropionic acid; αCaMKII, *Ca^{2+}/calmodulin-dependent protein kinases II*; MAPKs/ERKs, Mitogen-activated protein kinases/Extracellular-signal-regulated kinases; PKA, Protein kinase A; PKC, Protein kinase C. Adapted from [126,127,128,131-133 http://www.mindsmachine.com/asf04.02.html]

To test the involvement of calcium for the KCl mediated Cdkl5 increase, DIV7 hippocampal neurons were maintained for 20 min in their medium supplemented with 2 mM EGTA (a chelator of calcium ions) before being treated with KCl. As shown in **Figure 4**, KCl treatment does not induce any increase in Cdkl5 levels in neurons treated with EGTA, demonstrating that the observed activation of Cdkl5 is calcium dependent. We proceeded analyzing a possible involvement of NMDA receptors (Rs), which are key molecules for the influx of calcium into the cells [90]. Thus, neurons were pretreated for 30 min with 100 μM AP5, a pharmacological antagonist of NMDA-Rs, before the standard KCl activation. Cdkl5 induction was found to be NMDA-R dependent since no change in Cdkl5 expression was observed after AP5 treatment. The importance of NMDA-Rs in the regulation of Cdkl5-expression in activated neurons was also confirmed by using CNQX, an AMPA channel antagonist, since the AMPA-Rs work in the glutamatergic synapse permitting and regulating the NMDA-R activation [91]. Cdkl5-expression remained unaltered when DIV7 hippocampal neurons were treated with 40 μM CNQX for 30 min before and during the KCl treatment.

The intracellular signaling cascades activated by calcium influx upon opening of NMDA-Rs involve various signaling molecules, protein phosphatases, and protein kinases such as mitogen activated protein kinases (MAPKs), the PKA (Protein Kinase A), PI3K etc. [92-99, 118-122]. The rapid and transient activation of MAPKs and PKA are essential for proper induction of long-term potentiation (LTP) [100-105, 113, 114]; indeed, they are associated with a specific regulatory pathway that can couple NMDA-R opening to the activation of translation initiation factors in the hippocampus, triggering protein synthesis during LTP via the MnK1 (*mitogen-activated protein kinase-interacting kinase 1*) activation and the phosphorylation of eIF4E (*eukaryotic translation initiation factor 4E*) [95, 115] (**Fig. 9**).

Therefore, we exposed hippocampal neurons to specific inhibitors of ERK-1/2 (10 μM UO126) and PKA (20 μM H89) for 20 and 30 min, respectively, before and during KCl treatment. Cdkl5 protein levels remained unaltered upon KCl treatment in the presence of these inhibitors (**Fig. 8**). Similarly, the lack of KCl-induced Cdkl5 increase was also observed when the hippocampal neurons were pre-treated with 2 μM wortmannin, a specific PI3K inhibitor (data not shown).

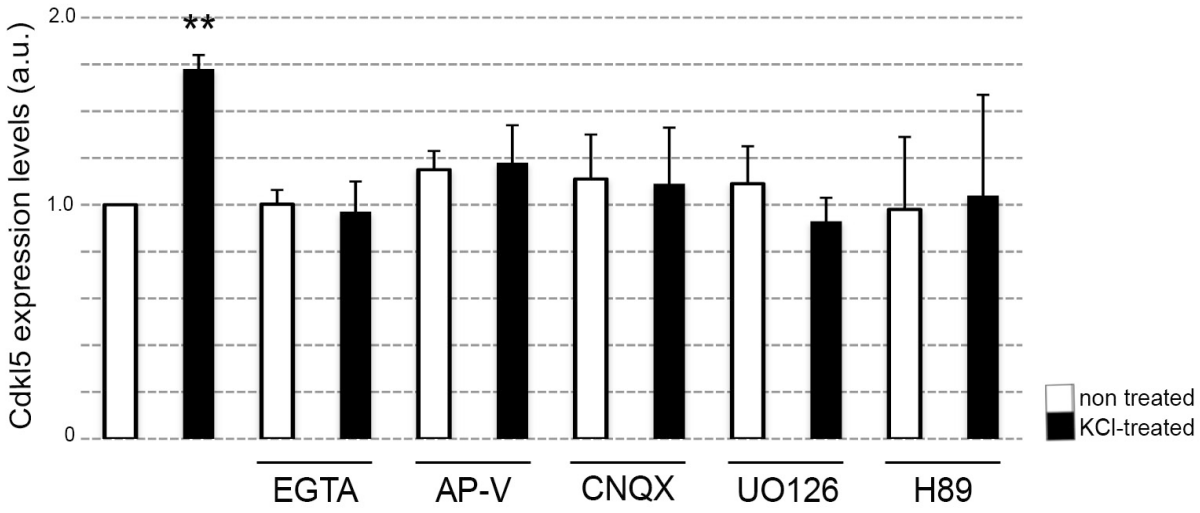


Fig. 8. **NMDA receptor-dependent signaling pathway(s) are involved in the KCl-mediated increase of Cdkl5 expression**. Cdkl5 protein levels in E18-DIV7 hippocampal neurons treated with 50 mM KCl for 5 min alone or upon pre-treatment with inhibitors of specific signaling pathways (EGTA 2 mM, AP-V 100 μM, CNQX 40 μM, UO126 10 μM, H89 20 μM). All values are expressed as the average of 3 different experiments ± standard error of the mean (SEM). The significance of results was evaluated by Student's *t* test and statistical significance was established as $p < 0.05$ (*) or $p < 0.01$ (**). Cdkl5 levels were normalized to β-tubulin III (TUJ1).

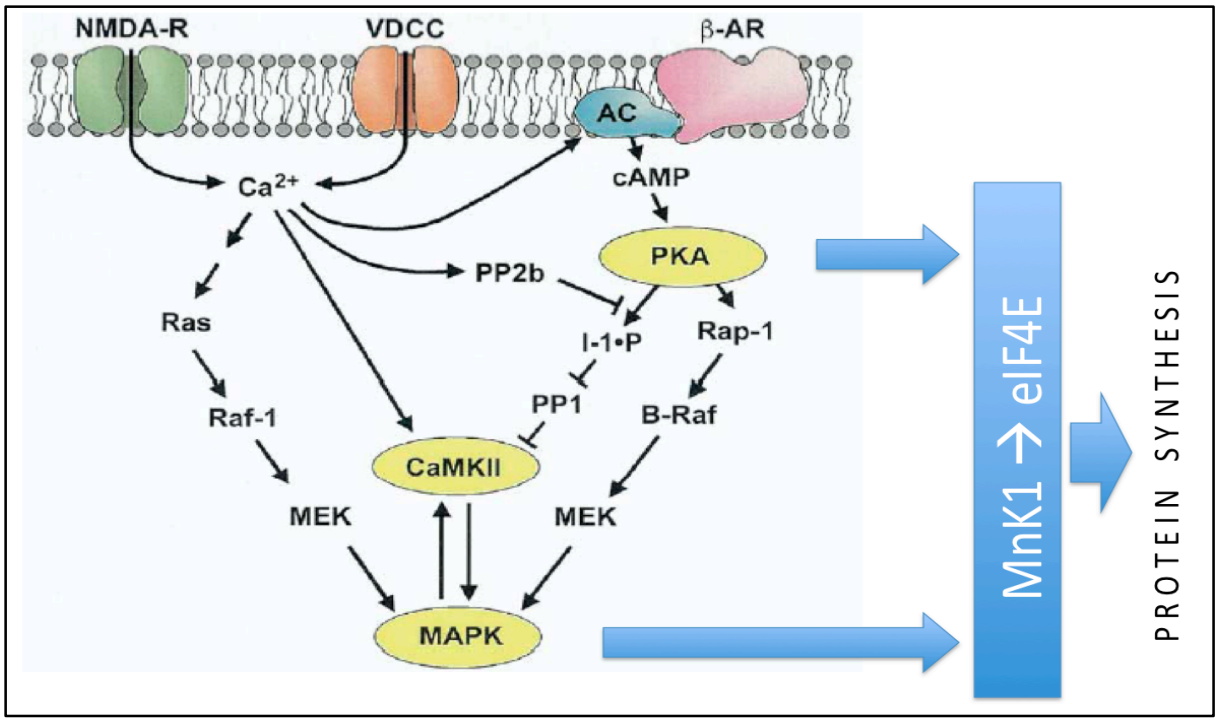


Fig.9 The interaction of three major signaling pathways in post-synaptic neurons during LTP, converging on the regulation of translation. The influx of calcium through NMDA-Rs or voltage-dependent calcium channels (VDCC) can engage signaling cascades that activate MAPKs and αCaMKII. PKA can be activated by

beta-adrenergic receptors (β-AR) and by the influx of calcium through NMDA-Rs. The intracellular calcium stimulates Ca/CaM (calcium and calmodulin) sensitive adenylyl cyclase increasing cAMP levels. Cytosolic cAMP can bind to regulatory subunits of PKA holoenzymes causing the release of the free catalytic subunits that translocate to the nucleus and phosphorylate the transcription factor CREB. αCaMKII, MAPKs and PKA are all required for the induction of LTP.

The activation of MAPKs and PKA are both required for the activation of MnK1-eIF4E, involved in activity dependent protein synthesis; for exemple, MAPKs are required for an increase in αCaMKII levels produced by LTP-inducing stimulation.

Abbreviations: NMDA, *N-methyl D-aspartate;* AMPA, *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;* VDCC, *voltage gated calcium channels*; AC, *adenylyl cyclase*; PP2b, *protein phosphatase 2b* (calcineurin); I-1●P, *phosphorylated protein phosphatase inhibitor-1*; Ras, Rap1, Raf-1, B-Raf, and MAPK/ERK kinase (MEK) are all components of the MAPKs cascade; αCaMKII, Ca^{2+}*/calmodulin-dependent protein kinases II*; MAPKs/ERKs, Mitogen-activated protein kinases/Extracellular-signal-regulated kinases; PKA, Protein kinase A. Adapted from [95,129,130]

The role of NMDA-Rs and PKA in the regulation of Cdkl5 expression was confirmed by using specific activators of these molecules. DIV7 hippocampal neurons were stimulated for 5 min with N-Methyl-D-aspartate (50 μM), an agonist of NMDA-Rs, or forskolin (100 μM), an adenylyl cyclase activator that activates PKA by increasing cAMP levels. These results confirmed the importance of NMDA and PKA for the increase in Cdkl5-expression (**Fig. 10A**, **10B**). It is also interesting to consider the response of Cdkl5 in cultured hippocampal neurons treated with BDNF (*brain-derived neurotrophic factor*), a neurotrophic factor that is a member of the "neurotrophin" family of growth factors. Interestingly, BDNF treatment of DIV12 neurons, when the TrkB receptors are likely to be expressed, caused an increase in Cdkl5 levels (**Fig. 11A, 11B**; N=2) [105,106,183]. To conclude, these results show that Cdkl5 is a target of intracellular cascades activated by NMDA-Rs or TrkB-Rs during early neuronal activation.

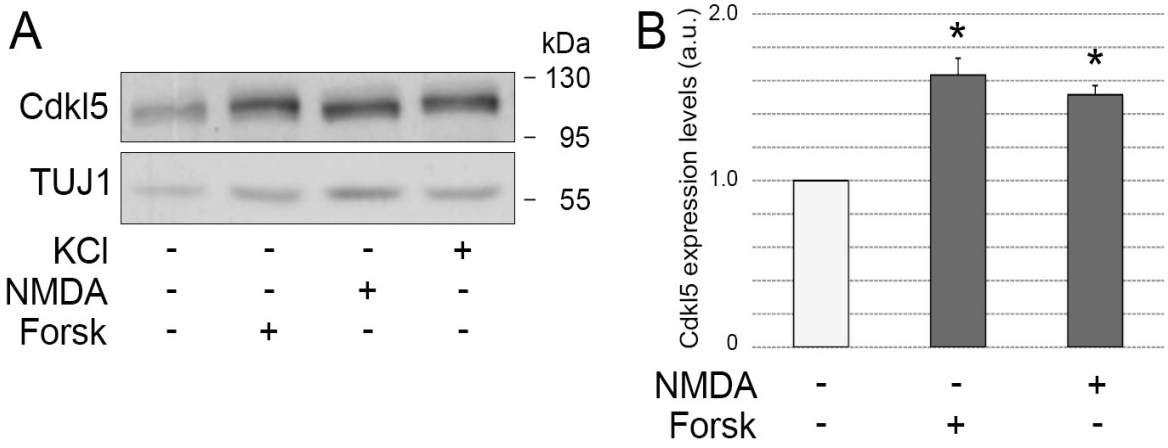


Fig.10 Cdkl5 expression is induced by activation of NMDA-Rs and PKA.

A) Immunoblot analysis of Cdkl5 expression in unstimulated DIV7 hippocampal neurons or in neurons treated for 5 min with 100 μM forskolin or with 50 μM NMDA.

B) Graphic illustration of Cdkl5 levels in treated versus untreated cells. Cdkl5 protein levels were normalized to β-tubulin III (TUJ1). All values are expressed as the average of different experiments (NMDA N=3; forskolin N=2) ± standard error of the mean (SEM). *t-test* statistical analysis, $p < 0.05$ (*).

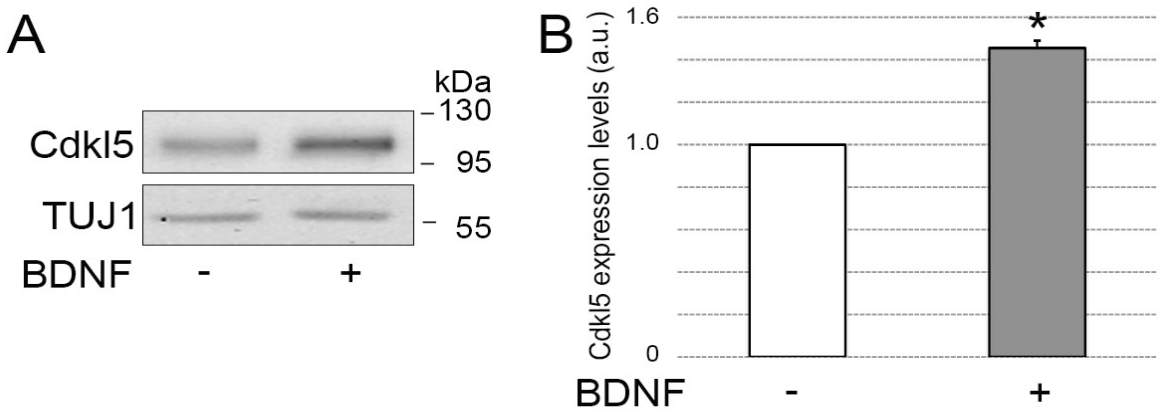


Fig.11 BDNF induces Cdkl5 expression in mature neurons. A) Western blot analysis of Cdkl5 expression in DIV12 hippocampal neurons after 5 min of treatment with 50 μg/ml BDNF. **B)** Graph showing Cdkl5 levels normalized to those of TUJ1 in neurons treated or not with BDNF. The values are expressed as the average of 2 different experiments ± standard error of the mean (SEM). $p < 0.05$ (*).

CDKL5 expression during the late phase of neuronal activation

Having demonstrated that *Cdkl5* gets activated soon after neuronal depolarization, we decided to evaluate what happens to its expression upon prolonged stimulation. Thus, we performed a

time-course analysis addressing the expression levels of the kinase during KCl-stimulation of DIV3 and DIV7 hippocampal neurons. Cells were directly collected in Laemmli buffer at different time points after KCl stimulation (5, 20, 40 min, 1 and 3 hrs) and Cdkl5 expression assayed by Western blotting. Interestingly, we found that in both cases a prolonged KCl treatment leads to a significant down-regulation of the expression of the kinase, leading to its disappearance after 3 hours of stimulation. However, a different kinetics can be observed depending on the neuronal maturation stage. Indeed, as already reported above, in more mature neurons (DIV7; **Fig. 12A, 12B**) Cdkl5 levels increase soon after KCl treatment (5 min) and then progressively diminish, becoming indetectable at 3 hrs. Conversely, in DIV3 neurons, Cdkl5 levels, which are lower in comparison to DIV7 neurons, increase with the same kinetics (5 min) but remain elevated for much longer; indeed, a significant reduction in the Cdkl5 signal appears only at 1 hour and a faint signal is visible even 3 hrs after KCl administration (**Fig. 12C, 12D**). The half-life of Cdkl5 (expressed exogenously in cell lines though) has been estimated to almost 6 hrs [53], we therefore reasoned that the observed down-regulation might probably be mediated by an active proteolysis of Cdkl5. The degradation of Cdkl5 could also be observed in frontal cortical slices of adult mice after prolonged KCl stimulation (1 hour) in comparison with control slices, while Erk-1/2 levels were not altered (**Fig. 12E, 12F**).

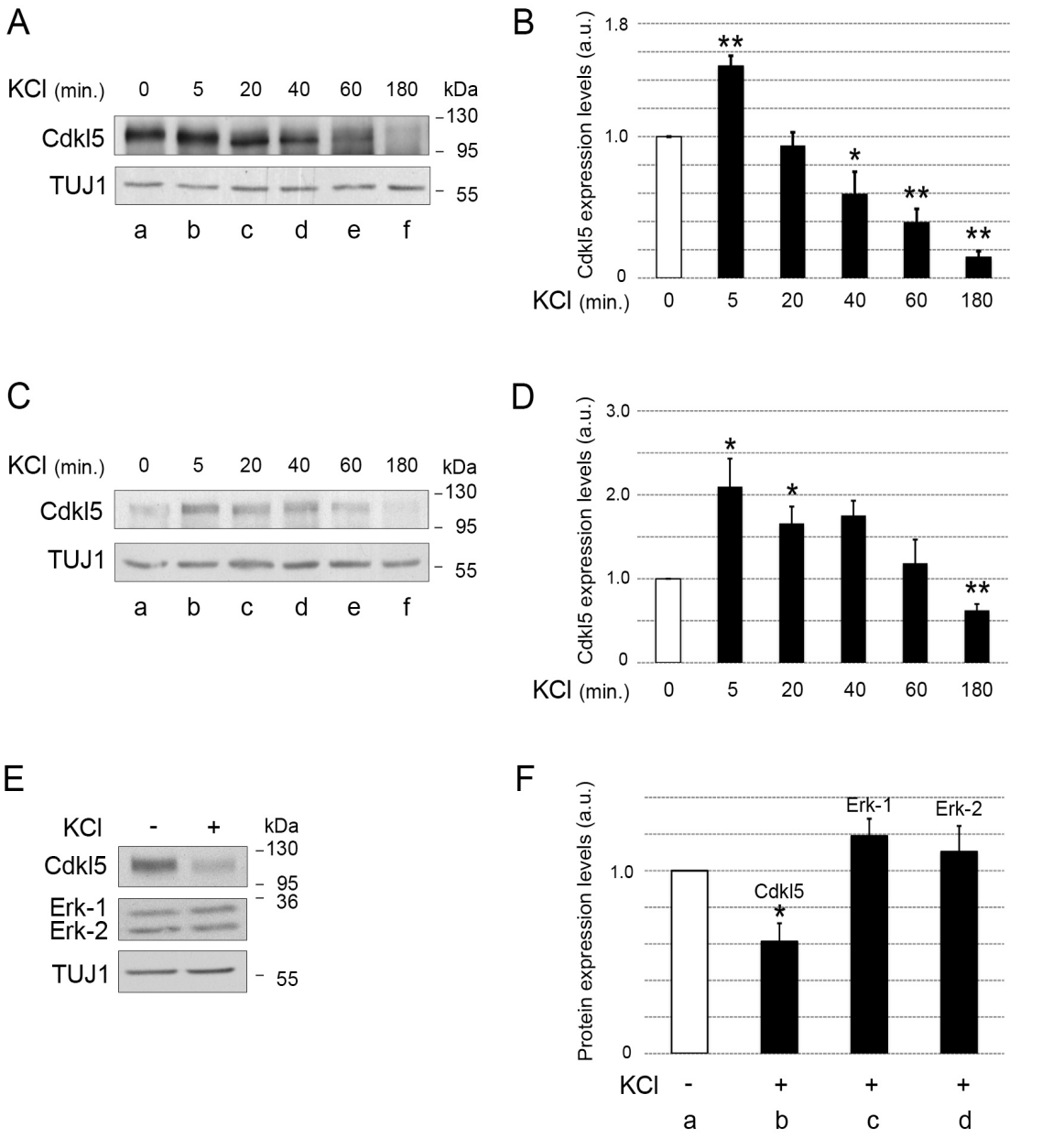


Fig.12 A prolonged KCl treatment down-regulates Cdkl5 expression in hippocampal neurons and in cortical slices. **A,C)** Western blot analysis of Cdkl5 expression in DIV7 (A) and DIV3 (C) hippocampal neurons after 5, 20, 40, 60 and 180 min of 55 mM KCl treatment. **B, D**) Statistical analyses of Cdkl5 expression in DIV7 (B), DIV3 (D) hippocampal neurons. **E**) Western blot showing Cdkl5 and Erk-1/2 levels in cortical slices after 1 hour of perfusion with 30 mM KCl solution (lane b) or with buffer alone (lane a). **F)** Statistical analysis of (E); expression levels of Cdkl5 (lane b), Erk-1 (lane c) and Erk-2 (lane d) in comparison with the control level in not treated slices (lane a). All values are expressed as the average of 3 different experiments ± standard error of the mean (SEM). The results were evaluated by Student's *t* test and the statistical significance was established as $p < 0.05$ (*) or $p < 0.01$ (**).

To determine the mechanisms by which Cdkl5 gets degraded during the late phase of neuronal depolarization, cultured hippocampal neurons (DIV3 or DIV7) were treated with 50 μM MG132, an inhibitor of proteasomal activity, for 3 hours before stimulating with KCl. In DIV7 hippocampal neurons, Cdkl5 degradation was partially prevented by the presence of MG132 (**Fig. 13A**). Indeed, by quantifying three independent experiments we found that, in the absence of MG132, 80% of Cdkl5 disappears upon the prolonged KCl stimulation, whereas by inhibiting proteasome degradation, more than 50% of Cdkl5 resists to the KCl treatment (**Fig. 13B**). It is worth noting that MG132 per se led to a significant increase in Cdkl5 levels, indicating that, in "mature" neurons, the kinase is a constitutive target of the proteasome that regulates its turn over.

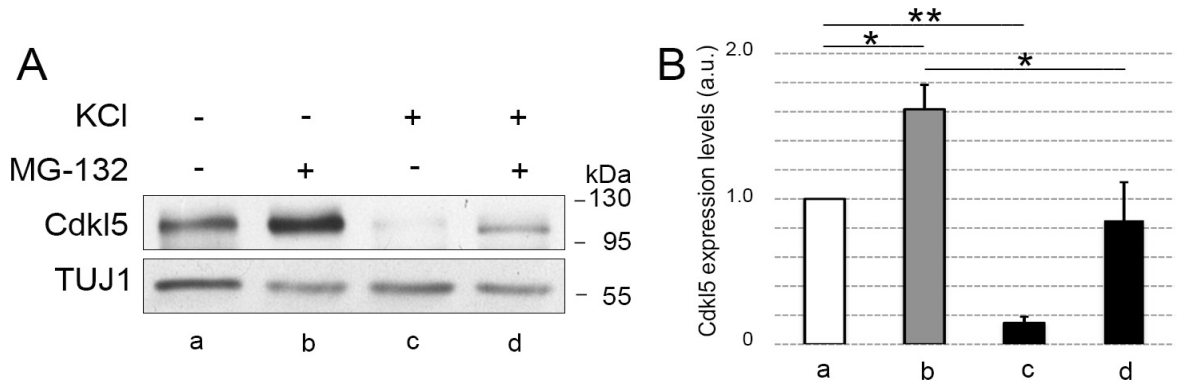


Fig.13 Cdkl5 turnover is regulated by the proteasome activity in DIV7 hippocampal neurons. A) Western blot analysis of Cdkl5 expression after 3 hours of treatment with 55 mM KCl preceded or not by a treatment of 3 hours with 50 μM MG132 (lanes d and c, respectively). As control, neurons were treated for 6 hours exclusively with MG132 (lane b) and compared with non-treated neurons (lane a). **B)** Statistical analysis; the letters below the bars correspond to panel A. All values are expressed as the average of 3 different experiments ± standard error of the mean (SEM). The significance of the results was evaluated by Student's *t* test; statistical significance was established as $p < 0.05$ (*) or $p < 0.01$ (**). Beta-tubulin III (TUJ1) was used as internal standard.

On the contrary, in immature neurons (DIV3), Cdkl5 levels are not regulated by the proteasome; indeed, its turn over, both in unstimulated or long-lasting depolarized conditions appear insensitive to MG132 (**Fig. 14A, 14B**). Therefore, summarizing the obtained results we suggest that during neuronal maturation the proteasome becomes one of the factors controlling Cdkl5 levels. The fact that 50% of the proteins gets degraded even when the proteasome has been inhibited suggests that in our experimental conditions the turnover of the

kinase might be also regulated by its synthesis and/or by lysosomal degradation.

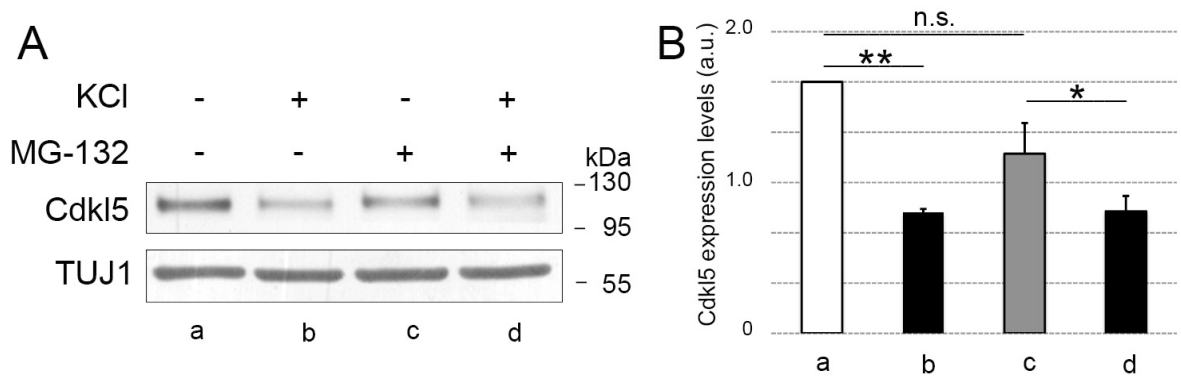


Fig.14 In immature hippocampal neurons Cdkl5 protein levels are not affected by the proteasome. **A)** Western blot analysis of Cdkl5 expression in control neurons (lane a) and after 6 hours of treatment with 50 μM MG132 (lane c), or 3 hours of treatment with 55 mM KCl pretreated or not for 3 hrs with MG132 (lane d and b). **B)** Statistical analysis (t-test, N=3) after W.B. densitometry (QuantityOne Biorad software). The lanes are the same described in (A). As above, significance of the t test was established at $p < 0.05$ (*) or $p < 0.01$ (**); *n.s.*: not significant.

Considering that a previous publication from our laboratory demonstrated that upon glutamate stimulation CDKL5 exits the nuclear compartment and then gets degraded [63], we evaluated the subcellular localization of endogenous Cdkl5 in non-treated and KCl-treated hippocampal neurons. However, no difference in the kinase localization was observed in any tested time point (KCl 5, 20, 40 min., 1 and 3 hours; data not shown).

Post-translational modification of CDKL5 during neuronal depolarization

While depicting the reactivity of Cdkl5 upon depolarization at different time points, we noticed that Cdkl5 was showing a faster electrophoretic mobility when neurons were treated with KCl for 20 min (Fig.8A). We reasoned that different levels of Cdkl5 phosphorylation might cause the observed change in mobility.

Importantly, few publications have already suggested a regulation of CDKL5 phosphorylation. In particular, it has been demonstrated that CDKL5, as some other kinases

belonging to the CMGC group, is capable of auto-phosphorylating its TEY motif [55,61]; however, the timing and duration of this phosphorylation, as well as the specific stimuli driving it and its functional meanings remain unknown. Of possible relevance, it is interesting to observe that CDKL5 shows some similarity, including the presence of the TEY motif, with the extracellular signal-regulated kinases (ERKs); in these kinases, the dual phosphorylation of the TEY motif is generally required for the activation of their catalytic site [108,117]. However, in ERK-7, a member of the family, this motif is constitutively phosphorylated by auto-phosphorylation and the kinase is constitutively activated [109]. Interestingly, Chen *et al*. have recently reported a transient increase in DIV5 rat cultured cortical neurons of CDKL5 threonine phosphorylation, showing the highest level 5 min after BDNF stimulation; these data indicate the presence of rapid post-translational modifications of the kinase during neuronal activation [56].

In order to investigate whether the observed change in Cdkl5 mobility is caused by its dephosphorylation, we performed an *in vitro* dephosphorylation assay. DIV7 hippocampal cultured neurons were collected directly in lysis buffer and the lysate was incubated with Lambda Protein Phosphatase (presenting activity towards phosphorylated serine, threonine and tyrosine residues; PPase). Samples were run on a 7% SDS-PAGE followed by immunoblotting. After Lambda PPase treatment, a "*fast-migrating*" band was detected using the anti-CDKL5 specific antibody, therefore corresponding to a dephosphorylated form of the kinase (**Fig. 15A**). These data suggest that in non-stimulated mouse hippocampal neurons, Cdkl5 is constitutively phosphorylated.

We proceeded by comparing the electrophoretic mobility of Cdkl5 after prolonged KCl treatment to that obtained by dephosphorylation. Thus, DIV7 hippocampal neurons were treated with KCl for 40 min and Cdkl5 migration analyzed by Western blotting. Interestingly, after prolonged depolarization Cdkl5 presents the same "fast-migrating band" as obtained with Lambda PPase treatment (**Fig. 15B**). These data suggest a KCl-dependent loss of Cdkl5-phosphorylation. Immunofluorescence experiments of KCl treated neurons showed that the overall ratio of Cdkl5 remains constant between the nucleus and the soma suggesting that the KCl-mediated dephosphorylation targets both nuclear and cytoplasmic Cdkl5 (**Fig. 15 C**).

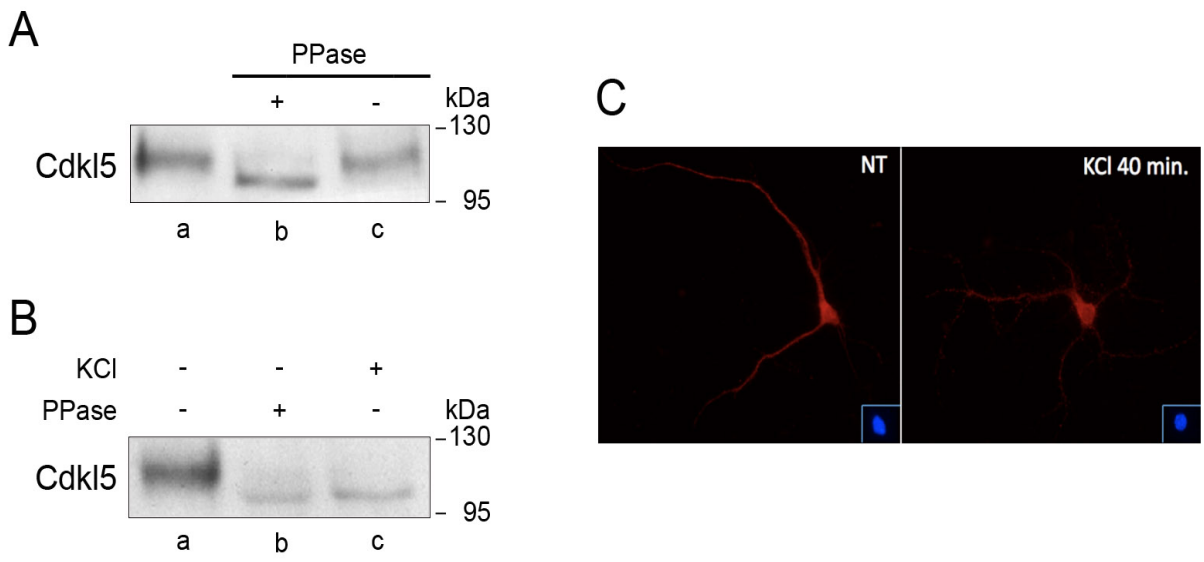


Fig.15 CDKL5 is dephosphorylated during the late phase of neuronal depolarization. A) DIV7 hippocampal neurons were collected in lysis buffer and treated with Lambda PPase as indicated. The samples were separated by 7% SDS-PAGE (acrylamide/bisacrylamide 77/1) and analyzed by Western blotting. The mobility of Cdkl5 is increased upon PPase treatment compared to control non-treated neurons (lanes b and c, respectively). Mock control: the extract was incubated as for lane b (3h, 30°C, Lambda PPase, buffer) but omitting the enzyme (lane c). **B)** After KCl-treatment (55 mM 40 min) of DIV7 hippocampal neurons, Cdkl5 migration is identical to that of the PPase treated sample (lanes c and b, respectively). Control: non-treated DIV7 neurons (lane a). **C)** Cdkl5 maintains its subcellular distribution after 40 min of KCl treatment. Representative immunofluorescence assays, using antibodies against CDKL5 (red) or DAPI staining (blue), of unstimulated hippocampal neurons at DIV7 and neurons exposed for 40 min to 55 mM KCl.

By analyzing CDKL5 mobility at different time points after KCl stimulation, we could observe that dephosphorylation starts already at 5 min of depolarization, when a doublet can be recognized (shown by arrows, **Fig. 16A**) and becomes more pronounced at 20 min of KCl-treatment (**Fig. 16B**). It is interesting to note that when tested in younger hippocampal neurons (DIV3), the KCl-dependent dephosphorylation of Cdkl5 was not apparent (**Fig. 16C**), suggesting that the mechanisms related to the dephosphorylation of Cdkl5 depends on neuronal maturation. We hypothesize that a relationship exists in DIV7 cultured hippocampal neurons between Cdkl5 dephosphorylation and the subsequent proteasome-dependent degradation of the kinase, both of which are absent in younger neurons (DIV3).

To summarize, CDKL5 appears to be constitutively phosphorylated in cultured mouse hippocampal neurons and is sensitive to KCl-treatment in terms of expression, post-translational modification and degradation.

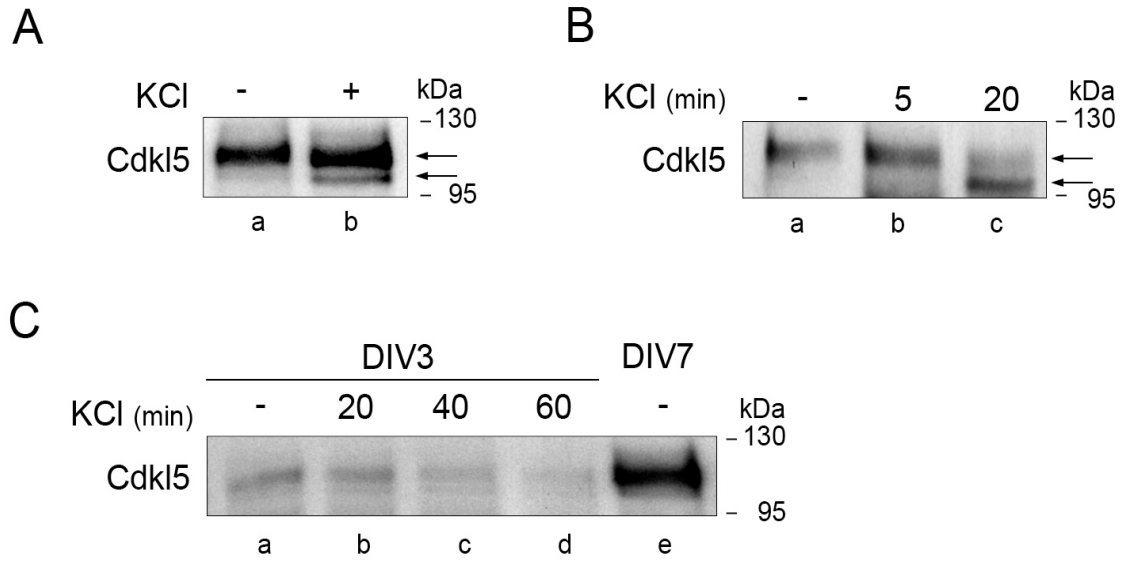


Fig.16 Cdkl5 dephosphorylation starts early after depolarization. **A)** DIV7 hippocampal neurons were treated or not with 55 mM KCl for 5 min and Cdkl5 migration analyzed by 7% SDS-PAGE followed by Western blotting. Arrows indicate the slow and fast migrating bands corresponding to phosphorylated and dephosphorylated Cdkl5 isoforms. **B)** The dephosphorylation of Cdkl5, hardly detectable after 5 min of depolarization, proceeds and becomes clearly visible after 20 min. **C)** Cdkl5 is not dephosphorylated in DIV3 hippocampal neurons when treated with 55 mM KCl for 20, 40 and 60 min (lanes b,c,d). The height of the band of Cdkl5 is the same in non-treated neurons at DIV3 (lane a) and DIV7 (lane e).

The phosphatases PP1/PP2A regulate the overall phosphorylation state of CDKL5

To identify the phosphatase(s) involved in Cdkl5 dephosphorylation, we treated DIV7 hippocampal neurons with specific inhibitors against tyrosin phosphatases and the serine/threonine phosphatases PP1/PP2A (sodium orthovanadate and okadaic acid, respectively) before treating with KCl. Whereas treatment with sodium orthovanadate (Na_3VO_4 for 1 hour) did not inhibit Cdkl5 dephosphorylation (**Fig. 17A**), the administration of okadaic acid for 45 min (O.A.) revealed three important features (**Fig. 17B**):

a) in unstimulated DIV7 hippocampal neurons, the state of Cdkl5 phosphorylation is influenced by the activity of PP1/PP2A phosphatases. Indeed, a retardation of Cdkl5 migration is evident in DIV7 hippocampal neurons treated with okadaic acid when compared to untreated neurons (lanes c and d, respectively). This band probably corresponds to a

"hyper-phosphorylated" form of Cdkl5 or to another phosphorylation dependent post-translationally modified isoform of the kinase.

b) the KCl-induced dephosphorylation, observed after 20 min treatment, is blocked by PP1/PP2A inhibitors (lanes e and f). We speculate that PP1/PP2A might be directly or indirectly involved. The basal conformation of Cdkl5 in resting neurons, regulated by these phosphatases, might represent a necessary condition for the intervention of other phosphatases. Furthermore, since in depolarized neurons treated with OA we could not detect any band corresponding to the slowest migrating band (lanes c and f), we speculate that other phosphatases are involved in this regulation and/or that a cross-talk between post-translational modifications exist, influencing the overall state of phosphorylation.

c) exploiting a long electrophoresis, a doublet of Cdkl5 becomes visible also after 5 min of KCl treatment that differs from the bands observed after 20 min of KCl treatment (compare lanes b and e). This suggests, once again, that a complex pattern of post-translational modifications occur on Cdkl5 upon specific stimuli. This double-band is also sensitive to O.A. treatment (lane a), indicating its dependence on PP1/PP2A phosphatases.

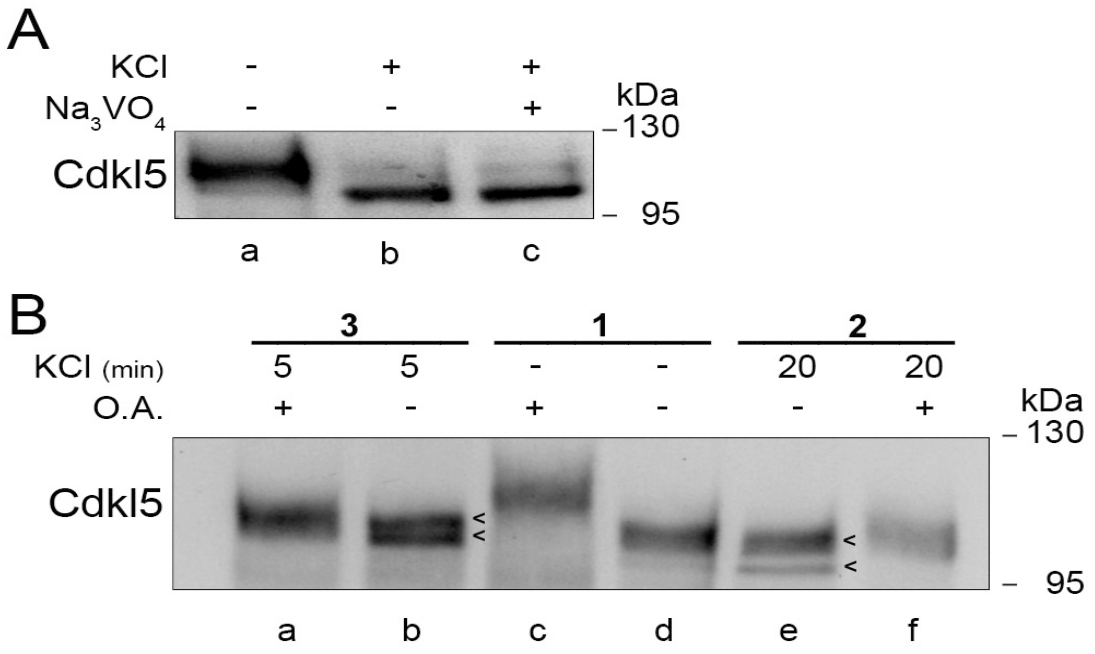


Fig. 17 The phosphatases PP1A/PP2A alter Cdkl5 phosphorylation during neuronal depolarization.

A) The KCl dependent dephosphorylation of Cdkl5 is not mediated by tyrosine phosphatases. Immunoblotting of DIV7 hippocampal neurons (lane a) treated as indicated with 1 mM Na_3VO_4 for 1h before the KCl-dependent

depolarization (20 min; lanes b,c).

B) DIV7 hippocampal neurons were treated as indicated with 1 μM okadaic acid (O.A.) for 45 min and KCl for the indicated time points. Cdkl5 migration was analyzed by Western blotting after separation of total extracts on 7% SDS-PAGE. Arrow-heads indicate the fast and slow migrating Cdkl5 isoforms.

DIV7 hippocampal neurons treated with okadaic acid compared to untreated neurons (lanes **c** and **d**, respectively).

The KCl-induced dephosphorylation, observed after 20 min treatment (lane **e**), is blocked by PP1/PP2A inhibitors (**f**).

The KCl-induced post-translational modification of Cdkl5 (lane **b**) is inhibited when PP1/PP2A are inhibited (lane **a**).

Summarizing the presented data concerning a direct or indirect involvement of PP1/PP2A in the regulation of Cdkl5 phosphorylation, we propose the following model (**Fig. 18**):

i) in non treated neurons, the basal level of Cdkl5 phosphorylation is affected by a constitutive action of PP1/PP2A;

ii) soon after neuronal depolarization, the levels of Cdkl5 phosphorylation decrease depending directly or indirectly on PP1/PP2A;

iii) upon sustained depolarization, Cdkl5 gets extensively dephosphorylated. This dephosphorylated form is the target of proteasomal degradation.

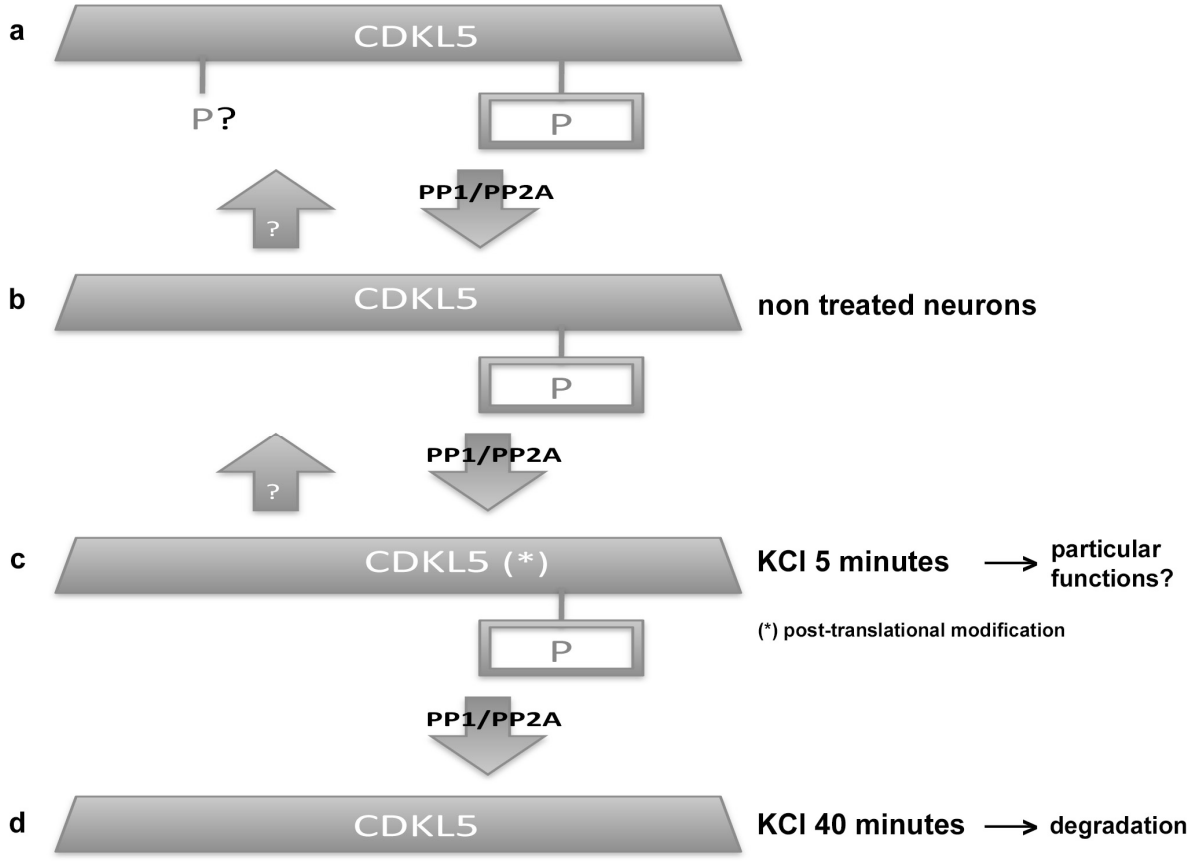


Fig.18 Schematic model of the cross-talk between Cdkl5 phosphroylation and degradation in depolarized hippocampal neurons. Our data suggest the presence of distinct Cdkl5 isoforms in hippocampal neurons:

a. a "*hyperphosphorylated form*" that is undetectable without inhibiting PP1/PP2A phosphatases; probably, it represents a very labile state of Cdkl5 appearing soon after synthesis or depending on stimuli that still remain to be discovered;

b. the "basal" form of Cdkl5, carrying at least one phosphate group, in unstimulated neurons;

c. the "modified form" of Cdkl5 appears soon after depolarization and is probably characterized by a reduction of its phosphorylation state; however, at least one phosphate group is bound to the kinase;

d. the "dephosphorylated form" of Cdkl5. Prolonged depolarization leads to a dephosphorylated form of Cdkl5 that constitutes the target of the proteasome.

Is CDKL5 involved in the regulation of genes associated to the early neuronal activation?

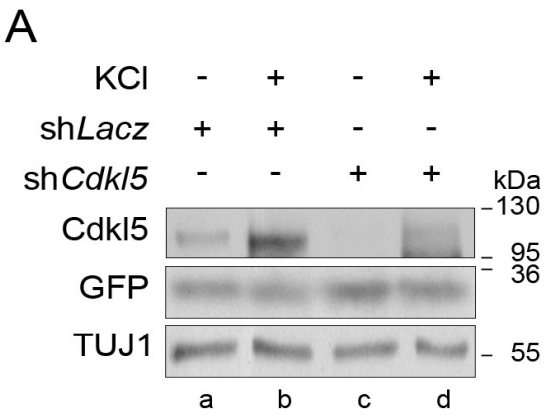
Considering that CDKL5 is an interactor of molecules related to the regulation of Immediate Early Genes expression, like MeCP2 [30, 41, 150] or DNMT1 [65, 297, 298], we decided to investigate whether Cdkl5 affects gene expression during neuronal activation. In particular, we started focusing on transcription during the first 5 min of KCl-treatment, when Cdkl5

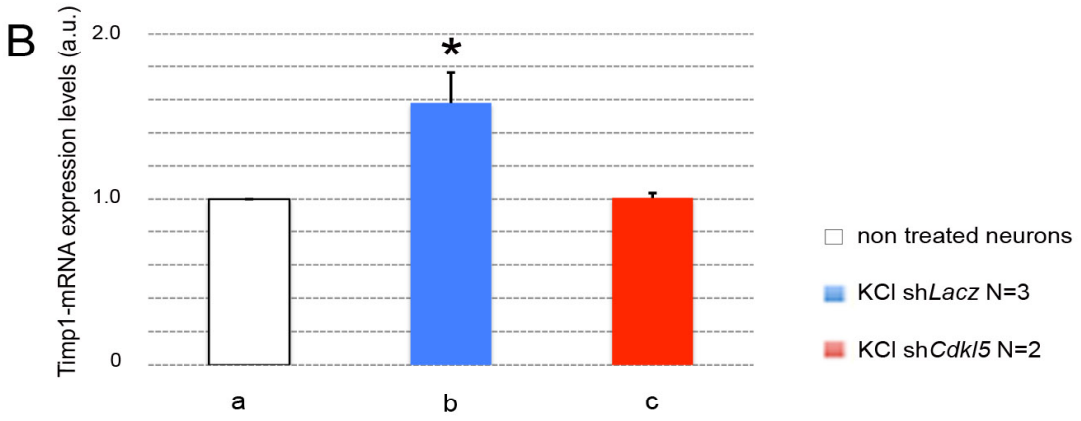
expression is up-regulated. Indeed, the transcriptional regulation of the iEGs (*immediate Early Genes*) results from the activation of pre-existing transcription factors and does not require protein synthesis [295, 296].

We used RT2 Profiler PCR Arrays to obtain the transcription profiles of 84 immediate early genes (iEG; listed in Fig. 19C) involved in synaptic plasticity, testing their expression in DIV7 control and *Cdkl5*-silenced hippocampal neurons before and after KCl treatment. To down-regulate *Cdkl5*-expression, DIV0 hippocampal neurons were infected with recombinant lentiviral particles produced using the pLentiLox 3.7 GFP plasmid and expressing a short-hairpin sequence against mouse *Cdkl5*-mRNA (sh*Cdkl5*). Similarly, DIV0 hippocampal neurons were infected with a control lentivirus carrying a short hairpin against the *β-galactosidase*-mRNA (sh*Lacz*). The efficient silencing of Cdkl5 was verified by Western blotting (**Fig. 19A**). The mRNA-expression levels were monitored by RT-qPCR following the indications of "RT2 profiler PCR Array Handbook" (QIAGEN), as described in "Experimental procedures". The ΔCt (Ct of iEGs - Ct of housekeeping genes) of 2-3 independent measurements was mediated and then ΔΔCt (Average ΔCt after KCl treatment - Average ΔCt in non treated neurons) was calculated. The Ct of 5 housekeeping genes was averaged and used as internal standard, as indicated by the manufacturers ("RT2 Profiler PCR Array PAMM-126ZA", QIAGEN).

An increase ($p \leq 0,05$; N=3) in the expression of some iEGs was evident after 5 min KCl treatment of control neurons: Akt1 (+20%), Egr1 (+30%), Grm2 (+45%), Rela (+10%), Timp1 (+60%) etc. (data not shown). In *Cdkl5*-silenced neurons, the expression of the same genes was similarly increased upon KCl treatment, while the induction of Timp1-gene (**Fig. 19B**) (*tissue inhibitor of metalloproteinases*) was inhibited, suggesting a role of Cdkl5 in regulating the expression of this gene, which is involved in the synaptic mechanisms underlying learning and memory [116].

In the future, it will be interesting to confirm these preliminary data and to understand the role of CDKL5 as a transcriptional co-factor involved in the early phases of neuronal activation.





C

Adam10	Grin1	Nos1	Crem	Homer1	Ppp1r14a
Adcy1	Grin2a	Nptx2	Dlg4	Igf1	Ppp2ca
Adcy8	Grin2b	Nr4a1	Egr1	Inhba	Ppp3ca
Akt1	Grin2c	Ntf3	Egr2	Jun	Prkca
Arc	Grin2d	Ntf5	Egr3	Junb	Prkcc
Bdnf	Grip1	Ntrk2	Egr4	Kif17	Prkg1
Camk2a	Grm1	Pcdh8	Ephb2	Klf10	Rab3a
Camk2g	Grm2	Pick1	Fos	Mapk1	Rela
Cdh2	Grm3	Pim1	Gabra5	Mmp9	Reln
Cebpb	Grm4	Plat	Gnai1	Ncam1	Rgs2
Cebpd	Grm5	Plcg1	Gria1	Nfkb1	Rheb
Cnr1	Grm7	Ppp	Gria2	Nfkbib	Sirt1
Creb1	Grm8	Ppp1cc	Gria3	Ngf	Srf
Ywhaq	Timp1	Tnf	Gria4	Ngfr	Synpo

Fig.19 KCl dependent induction of transcription is altered of neurons devoid of Cdkl5. **A)** Western blot of Cdkl5 and GFP from DIV7 hippocampal neurons infected at DIV0 with lentiviral particles expressing GFP and a shRNA against *Cdkl5* (sh*Cdkl5*) (lanes c, d) or *β-galactosidas*e (sh*Lacz*) (lanes a, b). At DIV7 the neurons were treated with KCl for 5 min (lanes b, d) and the expression of Cdkl5 was compared to neurons at basal conditions (lanes a, c). The signal of TUJ1 is used as loading control. **B)** Expression levels of Timp-mRNA in neurons expressing Sh-*Lacz* (lane b) or sh*Cdkl5* (lane c) treated for 5 min with KCl. The increase of expression in control Sh*Lacz* neurons (blue bar) is significant ($p \leq 0{,}05$; N=3) and has been compared with 1, the value of the expression of the relative gene in non KCl-treated neurons (lane a). In *Cdkl5*-silenced neurons, the expression of the same gene after KCl treatment is reported as the average of 2 measurements (red bar, N=2). **C)** List of the iEGs analyzed.

DISCUSSION

Rett syndrome is a postnatal progressive neurodevelopmental disorder that manifests in girls during early childhood. Its typical form is characterized by the appearance of neurodevelopmental arrest and regression, after a period of apparent normal psychophysical development up to 6–18 months of age. As the syndrome progresses, patients lose purposeful use of their hands and develop stereotypic hand movements. Loss of language and social skills, irritability, breathing anomalies and autistic features become apparent during this period of infantile life and will persist throughout adulthood. The severe mental retardation and the neurologic malfunctioning prevent patients from leading an independent social life.

Mutations in the *MECP2*-gene are associated to the classical form of Rett syndrome [3, 14]. MeCP2 is a nuclear protein that binds methylated DNA and recruits histone deacetylases and co-repressor complexes to suppress transcription. It belongs to the MBD family of proteins involved in the epigenetic regulation of gene-expression [27, 30]. MeCP2 is widely expressed but is significantly more abundant in brain, primarily in mature postmigratory neurons [3, 26, 33, 240]. Its expression in humans increases during the late fetal stage and in infancy [241]. Recently, new roles of MeCP2 as a transcriptional activator [30] and a splicing modulator [32, 230] have also been described. Interestingly, it has been proposed that in neurons MeCP2 could be considered a global chromatin structure regulator, since its abundance in nuclei is similar to one molecule every second nucleosome and its deficiency results in alterations of histone acetylation and doubling of histone H1 levels [33]. The role of MeCP2 in the regulation of chromatin structure could explain its putative involvement in the suppression of transcriptional noise [33] and in the inhibition of *Long interspersed nuclear elements-1* transcription [226].

Recently MeCP2 has been described as having a role in the regulation of dendritic arborization and spine morphogenesis. Indeed, membrane depolarization triggers MeCP2 phosphorylation at serine 421, allowing transcription of BDNF [28, 38]. It was demonstrated that MeCP2 S421 phosphorylation has a role in chromatin remodeling during neuronal activity and synapse development [35]. Furthermore, MeCP2 regulates the strength of synaptic response in hippocampal neurons by promoting the formation of glutamatergic synapses during early postnatal development [34]. During morphological differentiation of neurons and in adult stage, MeCP2 is involved in the regulation of dendritic arborization and dendritic spine density and regulates the expression of several synaptic proteins, including α/β CaMKII, AMPA, and NMDA receptors [242, 244, 245]. Mouse models have shown that

a phenotype similar to that of RTT syndrome can be caused by dysregulation in the expression of MeCP2 (underexpression or overexpression), confirming prior studies showing that either loss or doubling of MeCP2 results in postnatal neurodevelopmental disorders [34, 227-229, 244].

Atypical forms of Rett syndrome that deviate from the typical clinical presentation have been recognized since 1985 when a girl with infantile spasms and RTT-like features was described (Hanefeld variant). The atypical presentations of RTT are very different, and vary from milder phenotypes such as the "preserved speech variant" to more severe manifestations, such as the "congenital form" and the Hanefeld variant, with onset of intractable seizures before the age of 6 months [3]. The rare disorder Hanefeld variant was demonstrated related with mutations in the X-linked gene cyclin-dependent kinase like 5 (*CDKL5*). Mutations in the *CDKL5* gene have also been involved in a wider range of phenotypes including West syndrome, mental retardation and autism. In general, *CDKL5*-mutations are associated to the following clinical features: the onset of intractable seizures during the first months of life, infantile spasms and severe developmental delay and hypotonia [49, 231]. The product of *CDKL5* is a serine/threonine kinase that belongs to the CMGC family (named after the initials of some members: *cyclin-dependent kinases, mitogen-activated protein kinases*, *glycogen synthase kinases and CDK-like kinases*). Its catalytic domain, at the N-terminus, shares high homology with that of MAPK and CDK family members, while its long C-terminal tail does not share homology with other proteins. CDKL5 is a widely distributed protein expressed in a lot of peripheral districts, but highest levels are found in brain, particularly in the forebrain [50, 52, 53]. At the cellular level, the kinase is highly expressed in neurons, both in nucleus and in cytoplasm, whereas very low levels are present in glia. Similarly to MAPKs, the cytoplasmic form of CDKL5 is expressed in dendrites and somas of cortical and hippocampal neurons, a localization that suggests a role in postsynaptic functions [63, 305]. In non-neuronal cell lines the kinase shows a constitutive shuttling between the cytoplasm and the nucleus through an active nuclear export mechanism utilizing a signal in its C-terminal tail [41, 54, 56, 61, 63]. During development, the relative concentration of CDKL5 in the nuclear and cytoplasmic compartments of neurons varies (with an increase in the nuclear subpopulation during maturation) [54], as well as the expression levels in different brain areas. High expression levels were detected in the entorhinal cortex, in the hippocampus and in the most superficial cortical layers, involved in the intercortical connectivity, suggesting an involvement of the kinase in high cognitive functions (**Fig.20**) [52, 54].

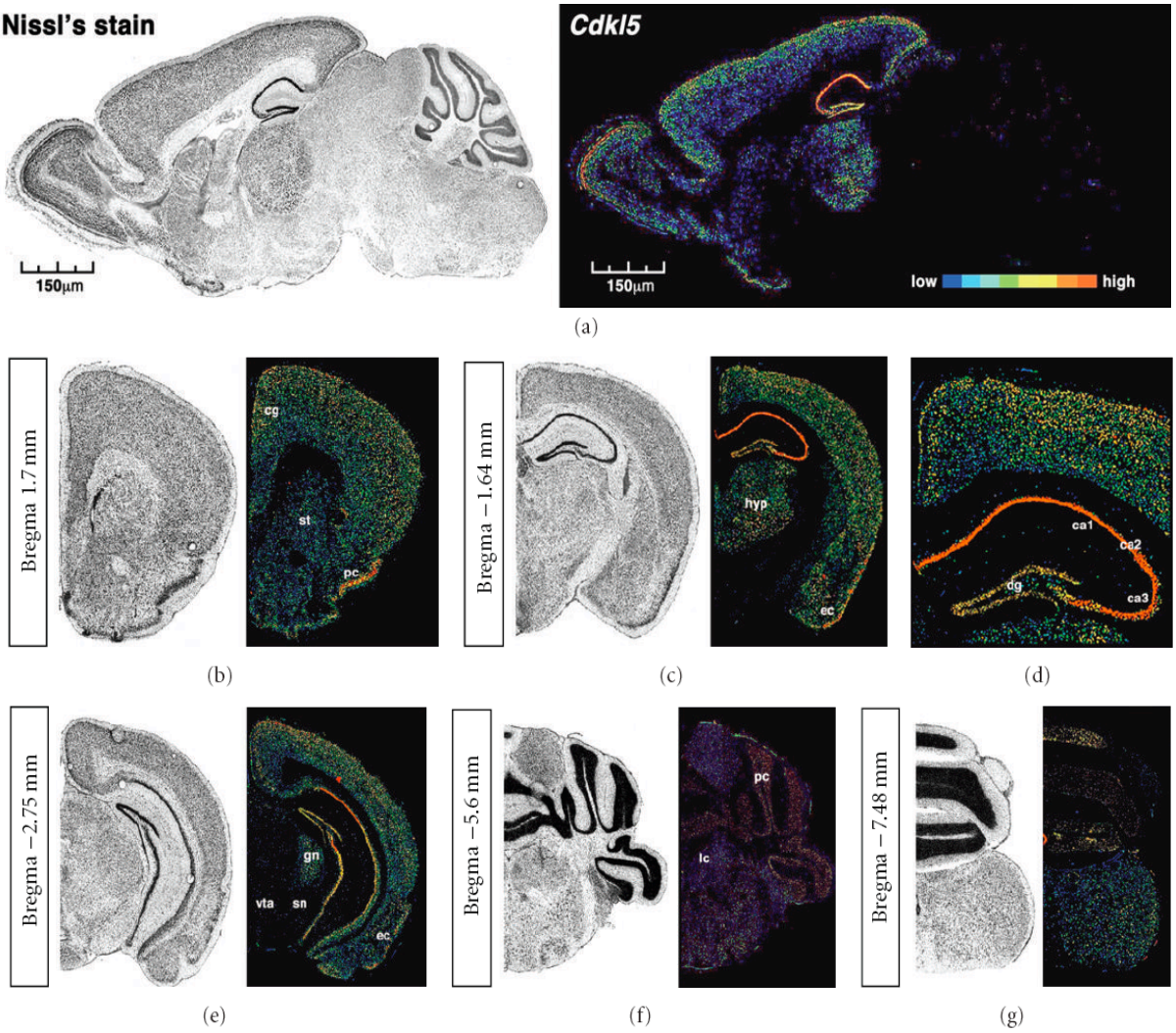


Fig.20 *Cdkl5* expression patterning in the adult male mouse brain (C57BL/6J, postnatal day 56). Each Nissl-stained section is coupled by a quantitative *in situ* hybridization screening at different sectioning levels. The color bar in (a) might be used to follow transcription level intensity. (b), (c), (d), (e), (f), and (g) are representative of different brain levels, therefore, different brain areas.

Abbreviations: cg (cingulated cortex), st (striatum), pc (piriform cortex), hyp (hypothalamus), ec (entorhinal cortex), dg (dentate gyrus), ca1, 2, 3 (hippocampal CA fields), vta (ventral tegmental area), gn (geniculate nuclei), sn (substantia nigra), lc (locus ceruleus), pc (Purkinje cells). Adapted from [52].

The expression of CDKL5 in brain is generally induced during the late phase of pre-natal neuronal development and the early post-natal stage, suggesting an involvement of the kinase in the formation and in the maturation of dendritic branches and synapses [54]. Accordingly, it has been demonstrated that CDKL5 is a critical regulator of neuronal morphogenesis,

neurite growth and dendritic arborization [56]. The cytoplasmic localization of CDKL5 and its catalytic activities are essential for the kinase to acquire its functions [56]. Furthermore, in the cytosol, CDKL5 phosphorylates NGL-1 (*Netrin-G1 Ligand 1*), a transmembrane protein interacting with Netrin-G1, a regulator of the early synapse formation and subsequent maturation [67].

In spite of the clear importance of CDKL5 for the central nervous system, the exact functions exerted by this kinase and its regulatory mechanisms remain mainly unknown. In the nucleus, CDKL5 binds MeCP2 and is certainly able to phosphorylate it *in vitro* [55, 59]; it remains to be demonstrated whether in vivo CDKL5 is involved in MeCP2 and Dnmt1 (*DNA methyltransferase 1*) phosphorylation [65]. Furthermore, in the nucleus CDKL5 colocalizes with nuclear speckles and is probably involved in the regulation of mRNA splicing [66].

As mentioned, the levels of CDKL5 and its sub-cellular distribution gets modified during neuronal activation. A recent article from our group suggested that the expression and subcellular distribution of Cdkl5 appears tightly regulated in mouse hippocampal neurons by exposure to glutamate, the most abundant excitatory neurotransmitter in brain. Indeed, a glutamate bath induces a rapid exit from the nucleus (through an active nuclear export system, mainly occurring in glutamatergic neurons) and a massive proteasome-dependent degradation of the kinase [63]. The meaning of this response remains to be elucidated; it was speculated that CDKL5 could have different roles in the cytosol and in the nucleus during neuronal activation and that different stimuli could regulate its sub-cellular distribution. Furthermore, CDKL5 phosphorylation is sensitive to neuronal activation; indeed, in rat cortical cultures treated with BDNF for 5 min (minutes), the activation of the TrkB receptor leads to the phosphorylation of a CDKL5 threonine residue [56]. The intra-cellular mechanisms involved in the response of CDKL5 to the neuronal activation are not known hitherto; it has been shown that CDKL5 is capable of autophosphorylating its TEY motif [3, 55, 61] but according to the primary structure it is highly possible that CDKL5 contains a lot of phosphorylation-sites and many kinases or phosphatases could be involved in the post-translational modification of CDKL5. Accordingly, proteomic approaches, aimed at revealing in HeLa cells phosphoproteins related to the cell-cycle, identified the presence of 14 sites of phosphorylation in human CDKL5 (S306, S375, S377, S407, S476, S526, T528, S543, S646, S681, S720, S726, S761); in particular, S646 and S761 were found phosphorylated in mitosis [337], whereas S407 in G phase [338]. Furthermore, one site of phosphorylation was identified in tyrosine-171, belonging to the TEY motif [337].

Considering all above, during my Ph.D. activity I decided to start characterizing mechanisms that might regulate CDKL5 expression and subcellular localization in murine primary

cortical/hippocampal neurons and in cortical slices using KCl-bath as a depolarizing agent mimicking the KCl-LTP (*Long Term Potentiation*) model [307, 356-362]. To better understand our results and their interpretation, let me discuss what I consider relevant information about LTP and LTD (*Long Term Depression*) in culture.

KCl depolarization-induced LTP (KCl-LTP or Chemical-LTP) is an electrophysiological model of induction of synaptic potentiation in cultured neurons in which a brief depolarization (application of 90 mM KCl: 3 x 1 second) is used to induce an increase in synaptic activity in neurons; as a consequence NMDARs are activated by the release of endogenous L-glutamate.

The advantage of studying dissociated neurons cultures consists in the possibility of rendering more accessible single synapses and evaluating the activity-related mechanisms during the initial steps of synaptic maturation. We focused our attention on DIV3 and DIV7 since significant changes in synaptic maturity occur between these two stages. In a recent publication, Sohya *et al*. reported opposite effects of KCl-dependent depolarization on spine morphology of rat hippocampal cultured neurons at different stages of maturation, demonstrating that high KCl induces synaptogenesis early in development (DIV3) but not later (DIV6) [187]. Interestingly, in E15 mouse primary cortical neurons the expression of PSD-95, a protein belonging to a multi-protein complex important in positioning signaling molecules for induction of LTP and LTD, starts to be detectable only at DIV7 and shows a progressive increase in its levels during synaptic maturation [339, 340].

ERKs have different functions in the nucleus, where they regulate activity-dependent transcription [301, 305] and in the cytoplasm, where they promote synaptic transmission and filopodia formation by phosphorylating ion channels [300, 302]. In addition, ERKs are activated in the hippocampus following convulsions [300, 301, 305; *in vitro* models of epilepsy using long exposure to KCl: 308, 311]. Other protein kinase cascades are required in the induction of the early phase of LTP in the CA1 area [317], subsequently to the stimulation of NMDA-Rs [305]; indeed the induction of LTP is blocked by general inhibitors of ser/thr protein kinases or tyrosine kinases [317]; accordingly it is well-known that a SRC-dependent tyrosine-phosphorylation of NR2B starts within 1-5 min after LTP induction [305, 315, 317].

In our experiment, we interpret the observed KCl-induced MAPKs activation (both at DIV3, when precocious neuronal processes leading to the synapse formation are activated [184-186], and DIV7/DIV12, when the synapses are getting matured) as a sign of neuronal LTP-related processes induced by depolarization. We verified the KCl-induced neuronal depolarization following Erk-1/2 activation with an antibody detecting the phosphorylated form of these kinases. In 1997, English and Sweatt demonstrated that the induction of LTP in the CA1

region of hippocampus activates the ERKs, whereas, a selective inhibition of the MAPKs cascade markedly attenuates the induction of LTP, without affecting basal NMDA-Rs-mediated transmission [113, 300, 302, 305, 326, 331]. NMDA-Rs stimulation in response to LTP-inducing *high frequency stimulation* leads to ERK-2 phosphorylation on tyrosine 42 after only 2 min (LTP, induced in "artificial" models by extremely brief patterns of afferent stimulation, reaches a peak within 30-60 seconds and then declines over time) [299-305]; the maximal activation of ERKs occurs within 5 min [302], followed by a slight decrease. Indeed, ERKs activation remains observable for at least 2 hours of KCl-bath [301]. In such a prolonged KCl treatment (in our conditions up to 3 hours), the onset of processes of saturation/resource depletion or active homeostatic regulation might occur [363]. Generally, as described in different models, the induction of E-LTP starts soon after the stimulation [316] and 2 to 4 hours are required to transit from the Early to the Late phase of LTP [273, 274, 316], the transcription- and translation-dependent phase that lasts many hours [317, 343]. The decline may be due to a naturally occurring activity-dependent LTD ("*Long Term Depression*") rather than a strictly time-dependent decay [269, 271]. LTD is a form of synaptic plasticity [301] important during the activity-dependent "selection" of neuronal circuitry; it has been hypothesized that LTP may be responsible for memory formation, while LTD may be involved in active suppression of pre-established memory [301]. Experiments *in vitro* and *in vivo* demonstrated in the rat amygdala the correlation between the induction of LTD and a reduced degree of MAPKs-phosphorylation [324]. An increase in post-synaptic calcium probably to levels below those necessary to induce LTP is one condition to induce LTD.

Hippocampal LTP is one of the most studied models of synaptic plasticity since its first demonstration through high frequency stimulation of afferent nerve fibers [276, 279]. The interest for LTP is due to the close parallels between this molecular mechanism and the persistence of hippocampus-dependent memory [274]. The most studied form of LTP develops through the activation of NMDARs and the consequent increase in AMPARs expression at the post-synaptic membrane [275]. The rapid transient depolarization in KCl-LTP models is due to AMPARs expression on the neuronal surface and is given by an increase in mEPSC frequency [270, 278].

LTP and LTD are both models of neuronal activity proposed after electrophysiological studies, using respectively *high-* or *low- frequency* stimulations; thus, it is difficult to follow a parallel model of activation in KCl-induced depolarized neuronal cultures. In these conditions, in fact, we do not know "if" and "when" a prolonged exposure to KCl saturates the responsivity of the cell. However, some elements could be considered good indicators of

LTP and LTD, such as the MAPKs phosphorylation status [305, 331], the expression of iEGs (*immediate Early Genes*) [317, 334, 335] or the presence of phosphorylated/dephosphorylated isoforms of some proteins related to synaptic plasticity [259, 317, 336].
Considering the literature, we can define two different stages of synaptic potentiation during LTP:

1) "*short-term effects"* (or Early-LTP, E-LTP) due to post-translational modifications of synaptic proteins, as well as alteration of their availability in correspondence of postsynaptic density [273]. Accordingly, it was demonstrated that αCaMKII-phosphorylation in KCl-LTP models was necessary for the cell surface delivery of AMPARs in hippocampal cultures [266, 267, 270, 280].

2) "*long-term effects*" (or Late-LTP, L-LTP) due to activity-dependent transcription, which is coupled to a PKA/MAPKs signaling pathways [322], and protein synthesis [262]. The events occurring during E-LTP, such as the recruitment of calcium-permeable AMPARs, can explain the activation of signaling pathways that in L-LTP drives spine morphological changes [234, 235, 270, 281]. New proteins reinforce the synaptic changes initiated during E-LTP, leading to the strengthening of the inter-neuronal transmission.

The same stages are present also in LTD [316].
Some authors describe a very precocious stage, which lasts only 15 min and is called STP (Short Term Potentiation) [317].
In neurons, an activity-dependent protein synthesis has been found to be related to the late phase of LTP [148, 153, 159], a long-term memory formation [160, 161] and seizure [146, 157, 158]. Protein synthesis starts within the first minutes of neuronal activation, depending on a coordinated program of stimulated gene transcription and translation, and the new proteins exert their functions during Late-LTP [272, 273, 352, 353]. In 1986, Goelet *et al.* proposed that iEGs, such as *c-fos,* might mediate the late phase of memory acquisition [138, 143, 144, 146, 147, 175]. We now recognize that iEGs and specific transcription factors are the most important mediators between L-LTP and the functional/structural changes of synapses [188-192, 273, 159, 277]. The iEGs-induction has been well studied in many cell types [176-179] and appears to be regulated mainly by alteration in intracellular calcium ions [180-182, 189-191, 193-195], in response to a variety of external stimuli, including neurotransmitters, growth factors and membrane depolarization [139, 140, 141]. The modality of induction follows a similar pattern in different *in vitro* and *in vivo* models: mRNA

transcription occurs within 5 min and reaches the steady-state level in 30-45 min, while protein synthesis starts at least 30 to 45 min after the stimulus and the peak can be reached even 2 hours after the stimulation [137, 139, 140, 142, 232, 233]. NMDA-Rs are strictly involved in the activation signals of the early transcriptional events; indeed specific inhibitors prevent glutamate-mediated induction of iEGs-mRNA both *in vitro* and *in vivo* [141, 143-145, 148-152].

Interestingly, new evidences suggest that LTP requires an initial period of activity-dependent translation of pre-existing mRNA which reinforces the switch from Early to Late LTP mechanisms [159]. Some authors described this reinforcement of E-LTP mechanisms as an "intermediate" phase [159, 282]. Pre-existing mRNA translation is essential for the late phase of LTP in the CA1 region also in spines and "isolated" dendritic preparations [115, 260-264, 306, 307, 312-314]. NMDA-Rs directly promote the translation of pre-existing mRNAs [95, 233] mainly in the soma but also in the dendritic processes. The activity-dependent translation has been described for some iEGs, such as *Arc* and *αCaMKII*, highly abundant in dendrites [115, 143, 154, 155, 233, 234, 236, 239, 252-254], permitting a rapid increase of their protein expression levels 3-10 min after NMDA-Rs activation. The new synthesis in loco of Arc and αCaMKII is important to regulate L-LTP, while the post-translational modification of αCaMKII (autophosphorylation after 1 min of neuronal activation [270, 276]) is important to permit and regulate E-LTP related processes [233, 234, 252-254, 270, 159, 276, 280, 283-286, 292-294]. Indeed, the activated αCaMKII is translocated to the PSD and phosphorylates AMPA-Rs [317]. Both, the new protein synthesis *in loco* and the targeting to synapses of proteins synthetized in the soma are important to guarantee the "synapse-specificity" of plasticity.

Molecularly, the translation of pre-existing mRNA during E-LTP is given by an increase in the phosphorylation of the *eukaryotic initiation factor 4E* (eIF4E), mediated by the opening of NMDA-Rs [95, 233, 237,307].

Some recent papers confirmed the link between neuronal activation and changes in both the expression level and the sub-cellular distribution of CDKL5 [63, 64]. Therefore, we wanted to decipher both of these aspects during KCl-stimulation of neurons *in vitro* and *ex vivo*.

In non-stimulated cultured neurons, Cdkl5 expression level increases during neuronal maturation and distributes between the nucleus and the cytosol [54, 63]. We found that, in cortical and hippocampal cultured neurons at different stages of maturation (DIV3, DIV7 and DIV12), as well as in adult mouse cortical slices (P35-P38), a KCl-bath of 5 min induces the expression of Cdkl5 (+60%). This increase is due to a new synthesis of the kinase since it is abolished by blocking translational elongation through cycloheximide treatment. According to

literature, other proteins, such as Erk-1/2 and c-Fos, did not show any change in their expression level after 5 min of treatment. Similarly, Cdkl5 induction was observed stimulating for 5 min at DIV12 cultured hippocampal neurons (characterized by receptorial maturity [183]) with BDNF, which is known to regulate Early-LTP processes by activating the peripheral translation of mRNA and the trafficking of eIF4E into spines [159, 252-254, 287-289].

This early induction of CDKL5 might suggest a requirement of novel synthesis of the kinase for early/intermediate phase of LTP. This hypothesis has to be confirmed through electrophysiological experiments performed on stimulated neurons ablated for Cdkl5.

In hippocampal cultured neurons, calcium entry and the activity of NMDA-Rs and AMPA-Rs, as well as the PKA and MAPKs activity, are required for observing the Cdkl5 response to KCl.

Since we were interested in examining whether *Cdkl5*-transcription was increased as well. *Cdkl5*-mRNA was quantified in KCl-depolarized neurons. An increased expression of *Cdkl5*-mRNA and *c-fos*-mRNA was found after 5 min of KCl administration, suggesting a reactivity of *Cdkl5* to neuronal activation similar to that of iEGs. The augmented level of *Cdkl5*-mRNA was probably due, similarly to *c-fos*, to increased transcription of the gene, since it was abolished by pre-treating neurons with the transcriptional repressor actinomycin-D. Interestingly, in analogy with the *Arc*-gene [233], we identified in the regulatory sequences of *Cdkl5* a CRE motif (*cAMP response element;* TGAGCTCA) and in the future it would be interesting to investigate whether it has a role in the transcriptional activation. Summarizing, we can conclude that after 5 min of exposition of cultured neurons to an elevated level of extracellular K^+, or BDNF, there is an increase in Cdkl5 expression levels and the activation of its transcription. The precocious mRNA-translation is mediated by the opening of NMDA-Rs and the subsequent activation of PKA and MAPKs, probably through the involvement of specific regulators, such as Mnk1 and eIF4E.

Generally, factors induced during LTP increase their expression at the beginning of stimulation, reach a plateau and then return to control levels [188, 196, 197]. Therefore, we wanted to investigate whether CDKL5 was characterized by a similar behavior. DIV3/7 hippocampal neurons were exposed to KCl for different time points and we observed such a triphasic profile by western assay, whereas no changes in the subcellular distribution of Cdkl5 were detected by immunofluorescence.

Interestingly, after returning to basal levels, Cdkl5 expression decreased rapidly and dramatically, suggesting an active degradation in depolarized neurons, both at DIV3 and DIV7. A significant decrease of Cdkl5 expression level was evident also in cortical slices

treated for 1 hour with KCl, while the same treatment did not cause the degradation of the LTP-related proteins Erk-1 and Erk-2. We demonstrated that a KCl-dependent degradation mediated by the proteasome is present only when the synapses are getting matured (DIV7) but not before this stage. Accordingly, at DIV7, but not at DIV3, Cdkl5 shows a proteasome dependent constitutive turnover.

The ubiquitin-proteasome system is the major pathway for protein degradation in cells [266]. It is important to note that the expression of proteasome is very precocious in neurons, probably being already present at DIV3 in neurons [198, 199]. Furthermore, synaptic activity promotes proteasome sequestration within spines for the consolidation of the Early-LTP into Late-LTP, similarly to the activity-dependent mRNA-translation [233, 255, 257, 258, 262, 263, 264, 266, 290].

Both L-LTP and the late phase of the NMDA-Rs-related LTD are dependent on both protein synthesis and proteasome activation [159]. Although LTP and LTD specific gene expression profiles have not been fully characterized so far [159], there are important suggestions regarding a balance between protein-synthesis and protein-degradation during synaptic activation, mainly involving some plasticity-related proteins in the synapses. This balance could explain the restore of L-LTP when both proteasome and protein synthesis inhibitors are co-applied [262, 291].

Considering all above, we can hypothesize that CDKL5 levels could be yet another important element involved in the balance of synaptic protein composition during both Early- and Late-LTP. A further indication might be given by a recent publication demonstrating a co-localization of CDKL5 with PSD95 and excitatory synapse markers [64].

Eventually, we found that neuronal depolarization affects Cdkl5 post-translational modifications. In particular, electrophoretic mobility studies suggested that Cdkl5 in non-stimulated neurons and cortical slices is constitutively phosphorylated, similarly to ERK-7, a member of the MAPK family [109]. Indeed, KCl treatment leads to a rapid and progressive dephosphorylation of Cdkl5. So far, very little is known about the "phosphorylation status" of CDKL5. The kinase is capable of autophosphorylating its TEY motif [55, 61], but the involved stimuli are unknown.

Increased phosphorylation of MAPKs and other plasticity-related proteins has been associated to LTP, whereas their dephosphorylation has been linked to LTD. Particularly, LTP and LTD are related respectively to the inhibition or to the activation of protein phosphatases, such as PP1/PP2A (*Phosphoprotein phosphatase 1*; *Phosphoprotein phosphatase 2A*), as well as protein kinases [321], regulating the reversible phosphorylation of synaptic proteins, such as

αCaMKII, AMPA-Rs and NMDA-Rs [200, 204, 208, 209-211, 213, 214, 255-264, 301, 321, 322].

After synaptic maturation, NMDA-Rs activation results in the recruitment of PP1 to the synapses [219] where presumably it can target synaptic proteins, raising the intriguing possibility that the pattern of synaptic activity controls the phosphorylation status of Cdkl5, a kinase localized at excitatory synapses [64]. Thus, we wanted to study the putative involvement of endogenous phosphatases in this activity dependent dephosphorylation of Cdkl5. The PP1/PP2A mediate a basal process of dephosphorylation of Cdkl5 and, when inhibited by okadaic acid (O.A.), the kinase is "hyperphosphorylated". The PP1/PP2A are also involved, directly or indirectly, in the KCl-dependent dephosphorylation of Cdkl5, since the "large double band", corresponding to a progressive dephosphorylation of Cdkl5, is not detectable if the inhibition of the phosphatases precedes KCl treatment, while tyrosine phosphatases appear to be unrelated with this process. Interestingly, KCl-dependent dephosphorylation of Cdkl5 occurs in neurons at DIV7 but not in less mature neurons at DIV3, therefore correlating with the development of more complex systems related to synaptic transmission. Considering that PP1, which regulates many processes in young neurons (the formation of filopodia, the maturation of neuronal filopodia into dendritic spines, the functional maturation of excitatory synapses, the axonal targeting [217, 218]), is precociously and ubiquitously expressed in mouse embryo brain (starting from E15.5) and in plated cortical neurons [215, 216], we speculate that PP1 is present at DIV3 hippocampal neurons but the maturation of other interacting proteins bridging PP1 to Cdkl5 (probably in the *PSD fraction*) is required to trigger the activity dependent dephosphorylation of Cdkl5 observed at DIV7. Furthermore, we hypothesize that Cdkl5 dephosphorylation represents a signal for the activation of its proteasomal degradation, indeed Cdkl5 becomes a target of the proteasome, both in basal condition and during depolarization, only in DIV7 neurons.

Chen *et al*. reported a transient increase in CDKL5 phosphorylation 5 min after BDNF stimulation of DIV5 rat cultured cortical neurons, suggesting the presence of rapid post-translational modifications of the kinase during neuronal activation [56]. Thus, we investigated the presence of post-translational modifications, presumably activating the kinase and indicating a putative role during E-LTP, soon after KCl treatment of DIV7 hippocampal neurons. Interestingly, after a long gel running, we identified the presence of a different isoform of Cdkl5, which remains to be characterized but is certainly regulated, either directly or indirectly, by PP1/PP2A.

In conclusion, considering some similarities, during neuronal activation, between the behavior of CDKL5 and other molecules known to have a role in LTP (NMDA-Rs related intra-cellular

pathways, transcriptional/translational activation, post-translational modifications, sensitivity to the proteasome), we propose CDKL5 as a new kinase involved in LTP (**Fig. 21**). New insights in the future are required to confirm the role of CDKL5 in LTP using both electrophysiological approaches and studies addressing whether the kinase interacts with other LTP-related molecules (ion channels, membrane receptors, transcription factors etc.). A role of the kinase in LTD-processes, depending on its sensitivity to PP1/PP2A activity, is also plausible [330].

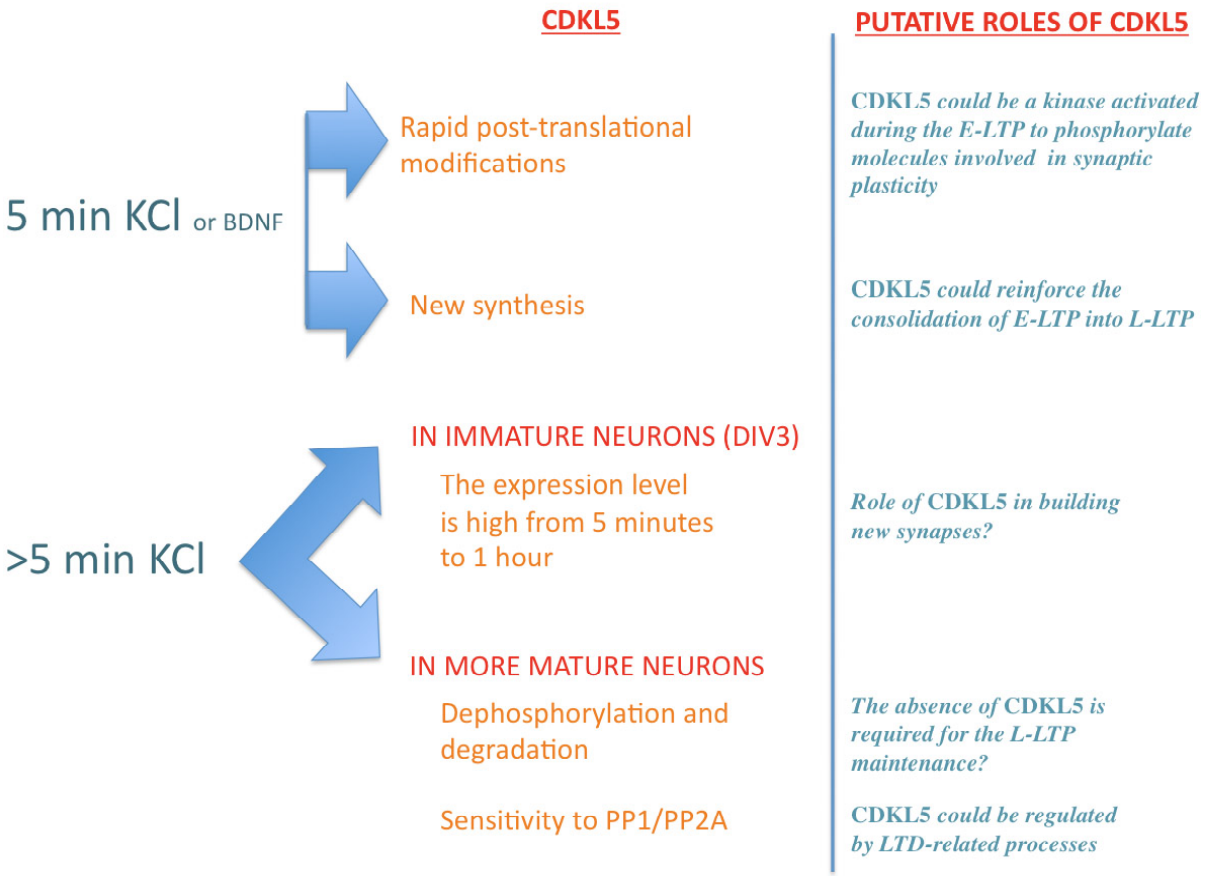


Fig. 21 CDKL5 is sensitive to neuronal activity and shows similarities to some molecules involved in synaptic plasticity, suggesting a role of the kinase in the LTP. During the first minutes of stimulation, CDKL5 expression levels increase and the protein undergoes rapid post-translational modifications, compatible with a role during the E-LTP. After more than 5 minutes of stimulation, the behavior of CDKL5 in KCl treated neurons is different depending on the maturational stage of the cells: in immature neurons, the expression levels increase from 5 min to 1 hour; conversely, in mature neurons, a mechanism involving CDKL5 dephosphorylation and degradation is active, suggesting that low levels CDKL5 are required during L-LTP at this stage. At last, the sensitivity of CDKL5 to PP1/PP2A could indicate an involvement of CDKL5 in an LTD-related process.

EXPERIMENTAL PROCEDURES

Primary cortical and hippocampal cultures were prepared from brains of CD1 mouse embryos at 18 days (E18). The mice were sacrificed by cervical dislocation; brains were removed from the embryos and the cortex and hippocampus rapidly dissected. After two washes in HBSS (GIBCO), neurons from the cortex and the hippocampus were dissociated by 15' incubation at 37°C in 0,25% trypsin (Sigma Aldrich). The cells were suspended with "dissection medium" [D-MeM with GlutaMAX-I (Gibco), horse serum 10%, 2 mM-L-Glutamine (Sigma), 1 mM NaPyruvate (Gibco)] to block the action of the trypsin. Finally, counting the cells in a *Bürker* chamber, the definitive Neurobasal medium (Gibco) was added to the cells (supplemented with B27 (Gibco) and 2 mM-L-Glutamine) that were plated on Poly-L-lysine hydrobromide (Sigma P2636) coated dishes (0.1 mg/ml) or glass coverslips (1 mg/ml). The number of the plated cells is reported in the following table:

	number of cells
cortical neurons, dishes	26.000 cells/cm^2
cortical neurons, glass coverslips	2.500 cells/cm^2
hippocampal neurons, dishes	16.000 cells/cm^2
hippocampal neurons, glass coverslips	2.500 cells/cm^2

After 3 days in vitro (DIV), cytosine-1-b-D-arabinofuranoside (Ara-c, Sigma Aldrich), at the final concentration of 2 μM, was added to prevent astroglial proliferation.

Treatments of cultured cells:

Neurons were treated after 3, 7, and 12 days in vitro (DIV) with KCl at a final concentration of 50-55 mM. The Neurobasal medium contains 5 mM KCl, which corresponds to the physiological range. We used two different protocols of KCl-stimulation:

1) 5 min (minutes) KCl treatment: the cells were stimulated in Krebs-Ringer solution (KRH; KH_2PO_4 1.2 mM; $MgSO_4$ 1.2 mM; $CaCl_2$ 2 mM; Hepes pH 7.5 25 mM; glucose 1.1 mg/ml) with KCl 50 mM and NaCl 85 mM. The "control cells" were simultaneously treated with an isotonic bath in KRH with 5 mM KCl and 130 mM NaCl.
2) Time course (5, 20, and 40 min; 1 hour, 3 hours) with 55 mM KCl.

Treated and control cells were collected directly in Laemmli buffer.
When necessary, KCl-dependent depolarization was anticipated by incubating with EGTA (2 mM, 20 min), UO126 (10 μM, 20 min; Promega), H-89 (20 μM, 30 min; Cell Signaling), wortmannin (2 μM, 30 min; Sigma Aldrich), AP5 (100 μM, 30 min; Sigma Aldrich), CNQX (40 μM, 30 min; Sigma Aldrich),
Actinomycin-D (20 μg/ml; 1 hour; Sigma Aldrich), cycloheximide (40 μM; 30 min; Sigma Aldrich).
Neurons were also exposed to specific activators like Forskolin (100 μM, 5 min; Sigma Aldrich), NMDA (50 μM, 5 min; Sigma Aldrich) and BDNF (50 μg/ml, 5 min; Sigma Aldrich). Treated and control cells were collected directly in Laemmli buffer.

KCl-treatment of mouse brain slices

WT C57Bl6J female mice were anesthetized at P35-P38 with halothane (Sigma-Aldrich, Milan, Italy) and decapitated. The brain was quickly removed and put in ice-cold cutting solution (NaCl 125 mM; KCl 2.5 mM; NaH_2PO_4 1.25 mM; $NaHCO_3$ 26 mM; glucose 10 mM; $CaCl_2$ 2 mM; $MgCl_2$ 1 mM) saturated with 95% oxygen and 5% CO_2 (pH 7.4). 200 µm-thick cortical-subcortical coronal frontal slices (anteriorly to the lateral ventricle) were then cut on the vibratome Leica VT1000S and maintained at 32°C for 30 min and at 25°C for other 30 min in the same buffer to allow functional recovery.
Recovered slices were incubated in perfusion chamber and perfused (1 ml/min) at room temperature for 5 min, 20 min and 1 hour with gassed (95% 0_2/5% CO2) solution (KH_2PO_4 1.25 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 17.6 mM, D-glucose 10 mM, pH 7.4) containing isomolar low or high [K^+] (NaCl 125 mM, KCl 3 mM or NaCl 98 mM, KCl 30 mM, respectively) in accordance with a previously published protocol [222]. After perfusion,

the slides were rapidly lysed in a potter with 200 ul of lysis buffer (PMSF 1 mM, Tris HCl pH 7,4 50 mM, NaCl 150 mM, Triton X-100 1%, EDTA 2mM, DTT 1mM, NaF 1mM, Na_3VO_4 1mM, Phosphatse Inhibitor Cocktail (Roche), Protease Inhibitor Cocktail Sigma)) and the protein content of the samples was measured using the Bio-Rad Bradford method. Finally, the samples were conserved in Laemmli buffer and processed by immunoblotting.

***Cdkl5* silencing**

The lentiviral knock-down construct, sh-*Cdkl5,* were generated by cloning a short hairpin sequence against *Cdkl5*-mRNA (5'-CTATGGAGTTGTACTTAAAT-3') into pLentiLox 3.7 that also expresses GFP from an independent promoter. As control, a shLacZ construct, directed against β-galactosidase, was used. Recombinant lentivirus was produced by cotransfecting the pLentiLox 3.7 plasmid with the packaging vectors pREV, VSVG and pMDL into 293T cells. The viral particles were collected 36 hours post-transfection and concentrated by ultracentrifugation at 20.000 rpm for 2 hours. The viruses were resuspended in PBS and stored at -80°C.

Cultured hippocampal neurons were infected at DIV0 (2 hours after plating) and collected at DIV7 after pharmacological/KCl treatment. Samples (300.000 cells silenced with 3 μl of vector concentrated stock [10^9 viral particles/ml] [223-225]) were collected directly in Laemli buffer 1X and processed by Western blot to confirm GFP expression and *Cdkl5* silencing. For RT-qPCR analysis, cells were collected in RLT buffer (RNeasy Mini Kit Cat. No. 74104, QIAGEN) and analyzed as described in the section "Gene expression analysis".

Gene expression analysis:

*** RNA preparation and real-time qPCR**

Total RNA was isolated from treated neurons using the "miRNeasy mini Kit" (Cat.No.217004; QIAGEN) according to the manufacturer's instructions. Contaminating DNA was removed with DNA-free reagent (RQ1 RNase-free DNase Cat.# M6101; Promega) and extracted RNA was quantified with Biotech Ultrospec 2000 UV visible spectrophotometer and its quality assessed through agarose gel electrophoresis (denaturing 1% gel with EtBr).

cDNA was synthetized from 200 ng of RNA using the "SuperScript II Reverse Transcriptase"

Kit (Cat. No. 18064-014; Invitrogen) as indicated by manufacturers. Quantitative Real-Time PCR was performed using 10 ng of cDNA mixed with the "GoTaq® qPCR Master Mix" (Ref.A6002, Promega) and the following primers:

mCDKL5 forward:	TTCCCAGCTGTTAACCATCC
mCDKL5 reverse:	AAGGAGACCGGTCCAAAAGT
c-Fos forward:	GGCAAAGTAGAGCAGCTATCTCCT
c-Fos reverse:	TCAGCTCCCTCCTCCGATTC
mGAPDH forward:	AAGGTCGGTGTGAACGGATTTG
mGAPDH reverse:	GCAGTGATGGCATGGACTGTG

The RT-qPCR reaction was performed according to the following conditions:

1) Mix:
GoTaq® qPCR Master Mix: 1X
Primer Mix 0,6 μM
cDNA: 10 ng
H_2O (total volume 25 μl)

2) Program:
95°C 3 min
Loop: 40 cycles (95°C 15 sec, 60°C 30 sec, 72°C 30 sec.)

The reactions were performed with Multiplate PCR plates™ Low 96-Well White (Cat.No.MLL9651, Bio-Rad) using a real-time cycler BioRad/MJ Research Chromo4.
Each sample was assayed in triplicate, and the experiment was repeated three times. The data were analyzed using the DDCT method: replicates were averaged and compared to the mean value of the normalizer GAPDH in the same sample. A melting curve was automatically generated for each sample and confirmed that a single amplicon was generated in each reaction.

*** RT2 Profiler PCR arrays**

Total RNA from DIV7 hippocampal cultures was isolated as above using the RNeasy® Mini Kit (Cat. No. 74104, QIAGEN) and purified from contaminating DNA directly in the RNeasy Spin Column Membrane (DNase I Stock Solution QIAGEN). *Cdkl5*-silenced and control

hippocampal neurons treated with KCl at DIV7 were collected (300.000 cells per sample) directly in RLT buffer with 40 uM DTT (RNeasy Mini Kit QIAGEN) and processed as following. Extracted RNA was quantified with Varian Cary 50 scan UV-visible spectrophotometer and the quality assessed calculating the O.D. 260/280 ratio after spectrophotometer quantification and through a denaturing 1% agarose gel (with EtBr).
350 ng of RNA was retro-transcribed using "RT2 First Strand Kit" (330401; QIAGEN) and gene transcription profiling was analyzed using the "RT2 Profiler™ PCR Arrays" technology (QIAGEN), following the procedures illustrated in the "RT2 Profiler™ PCR Array User Manual". cDNA was added to "RT2 SYBR Green ROX™ qPCR Mastermix" (330522, QIAGEN) and the mix was aliquoted into "RT2 Profiler PCR Array Format C" (96 wells) (PAMM-126ZA, QIAGEN). Next, the real-time PCR cycling program was run into a Bio-Rad/MJ Research Chromo4 real-time cycler, programmed according to the following table:

Cycles	***Duration***	***Temperature***
1	10 min	95°C
40	15 sec 35 sec 30 sec	95°C 55°C 72°C

Data analysis was performed as indicated in the RT2 Profiler™ PCR Array User Manual. Results were obtained analyzing three DIV7 hippocampal cultures and 2 *Cdkl5*-silenced DIV7 hippocampal cultures treated or not with KCl.

In vitro dephosphorylation assay

For analyzing the phosphorylation status of endogenous murine Cdkl5, we collected hippocampal DIV7 neurons (300.000) before or after KCl treatment, directly in lysis buffer (Protease Inhibitor Cocktail Sigma, PMSF 1 mM, NP40 1%, Tris-HCl pH8 50 mM, EDTA 1 mM, NaCl 150 mM, DTT 0,5 mM, SDS 0,1%) with or without phosphatase inhibitors (NaF 1 mM; $NaVO_4$ 1 mM; Phosphatase Inhibitor Cocktail (Roche)). Protein concentration was estimated using the Bradford method (Bio-Rad). An amount of 50 μg of total protein from the lysate was exposed to 800U of lambda phosphatase (Lambda-PPase, BioLabs) in PPase Reaction Buffer (Tris-HCl 50mM; DTT 5mM; EDTA 0,1mM; Brij 35 0,01%; pH 7.5), supplemented with 2 mM $MnCl_2$, for 3 hours at 30°C. The reactions was stopped by

incubation at 65°C for 15 min and the samples analyzed by immunoblotting.

PP1/PP2a inhibition assay

Cultured hippocampal neurons (DIV7) were treated with 1 μM okadaic acid (O.A.; Sigma Aldrich) for 45 min or with 1 mM sodium orthovanadate (Na_3VO_4, Sigma Aldrich) for 1 hour and then depolarized with the addition of 50 mM KCl for 5 and 20 min.

Proteasome inhibition assay

Hippocampal neurons (DIV7 and DIV3) were treated with MG132 (50 μM; Sigma Aldrich), a proteasome inhibitor, for 6 hours. Treated and control cells were collected directly in Laemmli buffer. When necessary, 3 hours-KCl treatment was anticipated by MG132 (50μM; Sigma Aldrich) pre-treatment for 3 hours.

Western Blotting Analysis

Western blot analysis was performed using standard methods. 30 μl of the lysate was heated (at 70°C when we wanted to preserve phosphorylation, otherwise at 98°C), separated by 8% SDS-PAGE (Acrylamide solution Euro-Clone, Mix 37,5:1) and transferred to nitrocellulose membranes. To enhance the separation of phosphorylated and non-phosphorylated Cdkl5 isoforms, a 7% SDS-PAGE was prepared with a particular concentration of the two forms of acrylamide (acrylamide/bisacrylamide Mix 77:1). Filters were developed by using a chemiluminescence-based detection system (SuperSignal West Pico Chemiluminescent Substrate Pierce; GE Healthcare, Piscataway, NJ) and quantified by scanning densitometry using the QuantityOne software package (Bio-Rad, Hercules, CA, USA). To evaluate phosphorylation, we made a ratio between the normalized phospho-protein and the normalized total isoforms of the same protein (phosphorylated and unphosphorylated).

Immunofluorescence

Hippocampal E18 neurons were seeded on glass coverslips. At DIV7 the cells were treated with KCl and fixed by 4% paraformaldehyde (PFA) in HBSS (15 min), washed and subjected to immunofluorescence. After 1 hour in permeabilization-blocking buffer (0,2% triton X-100, 5% foetal bovine serum in PBS) at room temperature the neurons were incubated overnight at

4°C with primary antibodies in 5% foetal bovine serum and 0,1% triton X-100. After 24 hours, cells were rinsed in PBS three times and incubated with the secondary antibodies (anti-rabbit Alexa Fluor 555 and anti-mouse Alexa Fluor 488, Invitrogen) in blocking solution for 1 hour at room temperature. Nuclei were stained with DAPI (Sigma) and the signal was analysed with an Olympus BX51 fluorescence microscope.

Antibodies

The following antibodies were used for western blotting and immunofluorescence experiments

- Rabbit polyclonal anti-CDKL5 antibody (Sigma Prestige HPA002847; 0,37mg/ml)
- Purified Mouse monoclonal α-tubulin Sigma T6074 (specific for human, mouse, rat)
- Affinity isolated rabbit polyclonal antibody anti-MeCP2 Sigma M9317 (a.a. 465-478); 0,6 mg/ml
- Polyclonal rabbit antibody anti-Erk-1l2 (C-terminal 35 a.a. in human and mouse) Millipore Cat.#06-182
- Rabbit polyclonal anti-phospho-p44/42 Erk (Thr202/Tyr204) antibody, Cell Signaling #9101
- Mouse monoclonal anti-GAD67 Millipore Cat.#MAB5406
- Anti-GFP mouse monoclonal antibodies Roche Cat.No.1814460
- Monoclonal mouse anti-neuronal class III-beta-tubulin (clone TUJ1) antibodies, Covance MMS-435P; 1mg/ml
- Rabbit polyclonal cleaved caspase-3 (Asp175) antibody, Cell Signaling #9661
- Rabbit polyclonal anti-GAPDH antibody Millipore AB2302
- HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies for Western blotting from Thermo Scientific
- Secondary Alexa Fluor anti-rabbit and anti-mouse antibodies (and DAPI) for immunofluorescence experiments were from Invitrogen

Statistical Analysis

All values are expressed as the average of at least three different experiments ± standard error (S.E.M.). The significance of results was evaluated by Student's *t* test, and statistical significance was established as $p < 0.05$.

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