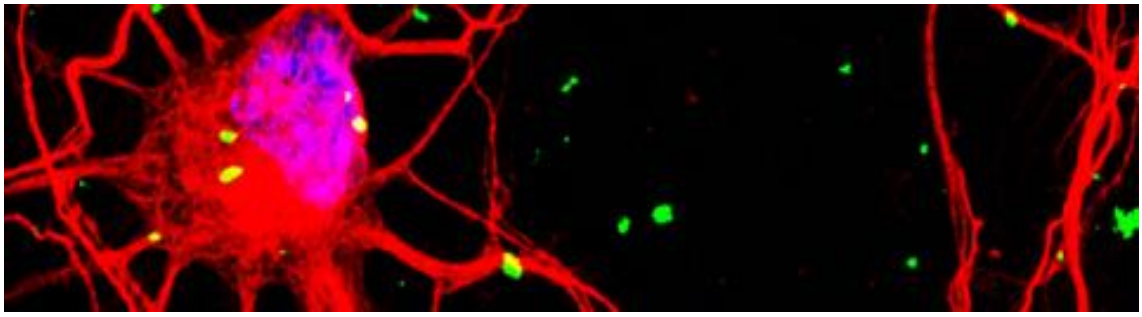

Corso di Dottorato in Neuroscienze
Curriculum Neuroscienze e Neurotecnologie
Ciclo XXXI

Role of Ca^{2+}/Calmodulin dependent protein kinase IV on REST activity

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1 ABSTRACT

The transcriptional repressor REST (Repressor Element 1-Silencing Transcription factor) is a zinc finger domain repressor protein which binds to the specific RE1 regulatory sequence in its target genes and limits their transcription by histone deacetylases and histone methylases recruitment. REST is involved in many key biological processes, including neural differentiation, neurogenesis, and titration of REST levels occurs during these processes.

As key regulators of protein biology, post-translational modifications modulate protein structure, activity and localization. It has been demonstrated that REST protein degradation during neural differentiation could be regulated by ubiquitin-mediated proteolysis through a conserved phosphodegron, stressing the importance of the regulation of REST phosphorylation levels. This phosphorylation-mediated control of REST levels appears to be important also in pathologies.

The principal aim of this study was to investigate whether REST expression may be modulated by calcium-signalling, a pivotal second messenger that regulates a variety of cellular processes, with special attention to calcium-dependent enzymes.

By a computational bioinformatics analysis, we identified five phosphorylation sites for Ca2+/calmodulin-dependent kinases (CaMKs), a family of Serine/Threonine protein kinases that is responsible for mediation of many intracellular responses to elevated Ca2+. By pharmacological and genetic approaches, we demonstrated that CaM-Kinase IV (CaMKIV) exerts a negative post-translational regulation on REST protein stability in cortical neurons.

We employed in our study a RESTGTinv-conditional animal model in which the expression of REST and its truncated forms are totally abolished upon Cre-recombinase activity. By specifically removing the expression of this transcription factor and/or interfering with kinase activities at the same time, we gain deeper details in REST functions and its possible regulation by CaMKIV. Our result shows that REST directly regulates synapse formation and activity, not impacting early stages of neurodevelopment. We also identified a role for both CaMKIV and REST in tuning the autophagic pathway, acting as negative and positive regulators respectively.

Altogether, our data highlight an important interplay between CaMKIV and REST, which can be a crucial control mechanism regulating different aspects relevant in the physiopathology of neurons in a calcium-related manner.

2 INTRODUCTION

2.1 RE-1 Silencing Transcription Factor (REST)

The Repressor Element-1 (RE-1) Silencing Transcription factor (REST), also known as Neuron-Restrictive Silencing Factor (NRSF), was identified as master regulatory gene of the neuronal phenotype in 1995 (Chong et al., 1995; Schoenherr & Anderson, 1995).

REST is a multi-zinc finger protein and was originally cloned as the repressor factor that binds the 21 bp repressor element 1 (RE1) or neuron-restrictive silencer element (NRSE) sequence in the promoter region of superior cervical ganglion 10 (SCGN10) and type II sodium channel (SCN2A). Genome-wide analyses predicted the complete set of RE1 sites and their associated genes, reporting that the RE1 consensus sequence is present in 1,892, 1,894 and 554 sites in the human, mouse and pufferfish genomes, respectively (Bruce et al., 2004).

Bioinformatics approaches and chromatin immunoprecipitation sequencing assays (ChIP-Seq) have revealed 40% of REST target genes are known to be expressed within the nervous system and encode for ion channels, synaptic proteins, cytoskeletal components and neural cell adhesion molecules (Fig. 2.1A). According to these data, REST has been defined as a transcriptional silencer of neuronal gene expression (Bruce et al., 2004).

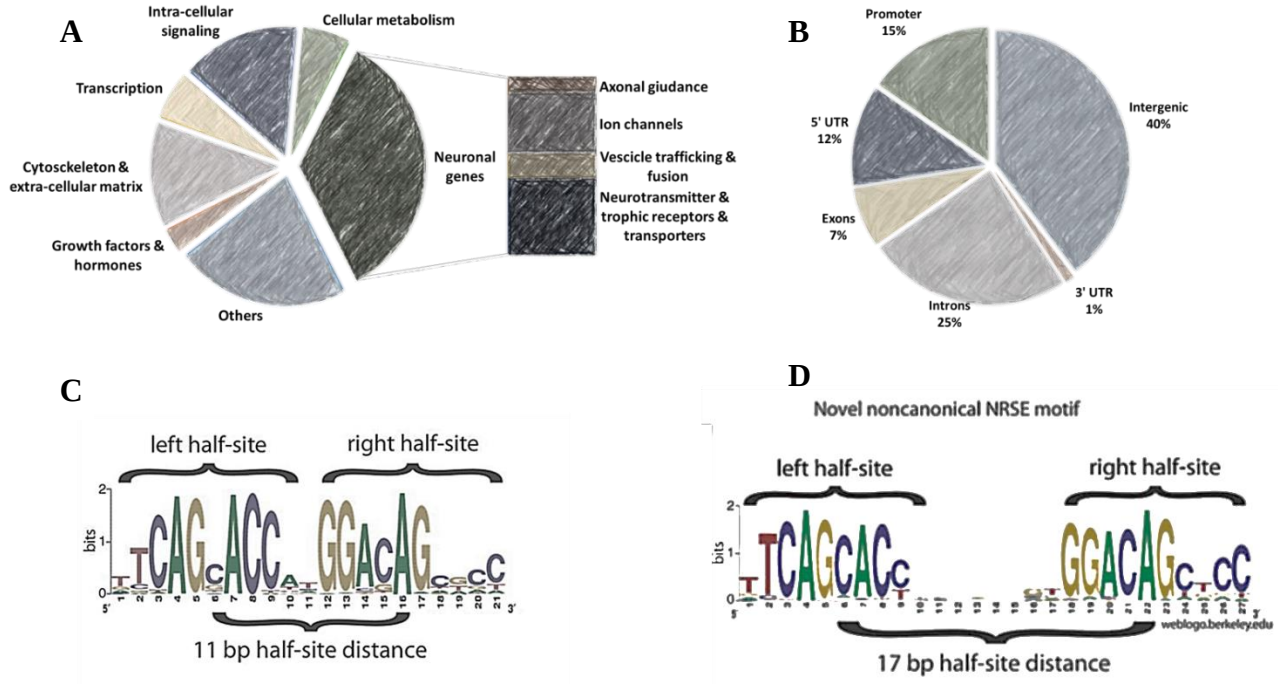

Figure 2.1. (A) Assignment of putative REST target genes within the RE1db database (adapted from Bruce et al., 2004); (B) Distribution of RE-1 binding sites across the genome (adapted from Jothi et al., 2008); (C) Canonical REST-binding motif and (D) noncanonical REST-binding motif (adapted from Johnson et al., 2007)

2.1.1 REST gene structure and isoforms

The human REST gene comprises 24 kb of genomic DNA. It is composed of three non-coding exons associated with different gene promoters, exons I-III, three coding-exons, exons IV-VI, and an alternatively spliced exon that contains a premature stop codon, exon N, located between exon V and VI. The exon and intron structure is conserved across human, mouse and rat (Palm, Metsis, & Timmusk, 1999; Coulson et al., 2000) (Fig. 2.2A).

Several REST splice variants have been identified and characterized: (i) hREST-N62 transcript, generated by the insertion of exon N; (ii) hREST-N4 transcript, due to alternative splicing-mediated addition of 4 bp within exon N; (iii) hREST-5FΔ transcript, resulting from exon V and exon N skipping; (iv) hREST1 transcript, composed only by exon 1 (Palm, Metsis, & Timmusk, 1999) and (v) sNRSF, identified in human SCLC, with a missing C-terminal repressor domain (Coulson et al., 2000).

The hREST-N62 and hREST-N4 transcripts encode for truncated REST isoforms, similar to the two REST isoforms (REST 4 and REST5) expressed in mature neurons of adult rat brain, that retains the N-terminal repressor domain and five of the nine zinc fingers (Palm, Metsis, & Timmusk, 1999). Acting in a dominant-negative fashion, it has been proposed that these isoforms antagonize REST-mediated gene repression leading to a de-repression of an RE-1 containing genes (Shimojo et al., 1999; Lee et al., 2000). Indeed, REST isoforms itself play a pivotal role in the physiological control of REST activity, as shown by Nakano and colleagues in which they highlighted the importance of alternative splicing-mediated REST action inactivation (Nakano et al., 2018). The authors showed that REST mRNA alternative splicing including frameshift-causing exon is essential for correct development of all mechanosensory hair cells in the ear, and in turn for a correct response to sound in mice and humans.

REST is subjected to extensive and context-dependent alternative splicing producing at least 45 mRNA variants predictive for structurally and functionally different REST protein isoforms (Chen & Miller, 2018). In light of this, REST mutations that affect the alternative splicing phenomena can impair the correct REST action tuning, leading to pathological conditions.

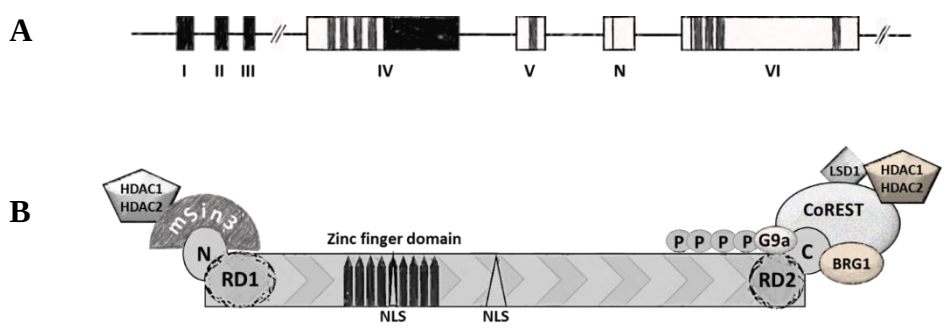

Figure 2.2. (A) REST gene structure and (B) Structure of the REST chromatin-modifying complex. REST-mediated gene repression is achieved by the recruitment of two separate corepressor complexes, mSin3 and CoREST. The mSin3 complex contains two class I histone deacetylases (HDACs), HDAC1 and HDAC2, which are thought to interact with histones, and several other proteins with unclear function. The class II HDACs HDAC4 and HDAC5 are also associated with the mSin3 complex. The CoREST complex contains HDAC1 and HDAC2, the histone H3K4 demethylase LSD1, and the chromatin-remodelling enzyme BRG1

2.1.2 REST Binding Site: Repressor Element-1 (RE-1)

REST represses gene transcription by binding a DNA sequence located in the regulatory regions of its target genes. This evolutionally conserved regulatory sequence of 21-23 bp is called Repressor Element-1 (RE-1) (Chong et al., 1995) (Fig. 2.1C).

Due to its relatively conserved sequence, many different approaches have been used over the years trying to identify the complete set of RE-1 sites and therefore REST target genes (Bruce et al., 2004; Johnson et al., 2007). The identified RE-1 sites are located in different regions of the human genome, spanning from promoter regions and exons to 3'UTR sequence, with the majority of them within the intergenic regions (40%) (Fig. 2.1B) (Jothi et al., 2008). Nevertheless, not all putative REST target genes are bind and regulated by the repressor.

The canonical 21 bp RE1 sequence can be subdivided in two non- canonical half sites, separated by a spacer sequence (Fig. 2.1D). REST has been shown to bind to the two non-identical and non-palindromic sites independently, but the binding affinity is weaker and less functional than full-site binding (Jothi et al., 2008). Indeed, some RE-1 sites are never bound by the transcription factor possibly due to their localization in the locus gene. Moreover, also the repressor affinity for the RE-1 sites can vary significantly depending on the target gene, complicating the comprehension of REST function and with consequences on the degree of targets repression (Mcclelland et al., 2014).

2.1.3 Regulation of REST

Specification of cellular identity during development requires a fine-tuned gene expression, which is regulated at transcriptional, translational and post-translational level.

In contrast to REST target gene regulation, little is known about REST regulation itself. Moreover, the identification of at least two functional promoters that drive REST transcription uniformly in neuronal and non-neuronal cells raise the possibility that REST transcription were similar in neuronal and non-neuronal cells (Kojima et al., 2001), suggesting that REST gene expression is not defined transcriptionally, but rather by post-transcriptional and/or post-translational modifications in a cell-specific manner.

Transcriptional regulation of REST has been described to involve both positive and negative elements, including cAMP response element-binding protein (CREB; Kreisler et al., 2010), Yin Yang 1 (YY1; Ravache et al., 2010), Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A; Canzonetta et al., 2008), Specificity Protein 1 (Sp1; Ravache et al., 2010), HIP1 protein interactor (HIPPI; Datta et al., 2011) and Wnt/TCF signaling (Nishihara et al., 2003). Moreover, REST mRNA stability is regulated by several microRNAs, including miR-153, miR-9 and miR-218 (Packer et al., 2008; J. J. Liu et al., 2016).

Recent studies have reported that REST activity is controlled by post-translational modifications. Although little is known about full-length REST glycosylation, experimental data on REST4 provided evidence that REST might be O-glycosylated on its C-terminal region, a post-translation modification not required for REST DNA-binding activity (Lee et al., 2000b). On the other hand, experimental evidence also showed that phosphorylation played a fundamental role in REST protein stability/degradation, a key point of regulation for its abundance.

In 2008, two interesting manuscripts by two independent groups were published claiming that REST protein degradation during neuronal differentiation is regulated by ubiquitin-mediated proteolysis (Westbrook et al., 2008; Guardavaccaro et al., 2008). They found that, upon REST degrons phosphorylation, beta-trasducin repeat containing E3 ubiquitin ligase (β-TrCP) binds and ubiquitinates REST targeting the transcription factor for proteasomal degradation. In their work, they showed for the first time a role of the phosphorylation status in REST protein stability through a conserved phosphodegron, stressing the importance of the regulation of REST phosphorylation levels (Westbrook et al., 2008). Starting from their work, different research groups have begun to study the importance of REST phosphorylation and their findings are summarized in the table below (**Table 2.1**).

Enzymes	Sites	Modifications	Effects	References
	S1024-S1027-S1030	Phosphorylation	Degradation by β-TrCP	Westbrook et al., 2008
CK-1	S1024-S1027-S1030	Phosphorylation	Degradation by β-TrCP	Kaneko et al., 2014
PLK 1	S1024-S1027-S1030	Phosphorylation	Degradation by β-TrCP	Karlin et al., 2014
	E1009-S1013	Phosphorylation	Degradation by β-TrCP	Guardavaccaro et al.,2008
CK-1	E1009-S1013	Phosphorylation	Degradation by β-TrCP	Kaneko et al., 2014
ERK 1/2	S861-S864	Phosphorylation	Degradation by β-TrCP	Nesti et al., 2014
CTDSP1	S861-S864	Dephosphorylation	Stabilization	Nesti et al., 2014
HAUSP	310-PYSS-313	Deubiquitination	Stabilization	Huang et al., 2011

Table 2.1 List of REST phosphodegrons and their regulators

As shown in the table above, different actors are involved in the phosphorylation-induced REST protein level tuning. Casein Kinase 1 (CK-1) was identified as one of the main upstream factors that regulate REST cellular abundance by the phosphorylation at two neighbouring phosphodegrons, although different factors are involved in this kind of regulation (Kaneko et al., 2014). Indeed, different phosphodegrons are present within the REST protein sequence and different kinases may phosphorylate the same residue, as shown by Karlin and colleagues (Karlin et al., 2014). Moreover, the same phosphodegron may be targeted by both kinases and phosphatases, highlighting a more dynamic and complex process (Nesti et al., 2014).

Furthermore, adding a new layer of complexity in this process, a Herpesvirus-associated ubiquitin-specific protease (HAUSP) has been shown to act as a deubiquitinase, counterbalancing β-TrCP activity (Huang et al., 2011). REST possess a specific consensus site for HAUSP within its sequence (310-PYSS-313) and, like β-TrCP, also HAUSP plays a pivotal role in REST titering during neural differentiation. Indeed, the concomitant downregulation of HAUSP and upregulation of β-TrCP in neuronal progenitors lead to a downregulation of REST protein level, allowing the neuronal differentiation (Huang et al., 2011).

Notably, several evidence has been collected showing that dysregulation of REST phosphorylation leads to different pathologies. Karlin and colleagues highlight the importance of REST phosphorylation-mediated degradation in the progression of triple-negative breast cancer (TNBC) (Kristen et al., 2014). Similarly, Dong's group describes a similar mechanism in treatment-induced neuroendocrine prostate cancer (t-NEPC),

highlighting the phosphorylation impact on REST stability in physiological and pathological conditions (R. Chen et al., 2017).

2.1.4 Searching for REST: Subcellular Localization

The nuclear localization of transcription factors is a key process in the transcriptional regulation of genes, therefore the translocation of REST into the nucleus is tightly regulated and represent a pivotal layer in the regulation of REST activity.

The nuclear targeting of this transcription factor is dependent on a canonical NLS and a non-canonical NLS involving zinc finger 5 of the DNA binding domain (Grimes et al., 2000; Shimojo et al., 2001). Among the two different NLS, the non-canonical one appeared to be fundamental for the transcription factor localization, as truncated forms of REST that lack the canonical NLS appears to be localized in the nucleus anyway (Shimojo et al., 2001).

The nucleocytoplasmic shuttling of REST appears to be a process tightly regulated, involving different proteins. REST directly interacts with REST-interacting LIM domain protein (RILP), a nuclear translocation receptor, and together they form a complex with the Huntington protein and dynactin $p150^{Glued}$ (Shimojo & Hersh, 2003; Shimojo, 2008). According to this model for REST nuclear translocation, both RILP and Huntingtin directly interact with dynactin $p150^{Glued}$, initiating the complex formation. REST binds to this complex through direct interaction with RILP, allowing REST nuclear transport. Finally, a further protein is involved in controlling the subcellular localization of REST in neuronal cells, the huntingtin-associated protein-1 (HAP1) (Shimojo, 2008). This protein is expressed predominantly in neuronal cells where it can bind to huntingtin and causes the retention of the complex in the cytosol while, in non-neuronal cells, the absence of HAP1 cause the correct traffics of REST to the nucleus leading to neuronal gene silencing in non-neuronal cells.

2.1.5 REST in action: Gene Regulation by REST

REST is a transcriptional repressor involved in silencing of neural genes in non-neuronal cells and, given its domain organization, this transcription factor acts as a scaffold recruiting various corepressor. These two separate repressor domains, located at the N- and at the C-terminus of the protein, recruit mSin3 and CoREST respectively (Grimes et al., 2000; Andres et al., 1999). The two major co-repressors proteins further engage proteins involved in chromatin remodelling and histone modification providing multiple layers of control that drive robust repression of its target genes. Indeed, mSin3 interacts with different Histone Deacetylases (HDACs) and the methylated CpG binding protein MeCP2, whereas CoREST engages histone methyltransferases, HDAC and chromatin remodelling complex such as Brg1 and G9a, and others as overall described in Ooi & Wood, 2007 (Fig. 2.3). Together these complexes modify the chromatin status adding markers for gene repression, as H3K9me2, and/or removing markers for gene activation, as H3K4, leading to a more condensate and less accessible chromatin.

In addition to this mechanism, REST also plays a direct role in repressing transcription by interacting with TATA-binding protein (TBP) and small CTD phosphatases (SCPs), two proteins involved in the transcription machinery (Murai et al., 2004; Yeo et al., 2005). In the first case, the interaction between REST and TBP inhibits the formation of the transcription preinitiation complex, while in the latter case REST localized SCPs to the carboxyl-terminal domain (CTD) of RNA polymerase II leading to its dephosphorylation and polymerase inactivation.

Finally, it is worth to say that both REST action and corepressor binding appears to be cell-specific and context dependent. Indeed, depending on the tissues, REST can mediate both transient and long-term silencing, by remaining associated at the RE-1 sites or leaving associated CoREST after its dissociation. Therefore, this mechanism represents an effective tuning of gene regulation through different RE-1 sites.

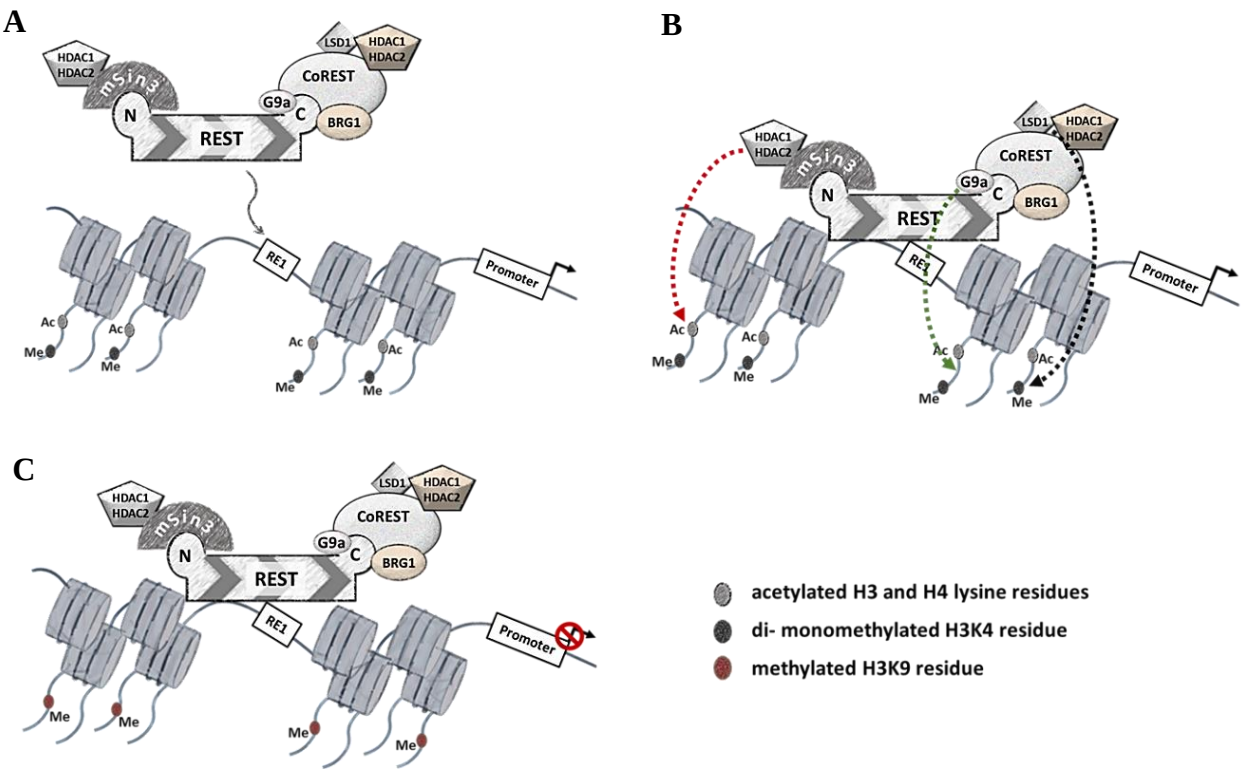

Figure 2.3. (A) The REST-repression complex is recruit to RE-1 sites and interaction with DNA is stabilized by BRG1, a chromatin-remodelling enzyme. (B) The N and C-termini of REST interact with the mSin3 and CoREST complex respectively, recruiting both histone deacetylases HDAC1 and HDAC2, H3K4 demethylase LSD1 and the H3K9 methylase G9a. Removal of acetylation from H3K9 stimulates LSD1 activity, which in turn removes di- and monomethylation from H3K4. Removal of H3K9 acetylation by HDACs also provides a substrate for G9a-mediated methylation. (C) As a result of REST recruitment, several modifications that are associated with active gene transcription are removed, and at least one mark that is associated with gene repression is added, providing gene repression chromatin landscape.

2.1.6 Like a Master: REST and Regulation of Neurogenesis

Among the many key biological processes in which REST is involved, it has been shown that it plays a pivotal role as a determinant of neuron-specific gene expression, as well as a regulator of neuronal gene expression during early embryogenesis. Indeed, REST-full knockout embryos develop normally until embryonic day 9.5, and after this stage, they undergo cellular disorganization and widespread apoptotic cell death. These

phenomena result in malformations of the developing nervous system and insufficient growth, leading to death at embryonic day 11.5 (Chen et al., 1998).

Consistent to its role as master regulator of neural differentiation, REST is highly expressed in stem cells, while its levels decline in neural progenitors and are maintained low in differentiated neuronal cells. The low expression of REST allows the transcription of a large panel of genes, all physiological targets of REST repression, which are necessary for the acquisition of the unique phenotype of neural cells (Ballas et al., 2005; Negrini et al., 2013). In addition, it has also been proposed that REST plays a role in controlling neurogenesis in adult hippocampus, especially in maintaining the adult neural stem cell pool and orchestrating stage-specific differentiation by the coordinated regulation of neuronal, ribosome biogenesis and proliferation genes (Gao et al., 2011; Mukherjee et al., 2016).

2.1.7 REST in animal models

REST is involved in brain development and activity as well as in the establishment of neuronal specificity. This transcription factor also plays a direct role in the pathogenesis of several neurological disease and cancers, therefore numerous animal models have been developed to elucidate its functions. Table 2.2 presents a summary of the animal models in which REST expression has been genetically modified, interfering with its function or its expression, as well as through its complete genomic ablation.

REST/NRSF-impaired function models

Animal Model	Tool	Findings	References
Chick embryo fibroblasts (CEFs)	dnREST injection	REST functions *in vivo* to control the proper spatial and temporal expression of neuronal genes	(Chen et al., 1998)

Chick embryo	Electroporated full-length mouse NRSF cDNA	Down-regulation of REST is necessary for the proper development of, at least, some classes of neurons in vivo	(Paquette et al., 2000)
Xenopus laevis embryo	dn-protein or antisense oligonucleotides injection	REST may be used to activate or repress transcription of neuronal genes in distinct cellular and developmental contexts	(Armisen et al., 2002)
Xenopus laevis embryo	Inducible dn-protein or antisense oligonucleotides injection	REST/ function is required *in vivo* for the acquisition of specific ectodermal cell fates	(Olguin et al., 2006)
Drosophila melanogaster	---	Charlatan (chn), analogous to REST in Drosophila, ensures robust development of sensory neurons	(Yamasaki et al., 2011)
Zebrafish	Zinc-finger nucleases (ZFNs)	- REST is not required to maintain pluripotency or self-renewal of developing zebrafish blastomeres - Dynamic roles for REST-mediated transcriptional regulation on complex behaviours in zebrafish - Maternal REST represses snap25a/b to modulate larval behaviour and early REST activity has lifelong behavioural impacts	(Kok et al., 2012); (Moravec et al., 2015); (Moravec et al., 2016)
Zebrafish	Morpholinos injection	REST (especially maternal supplied REST) is required for gastrulation and neurogenesis during zebrafish early embryogenesis	(Wang, et al., 2012)
Human ESC	Doxycycline-induced expression of shRNAmir	New role for REST in the regulation of growth and early differentiation decisions in human embryonic stem cells	(Thakore-Shah et al., 2015)

Overview of REST mutants and their phenotypes			
Cell types	**Permanent or Conditional**	**Findings**	**References**
Mouse embryonic stem (ES) cells	Permanent KO	- REST KO leads to embryonic death (E11.5) - REST functions *in vivo* to control the proper spatial and temporal expression of neuronal genes	(Z. Chen et al., 1998)
ESCs (Mouse)	- Conditional KO (transient transfection of Cre-Recombinase) - Conditional inducible (doxycycline-dependant)	REST is not required for maintenance of pluripotency, but it is involved in the suppression of self-renewal genes during early differentiation of ESCs	(Yamada et al., 2010)
Neurons (Mouse)	Conditional KO (neuron-specific enolase, NSE-Cre)	- REST, specifically REST4, may protect the developing brain from ethanol - Evidence that REST can be a therapeutic target in foetal alcohol syndrome (FAS) - REST protective roles in pentylenetetrazol (PTZ)-induced seizure - REST cKO mice are more vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease mode - REST cKO in neurons causes the disturbance of the homeostasis of neurotransmitters like DA and 5-HT, dopaminergic neurons in the SN	(Cai et al., 2011); (M. Liu et al., 2012); (Yu et al., 2013)
Adult Neural Stem Cell (NSC)(Mouse)	Conditional KO (Nestin-Cre)	REST is required to maintain the adult NSC pool and orchestrates stage-specific differentiation	(Zhengliang Gao et al., 2011)

Excitatory neurons (postnatal mouse forebrain)	Conditional KO (CaMKIIa-Cre)	REST functions as an intrinsic repressor of limbic epileptogenesis	(Hu et al., 2011)
Retinal Progenitor Cell (RGC) (Mouse)	Conditional KO (Six3-Cre)	*In vivo* role for REST in mammalian retinogenesis preventing premature retinal ganglion cells (RGC) genes expression in RPCs	(Mao et al., 2011)
ESCs (Mouse)	Conditional KO (required doxycycline)	REST plays a role in suppressing the expression of neuronal genes in cultured neuronal cells *in vitro*, as well as in non-neuronal cells outside of the central nervous system, but it is dispensable for embryonic neurogenesis *in vivo*	(Aoki et al.,2012)
Primordial germ cells (PGC) (Mouse)	Conditional KO (TNAP-Cre)	REST promotes PGC survival via regulation of the Mek5 expression	(Okamura et al., 2012)
Neural Crest cell (NCC) (Mouse)	Conditional KO (Wnt1-Cre)	- REST controls the acquisition of the specific NC cell lineage - Failure of gut function by underdeveloped cholinergic transmission in the enteric nervous system - Expression of REST during the early neural crest specification stage is necessary for the normal development of melanoblasts to cover all of the skin	(Aoki et al., 2014); (Aoki et al., 2015)
Cultured astrocytes (Mouse)	Conditional KO (transduction with Cre-encoding adenovirus)	Temporal hierarchy for cell fate change during neuronal reprogramming	(Masserdotti et al., 2015)
Quiescent neural progenitors (QNPs) (Mouse)	Conditional KO (transduction with Cre-encoding lentiviral)	- REST regulates both QNPs and TAPs, and importantly, ribosome biogenesis, cell cycle and neuronal genes in the process.	(Mukherjee et al., 2016)

		- Novel REST targets to maintain the quiescent neural stem cell state.	
Neurons (Mouse)	Conditional KO (Nestin-Cre)	- REST-mediated chromatin remodelling is required in neural progenitors for proper S-phase dynamics - REST repression protects the integrity of neuronal genes whose expression must be delayed until terminal differentiation	(Nechiporuk et al., 2016)
Neurons (Mouse)	Knock-in (Nestin-Cre)	- Overexpression of REST in DRD2-expressing neurons lead to spontaneous locomotion deficits	(L. Lu et al., 2018)

Table 2.2 List of REST in animal models

Among the various REST transgenic mouse lines, the $REST^{GTinv}$ mice developed by Nechiporuk and colleagues is the only model in which REST expression is totally abolished, while in other animal models a C-terminal REST peptide is still expressed (Nechiporuk et al., 2016; Zhengliang Gao et al., 2011).

REST is an important negative regulator of neuronal differentiation because differentiation of NSCs to mature neurons requires the activation of genes controlled by REST itself. In ES cells, downregulation of REST is sufficient to induce differentiation toward the neuronal lineage, increase the expression of mature neuronal markers, and decrease the expression of the astrocytic marker glial fibrillary acidic protein (GFAP) (Gupta et al., 2009). *In vivo* studies provide solid evidence supporting REST roles in neurogenesis, although different models showed different and apparently contradictory results.

In Xenopus embryos, REST inactivation induces abnormal neurogenesis including perturbations of the neural tube and decreased expression of neural crest markers (Olguin et al., 2006). In chicken embryos, studies have demonstrated that REST is necessary for proper development because its inhibition caused de-repression of neuronal tubulin as well as increases in the frequency of axon guidance errors (Paquette et al., 2000). In zebrafish, although a broad role for REST in the fine-tuning of neural gene expression

has been reported, the results seem controversial. Wang et al. reported that morpholino-mediated REST knockdown resulted in gastrulation delay or blockage and subsequent embryo lethality with deficient neurogenesis (Wang et al., 2012). Kok et al. reported that neurogenesis in zinc-finger nucleases-induced REST mutants was largely normal and only minor abnormalities were observed within the nervous system, while the surviving adult mutants showed abnormal behaviours such as atypical swimming patterns (Kok et al., 2012; Moravec et al., 2015). Finally, recent works in REST conditional mouse models have shown that this repressor is required to maintain the adult NSC pool and orchestrate stage-specific differentiation (Gao et al., 2011). Moreover, REST inactivation in neuronal progenitors shows that the absence of REST in proliferative cells that normally express it leads to DNA damage (Nechiporuk et al., 2016). These apparently controversial data about REST depletion may result from the fact that REST is primarily involved in the acquisition of neuronal phenotype. Indeed, the differentiation process is a highly regulated phenomenon with different actors and complex feedback mechanisms, therefore different results obtained with different models should be considered in light of the complexity of the organisms in which the experiments were carried out.

2.1.8 REST homeostasis: needs for a fine tuning

REST dysfunctions are implicated in a various number of diseases and cancers, both in the nervous system and non-nervous tissues. REST final action on its target genes is subject to many different factors, starting from the target itself, the cell context and the (dis)function of REST effectors. Perturbation of REST expression can be the consequence of impairment occurs at different steps in the pathway that regulates its functions (Table 2.3).

REST/NRSF Dysfunction and Related Diseases		
REST dysfunction	**Disease(s) Connected**	**References**
Genetic deletion	Colon Cancer; Foetal death	(Westbrook et al., 2005); (Mahamdallie et al., 2015).
Decreased expression	Schizophrenia; Down Syndrome; Foetal death; Human Epithelial Cancers	(Loe-Mie et al., 2010); (Canzonetta et al., 2008; (Lepagnol-Bestel et al., 2009); (Chen et al., 1998); (Westbrook, et al., 2008).
Enhanced expression	Drug addiction and mental disorders; Ischemic insult; Epilepsy; Medulloblastoma; Glioblastoma Multiforme (GBM); Pheochromocytomas; Autism spectrum disorder (ASD)	(Henriksson et al., 2014); (Noh et al., 2012); (Lawinger et al., 2000); (Kamal et al., 2014); (Alessandro et al., 2008); (Katayama et al., 2016).
Impaired function	SMCX-associated X-linked mental retardation; Parkinson's disease; non-SCLC; Prostate Cancer; Breast Cancer	(Tahiliani et al., 2007); (Yu et al., 2013); (Walker et al.,2006); (Tawadros et al., 2005); (Wagoner et al., 2010).
Subcellular mislocalization - lost from the nuclei -	Alzheimer's Disease	(T. Lu et al., 2014).
Subcellular mislocalization - accumulation in the nuclei -	Huntington's Disease	(Zuccato et al., 2003).
Truncated REST mutant	Colon Cancer; Small Cell Lung Cancers (SCLCs)	(Westbrook et al., 2005); (Coulson et al., 2000).

Table 2.3

As shown in table 2.3, a huge number of events could lead to a REST-mediated transcriptional regulation dysfunctioning and in turn to a broad spectrum of diseases.

In the brain, based on the cell type considered and the type of REST dysfunctioning, various pathologies are developed. A down-regulation of this repressor has been shown to be related to Schizophrenia (SZ). Indeed, alterations of Smarce1, Smarcd3 and SWI/SNF levels are associated with a decreased REST and, this environment leads to Smarca2 deregulation that in turn generates an abnormal dendritic spine morphology, an intermediate phenotype of SZ (Loe-Mie et al., 2010). Another psychiatric disease that is

associated with REST dysfunction is the SMCX-associated X-linked mental retardation. In this pathology a loss of SMCX (a demethylase) enzyme leads to impairment in REST-mediated downregulation, resulting in a dysregulated expression of SMCX/REST-regulated genes SCG10, BDNF and SCN2A that are implicated in mental retardation (Tahiliani et al., 2007).

In addition to these psychiatric diseases, some neurological diseases are also related with REST dysregulation, and this is the case of Alzheimer's and Huntington's diseases. In these pathologies, REST protein level and functions are not impaired, but the repressor loss its correct subcellular localization. Indeed, in Alzheimer's disease REST is lost from the nucleus and appears in autophagosomes together with pathologic misfolded proteins. This phenomenon leads to a derepression of genes that promote cell death and AD pathology, inducing also the expression of stress response genes (T. Lu et al., 2014). In a totally opposite fashion in Huntington's disease, aberrant accumulation of REST in the nucleus is the event that triggers the pathology (Zuccato et al., 2003). In this case, mutant huntingtin weakened the interaction between REST nuclear localization complex and HAP1, impairing the correct REST localization (Shimojo, 2008).

Finally, REST-related diseases can be trigger not only in a down-regulated/mislocalized-REST environment, but even an enhanced repressor expression can lead to pathological conditions, as in global ischemia insult. It has been demonstrated that in differentiated neurons global ischemia reduces CK1 and β-TrCP E3 ligase abundance, resulting in an enhanced REST expression and in turn to a down-regulation of pro-survival REST target genes (Noh et al., 2012).

In conclusion, given the above-reported evidence, understand in deeper details the molecular mechanisms that underlie REST functioning is a crucial step in identifying novel possible pathogenic pathways since alterations at any step of this process could lead to broad-spectrum diseases.

2.2 Calmodulin-dependent kinases (CaMKs): structure and function

Almost all physiological functions are founded on a multitude of signalling molecules, some of these interconnect cells over long distances, while some others coordinate the actions and information among neighbouring cells.

It is universally accepted that calcium (Ca2+) ions are the most ubiquitous and pluripotent signalling molecules, involved in a multitude of physiological and pathophysiological mechanisms. In many secretory cells, including nerve cells, Ca2+ finely tunes their secretory activity, while in muscle cells Ca2+ triggers contraction. Ca2+ is such a powerful signalling mediator because its concentration can undergo dramatic changes ranging from basal values of 50 nM in the cytosol to stimulated levels around 1–10 μM depending on the cell type (Shen et al., 2002).

Ca2+-dependent regulation occurs in very different temporal and spatial domains, varying within extremely rapid and localized events (e.g. neurotransmitters release) to long-lasting adaptive reactions (e.g. learning and memory). Such a fine regulation requires a large variety of exchangers, channels and pumps on both the plasma membrane and intracellular storage organelles (e.g., endoplasmic reticulum, mitochondria), as well as low-affinity, high-capacity cytoplasmic buffer proteins (e.g., calsequestrin, calreticulin) (Berridge, Lipp, & Bootman, 2000a). This large amount of control mechanisms cooperate to finally create a large gradient tending to drive Ca2+ into the cytosol across both the plasma membrane and the endoplasmic reticulum (ER). In this way, after the ligand-mediated opening of its channels, the concentration of Ca2+ may undergo a 10-20 fold-increase and activate Ca2+-responsive proteins inside the cell.

In light of this, it is not surprising that Ca2+-homeostasis is essential for life, and failure of such homeostatic cascades trigger universal cell death routines, which are firmly conserved throughout evolution (Nicotera P. et al., 2007).

2.2.1 Calmodulin (CaM)

Ca2+ is a highly reactive ion, therefore prolonged elevation in Ca2+ concentration inside the cell may result in high toxicity. In light of this, cells have to buffer and maintain Ca2+ levels at a basal and controlled state (50-100 nM concentration).

Among the numerous high affinity and specificity of Ca2+ binding-proteins, for several aspects calmodulin (CaM) is unique and it is responsible for transducing many of the second messenger effects due to elevated Ca2+ concentration inside the cell (Linse et al., 1991).

Calmodulin is a relatively small protein (17 kDa) ubiquitously expressed and its shape resembles a dumbbell. Its N- and C-terminal domains are globular-shaped and connected by a flexible α helix that allows CaM to hire different conformations when bound to different targets. Each terminal lobe contains two helix-loop-helix 'EF-hand' motifs responsible for CaM-Ca2+ binding (Chin & Means, 2000). When saturation state is reached (that occurs after binding to four Ca2+ ions), CaM undergoes a conformational change, exposing hydrophobic residues and promoting the interaction with and activation of different target enzymes (Chin & Means, 2000).

2.2.2 Calmodulin-dependent Kinases (CaMKs)

Of the many signalling proteins regulated by Ca2+/CaM complex, a family of Serine/Threonine (Ser/Thr) protein kinases named calmodulin-dependent kinases (CaMKs) is responsible for mediation of many intracellular responses to elevated Ca2+.

CaMKs are ubiquitously expressed in most mammalian tissues and, as well as CaM, are highly abundant in the brain. CaMKs are considered the master regulators of a variety of activity-dependent protein phosphorylation events that are crucial during synaptic plasticity, gene expression, and cytoskeletal remodeling (Chin & Means, 2000; Silva et al., 1992a-b; Matthews et al., 1994; Takemoto-kimura et al., 2010).

The overall domain organization of each member of the kinase family is similar, displaying an N-terminal bi-lobed catalytic domain followed by an autoinhibitory domain partially overlapping with the CaM-binding domain (Soderling & Stull, 2001). In conditions of low Ca2+ levels, the interaction between the catalytic domain and the autoinhibitory domain keeps CaMKs in an inactive state. This may occur either because the binding of the enzyme to its substrates is impeded, or because its catalytic domain is subjected to physical distortion and loses its functionality.

Although its name clearly implies Ca2+/CaM roles in the activation process of this kinase family, it is now clear that the different members of the kinase family are capable to become Ca2+/CaM-independent upon activation and/or require further modifications to achieve full activation (i.e. phosphorylation) (Fig. 2.4).

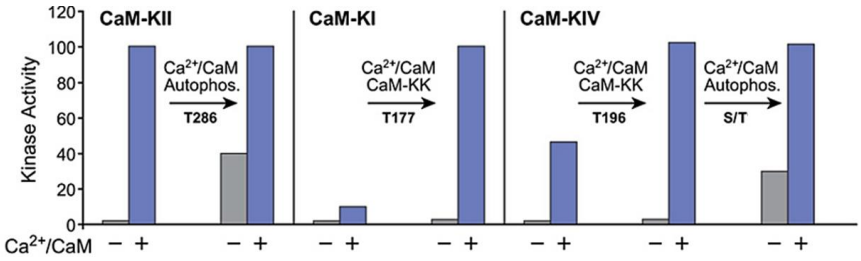

Figure 2.4. Modulation of CaMKs activity in relations with Ca^{2+}/CaM binding and/or phosphorylation on total kinase activity (Adapted from Wayman et al., 2008).

The family of CaMKs comprises "multifunctional kinases", which phosphorylate a wide range of substrates, and "dedicated CaM kinases", which phosphorylate a specific substrate. The former category is composed of CaM-Kinase Kinase (CaMKK), CaM-Kinase I (CaMKI), CaM-Kinase II (CaMKII) and CaM-Kinase IV (CaMKIV), while the latter by CaM-Kinase III (CaMKIII), Myosin Light Chain Kinase (MLCK) and phosphorylase kinase. Given their low substrate specificity and affinity, CaMKI/II and IV are believed to be ideal and powerful multivalent Ca^{2+} effectors in neurons.

2.2.3 Multifunctional CaMKs

Multifunctional CaMKs consist of a subset of kinases that phosphorylate a wide range of substrates. Members of this sub-family, such as CaMKII and members of the so-called 'CaM-K cascade' (CaMKK, CaMKI and CaMKIV), like their upstream regulator CaM, are present in the vast majority of mammalian tissues and are particularly enriched in the brain. Here, given their low substrate specificity and affinity, these kinases are considered ideal and powerful multivalent Ca^{2+} effectors in neurons.

2.2.3.1 CaM-Kinase Kinase (CaMKK)

As previously mentioned, once bound to Ca2+/CaM, some CaMKs need further modifications to reach their fully activated state. These components of the CaMK family require to be phosphorylated by another kinase that is itself dependent on the Ca2+/CaM

binding. This CaMK protein kinase is named calmodulin-dependent protein kinase kinase (CaMKK) which, together with its primary substrates, CaMKI and CaMKIV, forms the so-called CaMKs cascade (Lee et al., 1994; Tokumitsu et al., 1994).

CaMKK exists as two isoforms of about 60-70 kDa, coded by two different genes (CAMKKA/B) (Tokumitsu et al., 1995). The two isoforms are composed of two domains: a N-terminal catalytic domain and a C-terminal regulatory domain containing the auto-inhibitory domain and the Ca^{2+}/CaM binding domain. These kinases act as monomers and are highly expressed in the brain, where they respond to the dynamic oscillations of Ca2+ concentration.

Upon their binding to Ca^{2+}/CaM, they release their autoinhibitory domain and expose their competent catalytic domain, thus becoming active.

Finally, it is noteworthy that CaMKK may be a target for phosphorylation itself (i.e. from Protein kinase A), which may prevent the binding to CaM and therefore inhibit CaMKK activation and function (Wayman et al., 1997).

2.2.3.2 CaM-Kinase I (CaMKI)

There are four different isoforms of CaMKI coded by four different genes (CAMKIA/B/C/D) (Nairn et al., 1987; Takemoto-kimura et al., 2003; Ishikawa et al., 2003). These kinases act as monomers of around 370 aa (~40 kDa) and they are composed of two domains: a N-terminal kinase domain (until ~286 residue), comprising the 'activation loop', and a C-terminal regulatory domain that comprises the auto-inhibitory domain (residues 286-307) and a partially overlapping Ca^{2+}/CaM binding domain (residues 303-316) (Zha et al., 2012).

CaMKI is activated through a multi-step process and needs the binding of Ca2+/CaM complex for the release of its auto-inhibition. In an inactive state, this kinase is found in an ATP-unbound form (Autoinhibited state) where the activation loop adopts a unique helical conformation together with the autoinhibitory domain that sequesters Thr177 from being phosphorylated and occludes the substrate-binding site. When CaMKI is bound to ATP (that is called Pre-CaM binding state, as seen in the figure), its activation segment appears largely disordered and its CaM-binding segment protrudes out ready for binding

to CaM. Upon CaMKK-mediated Thr177 phosphorylation (Active state) the regulatory region is dissociated from the catalytic core and the catalytic site assumes an active conformation (Zha et al., 2012) (fig. 2.5).

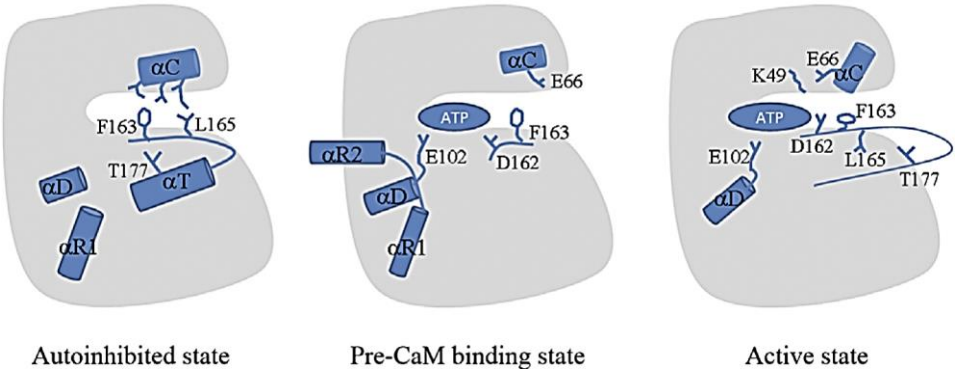

Figure 2.5. A schematic representation drawing the putative model of CaMKI regulation and activation (Adapted from Zha et al., 2012).

CaMKI family members may play a role during activity-dependent brain development tuning different processes involved in neuronal morphogenesis, such as growth cone mobility, neurite outgrowth and polarity formation (Takemoto-Kimura et al., 2010; Uboha et al., 2007; Wayman et al., 2004; Davare et al., 2009).

2.2.3.3 CaM-Kinase IV (CaMKIV)

CaMKIV is coded by one gene from which two isoforms of 65kDa are generated via alternative splicing (CaMKIVα and CaMKIVβ). These two variants show a slightly different expression pattern, with CaMKIVα mainly found in brain, thymus, CD4 T-cells and testis while CaMKIVβ in cerebellar granule cells (Bland et al., 1994; Sakagami et al, 1993). In details, focusing on brain tissues, CaMKIV isoforms are particularly enriched in neurons, with no expression in astrocytes and oligodendrocytes (Cahoy et al., 2008).

The basic structure of CaMKIV shares some similarities with that of CaMKI. More in details, CaMKIV displays a highly conserved N-terminal kinase domain, comprising the 'activation loop', and a relatively divergent C-terminal regulatory domain, that comprises

the auto-inhibitory, nucleotide-binding, serine/threonine phosphatase 2A (PP2A)-binding and CaM-binding domains.

These few structural differences may explain the slightly different regulation of CaMKIV, a multi-step activation process that initiates when Ca2+/CaM binds to CaMKIV. This event removes PP2A from the regulatory domain of CaMKIV and exposes the activation loop of the kinase. Subsequently, the CaMKK-mediated phosphorylation of Thr196 in the activation loop is an essential step that drives CaMKIV to enhance its enzymatic activity. Once phosphorylated, CaMKIV finally undergoes an intra-subunit autophosphorylation step at its Ser/Thr-rich N-terminal domain (such as Ser12 and Ser 13) (Chatila et al., 1996). This autophosphorylation step not only brings kinase enzymatic activity to its maximum but also generates Ca2+/CaM-independent activity that enables CaMKIV to maintain part of its functionality even after the initial elevation of Ca2+ has waned (Chatila et al., 1996) (Fig. 2.4 and Fig. 2.6).

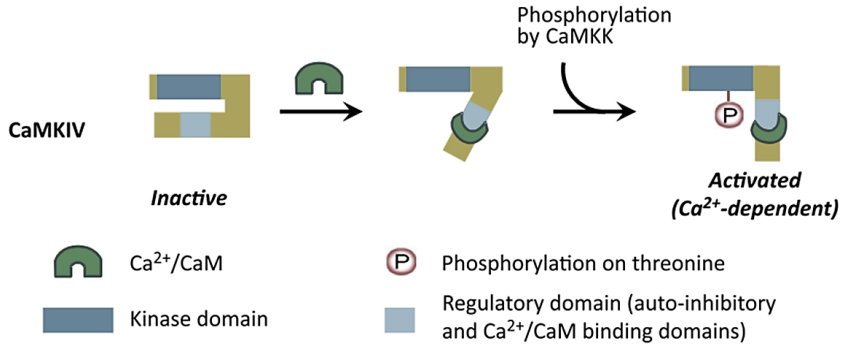

Figure 2.6. Cartoon showing the activation process of CaMKIV. Ca2+/CaM binding to CaMKIV leads to exposure of the kinase activation. CaMKK-mediated phosphorylation of Thr196 in the activation loop enhance enzymatic activity and, once phosphorylated, CaMKIV intra-subunit autophosphorylation brings kinase enzymatic activity to its maximum. (Adapted from Takemoto-Kimura et al., 2017)

PP2A plays a pivotal role in the process described above. This phosphatase stably associated with CaMKIV, generating a signalling complex that tightly controls phospho/dephosphorylation cycles of this kinase (Westphal et al., 1998). Moreover, CaMKIV shows an additional site of autophosphorylation at Ser332. Phosphorylation at this site represents a further level of self-regulation as it prevents any binding of

Ca2+/CaM to CaMKIV until Ser332-P is dephosphorylated, a reaction mediated by PP2A.

Considering that CaMKI and CaMKIV recognize a similar consensus sequence, it is possible to argue that they phosphorylate similar amino-acid sequences, leading to a partial overlapping of targets (Lee et al., 1994). Nevertheless, the functional redundancy between the two kinases is partially overcome by the fact that their sub-cellular localization is different so that they might involve different pathways and processes. Indeed, while CaMKI shows a cytosolic or membrane-anchored distribution, CaMKIV is predominantly enriched in the nuclei, where it is localized by an importin-α transport system (Lee et al., 1994; Kotera et al., 2005).

Among the targets of CaMKIV, a special consideration goes to several transcription factors, such as cAMP response element binding protein (CREB), myocyte enhancer factor (MEF), retinoic acid-related orphan receptor-α, serum response factor (SRF) and histone deacetylase 4 (HDAC4) (Matthews et al., 1994; Blaeser et al., 2000; Miranti et al., 1995; Miska et al., 2001). These molecular players are critically involved in numerous cellular processes, making CaMKIV a key factor tuning gene expression in a Ca2+-dependent manner. Indeed, transcriptional activation of CREB-dependent genes in a calcium/activity-related manner can participate in making long-term neuronal plasticity more persistent (Wayman et al., 2008).

However, it has been demonstrated that CAMKIV regulates also a variety of non-neuronal tissues processes, among which $CD4^+/CD8^+$ selection during T-cell expansion in thymus, and the regulation of blood pressure through the control of endothelial nitric oxide synthase activity (Mayya et al.,2009; Racioppi and Means, 2008; Santulli et al., 2012).

To better address the functional roles played by CaMKIV, three different strains of mice with a null mutation of this kinase have been created (Takao et al., 2010). Major results of the comprehensive characterization of these mice are listed in Table 2.4.

CaMKIV Knockout Mice - Phenotypes	
- deficiency in LTP in hippocampal CA1 neurons and a late phase of long-term depression in cerebellar Purkinje neurons	(Ho et al., 2000)
- infertility and impairment of spermiogenesis in late elongating spermatids of male null mice	(Wu et al., 2000)
- altered cerebellar function and development with locomotor defects and immature Purkinje neurons	(Ribar et al., 2000)
- decreased anxiety and stress-related behaviour	(Shum et al., 2005)
- impaired amygdala-related fear memory	(Wei et al., 2002); (K. H. Lee et al., 2009)
- defects in contextual and cued fear conditioning tests	(Takao et al., 2010)

Table 2.4 List of CaMKIV knockout mice phenotypes

The authors showed that CaMKIV KO mice exhibited a mild phenotype, characterized by specific defects in cerebellar development and functions as well as in amygdala fear-related memories, with no abnormalities in hippocampus-related spatial learning tasks. Overall, these data from *in-vivo* studies suggest that CaMKIV has a role in emotional behaviour, but cannot exclude its putative involvement in other contexts, that might be masked by compensation events occurring in the global KO mouse model.

2.2.3.4 CaM-Kinase II (CaMKII)

CaMKII exists as four distinct isoforms (α, β, γ, δ) coded by four different genes (CaMKIIA/B/C/D) expressing proteins around 50-70 kDa. The α and β isoforms are brain specific, with a specific enrichment in post-synaptic densities. Together CaMKII α and β isoforms constitute up to 2% of total protein in the rat hippocampus and up to 1% of total brain extract (Luczak & Anderson, 2014; Erondu et al., 1985).

Each CaMKII isoform shares the same domain organization and their basic structure is similar to that of CaMKI and CaMKIV. Nevertheless, unlike the previous CaMK members, CaMKIIs exist as holoenzyme in cells, with a C-terminus displaying an association domain that mediates the assembly of a twelve-subunit complex (Fukunaga et al., 1982) (Fig. 2.7A).

The activation process of this kinase appears more complex. When Ca2+/CaM binds to CaMKII, this binding triggers the release of the autoregulatory domain, which *per se* leads to maximal enzymatic activity. Finally, the subsequent autophosphorylation of the

Thr-286 residue confers a Ca^{2+}/CaM-independent activity state (Coultrap & Bayer, 2012) (Fig. 2.4).

Starting from a Ca2+/CaM-unbound and unphosphorylated form (Inactive), binding of Ca2+/CaM to CaMKII activates the kinase activity (Active-CaM Bound). Further binding of two Ca2+/CaM complexes to the adjacent enzymatic subunits triggers an intra-holoenzyme reaction leading to autophosphorylation of Thr 286 (Miller et al., 1986). These phosphorylation events generate autonomous activity and induce a remarkable increase in CaMKII affinity for Ca2+/CaM complex (Active-CaM Trapped), even when Ca2+ concentration falls (Meyer et al., 1992). After the dissociation of Ca2+/CaM, the enzyme displays a decrease in its activity, reaching 20–80% of its maximum (Active-Ca2+ Independent). More, the dissociation exposes additional sites in the regulatory domain (Thr 305 and Thr 306), which undergo autophosphorylation. At this point, phosphorylated CaMKII remains active at 20–80% of maximal activity because of pThr286 but it is incapable of binding Ca2+/CaM (Active-Capped); only after dephosphorylation, the kinase gets back to its basal state (Griffith, 2004) (Fig. 2.7B).

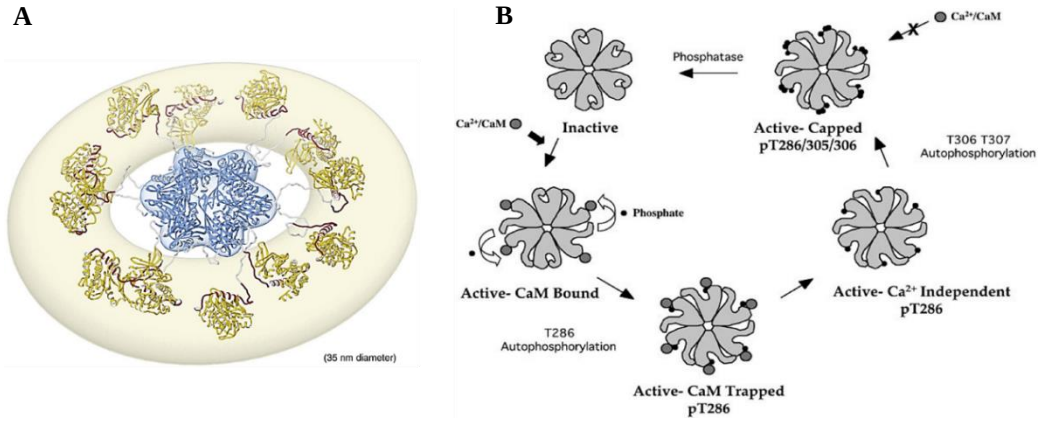

Figure 2.7. (A) Schematic representation of CaMKII holoenzymes structure and volume occupancy (maximum diameter: ~ 35 nm) (Adapted from Myers et al., 2017). (B) Representation of CaMKII activation process and phosphorylation-dependent kinase modulation (Adapted from Griffith, 2004)

As stated above, autonomous activity and CaM-trapping contribute to delineate an Active-CaM Trapped CaMKII form. In this condition, CaMKII remains at 100% of its maximal activity, also beyond the initial transient calcium elevation necessary for switching on the enzyme, a feature that defines CaMKII as a "memory molecule" (Lisman

et al., 2001). As a result of this property, and given its specific localization at postsynaptic densities, CaMKII is supposed to regulate mechanisms of activity storage at synapses, controlling processes such as memory, learning and cognition (Achterberg et al., 2014; Lisman et al., 2012).

2.2.4 Dedicated CaM Kinases

Dedicated CaMKs consist of a subset of kinases that phosphorylate only one known downstream target. Members of this sub-family include Myosin Light Chain Kinase (MLCK), phosphorylase kinase and CaM-Kinase III (also known as EF2K), which phosphorylates myosin light chain, glycogen phosphorylase and eukaryotic elongation factor-2 (eEF2), respectively.

MLCK exists as two distinct isoforms, known as skeletal muscle MLCK (skMLCK) and smooth muscle MLCK (smMLCK), coded by two different genes. smMLCK, the dimension of which ranges around hundred kDa in size, is not only localized in smooth muscle but can also be found in other tissues, including the brain (Robinson et al., 2004). Upon the phosphorylation of the regulatory light chain of myosin II, MLCK functions to initiate muscle contraction. Nevertheless, as stated before, smMLCK is present also in the brain, where it can regulate myosin-based transport in axons and nerve terminal, as well as Ca2+-mediated recruitment of vesicles in presynaptic terminals (Ryan, 1999).

Phosphorylase kinase is one of the largest (1.300 kDa) and structurally complex CaM-kinases. Working as a heterotetramer, this kinase is widely distributed in body tissues with the majority being present in liver and skeletal muscle, reaching 0.5-1% of total soluble proteins (Yoshitaka Tanaka et al., 2000). Phosphorylase kinase, through glycogen phosphorylase phosphorylation, regulates energy source for muscle contraction and contributes to blood-glucose homeostasis.

CaMKIII is present inside the cell as a monomer of approximately 100 kDa localized in the cytosol, where it couples the increase of cytosolic Ca2+ concentration to the suppression of protein synthesis (Nairn et al., 1985). Indeed, once activated, CaMKIII phosphorylates eEF2 leading to its dissociation from the ribosome and thus suppressing protein translation (Nairn et al., 1987).

3 AIM OF THE STUDY

The transcriptional repressor REST orchestrates neural differentiation, represses neuronal genes in non-neuronal cells and preserves the unique neural phenotype. REST is a protein highly expressed in stem cells and titration of its levels occurs as neurogenesis proceeds to release repression of neuronal genes. It has been demonstrated that during this process REST protein degradation is regulated by ubiquitin-mediated proteolysis in a phosphorylation-dependent manner, a post-translational control mechanism that appears to be relevant also in pathologies.

Given the established role of different phosphodegron motifs in controlling the stability of REST protein, the first aim of our study was to dissect new pathways linking the activity of various kinases to REST protein expression and function. This kind of post-translational modification appears to be a very attractive control mechanism in regulating REST protein stability considering that REST might be transcribed indiscriminately in both neuronal and non-neuronal cells, but REST protein is found at very low level in differentiated neurons.

In particular, we decided to focus our attention on the contribution of calcium in the processes that control REST activity. Indeed, calcium ions are essential second messengers in neuronal physiology and several kinases are regulated in a calcium-dependent manner. In this context, our aim is to address the undiscovered impact of calcium-dependent kinases on REST stability, a topic that is gaining increasing importance in literature.

Considering that neuronal physiology is highly regulated by calcium signalling, it is not surprising that calcium ion (un)balance is involved in a variety of neuronal processes that might be relevant for physiological as well as pathological conditions. Among these phenomena, we focused our attention on the autophagic pathway, a cellular process by which dysfunctional cellular components and proteins are degraded inside the cell. This topic results to be very attractive in neurophysiology since it has been demonstrated that autophagic-mediated degradation of intracellular material has a prominent role in many pathologies. Little is known about the putative contribution of REST in modulating the autophagic-dependent pathway and this is the reason that prompted us to concentrate on this subject. Therefore, the last aim of our study was to investigate the interplay between

calcium-dependent kinases and REST and its putative relevance on the regulation of autophagy in the physiopathology of neurons.

4 MATERIALS AND METHODS

4.1 Materials

All biochemical reagents and drugs were from Sigma-Aldrich (St. Louis, MO, USA) and Promega (Milano, Italy) unless otherwise specified. Tissue culture reagents and media were from Gibco-Invitrogen (Life Technologies Corp., Monza, Italy) or Sigma-Aldrich.

4.2 Experimental animals and housing

Wild-Type C57BL/6J were from Charles River (Wilmington, MA). Homozygous GTinvREST mice (Nechiporuk et al., 2016) were kindly provided by Gail Mandel and bred at the San Martino (GE, Italy) SPF animal facility. The colony was maintained on a C57BL/6J background and propagated in homozygosity. Two females were housed with one male in standard Plexiglas cages (33 × 13 cm), with sawdust bedding and a metal top. After two days of mating, male mice were removed and dams were housed individually in Plexiglas cages and daily checked. Mice were maintained on a 12 ∶ 12 h light/dark cycle (lights on at 7 a.m.). The temperature was maintained at 22 ± 1 °C, relative humidity (60 ± 10%). Animals were provided drinking water and a complete pellet diet (Mucedola, Settimo Milanese, Italy) *ad libitum*. Mice were weaned into cages of same-sex pairs. All experiments were carried out in accordance with the guidelines established by the European Communities Council (Directive 2010/63/EU of March 4th, 2014) and were approved by the Italian Ministry of Health (authorization n. 73/2014-PR and n. 1276/2015-PR).

4.3 Cell cultures

4.3.1 Continuous cell lines

Human Embryonic Kidney 293T (HEK293T, American Type Culture Collection, CRL- 1573) continuous cell line was used in the experiments. Cells were cultured in IMDM medium added with 10% (vol/vol) Fetal Bovine Serum (FBS, Life Technologies Corp), glutamine (2 mM), 100 U/ml penicillin and 100 mg/mg streptomycin (Life Technologies Corp) in 100 mm dishes (Falcon) at 37°C, 5% CO_2.

4.3.2 Primary Neuronal cells

WT primary neuronal cultures were prepared from day 17.5 embryos from WT mice of either sex as described previously (Banker & Cowan, 1977). The pregnant animals were killed with CO2, embryos were extracted and decapitated. Skulls were opened, and brains were dissected out and placed into HBSS. Cortexes were removed under a dissecting microscope and collected. After 30 min of incubation with 0.125% trypsin with DNase I at 37°C, the activity of trypsin was blocked by adding 5 ml of Neurobasal 10% FBS to the 15-ml tube and then mechanically dissociated. Neurons were plated on poly-L-lysine (0.1 mg/ml)-treated 18 mm glass coverslips at the density of 60,000 cells per coverslip for immunofluorescence protocols or 500,000 per wells for molecular biological and biochemical experiments. Cells were plated in Neurobasal added with 2% B27, 1% Glutamax and 1% Pen/Strep.

GTinvREST primary neuronal cultures were prepared from day P0 REST GTinv mice (Nechiporuk et al., 2016) of either sex. Pups were decapitated, skulls were opened and brains were dissected out and placed into HBSS. Cortexes were removed under a dissecting microscope and collected. After 6 min of incubation with 0.25% trypsin with DNase I at 37°C, the activity of trypsin was block by adding 5 ml of Dissection solution (HBSS, HEPES 10 mM, D-glucose 33 mM, Gentamycin 5 µg/ml, Albumin bovine 3%, $MgSO_4*7H_2O$ 5.86 mM, pH 7.4) supplemented with Trypsin Inhibitor (Sigma T9128-1G) to the 15-ml tube and then mechanically dissociated. Neurons were plated on poly-L-lysine (0.1 mg/ml)-treated 18 mm glass coverslips at the density of 60,000 cells per

coverslip for immunofluorescence protocols or 500,000 per wells for molecular biological and biochemical experiments. Cells were plated in Neurobasal-A added with 2% B27, 1% Glutamax and 1% Pen/Strep.

4.3.3 Primary Astrocytes

Mice were sacrificed by CO 2 inhalation, and 18-day embryos (E18) were removed immediately by caesarean section. Briefly, enzymatically dissociated cortical astrocytes were plated on poly-D-lysine-coated (0.01 mg/ml) cell culture flasks and maintained in a humidified incubator with 5% CO 2 for 2 weeks. At confluence, astrocytes were enzymatically detached using trypsin–EDTA and plated at the desired density, depending on the experiment.

4.4 Molecular Biology

4.4.1 RNA Extraction and retrotranscription and Real-time PCR

Total cellular RNA was extracted using TRIzol (Life Technologies). cDNA was synthesized starting from 0.25 μg RNA with SuperScript IV Reverse Transcriptase kit (#18090010; ThermoFisher) according to manufacturer's instruction and used for qRT-PCR.

Gene expression was measured by quantitative real-time PCR using C1000 Touch™ Thermal Cycler (Bio-Rad) on a CFX96™Real-Time System following the manufacturer's protocol. Relative gene expression was determined using the ΔΔCT method. The list of primers is provided in Table 4.1

Primers Name	Primers Sequence
F mCaMKIV	5'- CAGTTCATGTTCAGGAGAAT- 3'
R mCaMKIV	5'- AATGTAGTCAGCCGTTTC -3'
F mBDNF	5'- ATTACCTGGATGCCGCAAA -3'
R mBDNF	5'- TAATACTGTCACACACGCTCA -3'
F mCaMKI	5'- ATCAAGGAAGTCAGGGTTT -3'
R mCaMKI	5'- GCAGTGAAGAGTGAGAGG -3'

F mCaMKII	5’- ACTTCCTTCCACCACTTC -3’
R mCaMKII	5’- TGAGATACAGCATTCCATACA -3’
F mGAPDH	5’- AGGTCGGTGTGAACGGATTTG -3’
R mGAPDH	5’- TGTAGACCATGTAGTTGAGGTCA -3’
F mGusB	5’- TCGGGCTGGTGACCTACTGGATTTCTG -3’
R mGusB	5’- GTTGGCACTGGGAACCTGAAGTTGACC -3’
F mActin	5’- CTGGCTCCTAGCACCATGAAGAT -3’
R mActin	5’- GGTGGACAGTGAGGCCAGGAT -3’
F mREST	5’- GAACCACCTCCCAGTATG -3’
R mREST	5’- CTTCTGACAATCCTCCATAG -3’
F mLC3	5’- CACTGCTCTGTCTTGTGTAGGTTG -3’
R mLC3	5’- TCGTTGTGCCTTTATTAGTGCATC -3’
F mCTS	5’- GATGGGTGCTCTGAGAAT -3’
R mCTS	5’- GCAATGTCCGATTAGAGTATG -3’
F m CTB	5’- CTGCTGAAGACCTGCTTA -3’
R m CTB	5’- AATTGTAGACTCCACCTGAA -3’
F m HEXB	5’- GCTCCTGGTCTCCATTAC -3’
R m HEXB	5’- CGGCTACTGGTTCTTGTA -3’
F m LAPTM5	5’- GCCATTTACCACATAGTCAT -3’
R mLAPTM5	5’- GCATCTTGAAGAACCTACAG -3’

Table 4.1 List of primers used for qRT-PCR

4.4.2 List of siRNA and shRNA

siRNA against human, murine and rat CaMKI, CaMKII and CaMKIV were from Ambion® (Life Technologies). CaMKI siRNA (#1), s78770; CaMKI siRNA (#2), s78771; CaMKII siRNA (#1), s63280; CaMKII siRNA (#2), s201112; CaMKIV siRNA (#1), s63289; CaMKIV siRNA (#2), s63288. CaMKIV siRNA (#3) was from Takemoto-Kimura et al., 2007) and CaMKIa siRNA (#3) was from Ageta-Ishihara et al., 2009. scramble siRNA was used in the control conditions.

Starting from the candidate siRNA, short harping RNA (shRNA) sequences were synthesized from Sigma, annealed at the following conditions and cloned following Tyler Jacks Lab protocols (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html). In short, the consensus sequence should correspond to AAGN 18 TT. A 5’ guanine is required due to the constraints of the U6 promoter. Oligos were purchased from Sigma-Aldrichwith®, 5’ phosphates and PAGE purified and annealed as in Tyler Jacks Lab protocols.

Oligos Name	Oligos Sequence
shCaMKIa Sense oligo III	5'- TGCATTGTAGCCCTGGATGACTTCAAGAGA GTCATCCAGGGCTACAATGCTTTTTTC - 3'
shCaMKIa Antisense oligo III	5'- CTCGAGAAAAAAGCATTGTAGCCCTGGATGA CTCTCTTGAAGTCATCCAGGGCTACAATGCA - 3'
shCaMKIa Sense oligo IV	5'- TGGATCAAGCACCCCAACATTTTCAAGAGA AATGTTGGGGTGCTTGATCCTTTTTTC - 3'
shCaMKIa Antisense oligo IV	5'- CTCGAGAAAAAAGGATCAAGCACCCCAACATT TCTCTTGAAAATGTTGGGGTGCTTGATCCA - 3'
shCaMKIV Sense oligo II	5'- TGAGAGAATCTTCTTTATGCATTCAAGAGA TGCATAAAGAAGATTCTCTCTTTTTTC - 3'
shCaMKIV Antisense oligo II	5'- CTCGAGAAAAAAGAGAGAATCTTCTTTATGCA TCTCTTGAATGCATAAAGAAGATTCTCTCA - 3'
shCaMKIV Sense oligo III	5'- TGGTGTTAAAGAAAACAGTGGTTCAAGAGA CCACTGTTTTCTTTAACACCTTTTTTC - 3'
shCaMKIV Antisense oligo III	5'- CTCGAGAAAAAAGGTGTTAAAGAAAACAGTGG TCTCTTGAACCACTGTTTTCTTTAACACCA - 3'
scramble Sense oligo	5'- TGAGAGAATCTTCTTTATGCATTCAAGAGA TTGGGTTGAAGGTGGATCCCTTTTTTC - 3'
scramble Antisense oligo	5'- CTCGAGAAAAAAGGGATCCACCTTCAACCCAA TCTCTTGAATGCATAAAGAAGATTCTCTCA - 3'

Table 4.2 List of oligos for shRNA cloning

4.4.3 List of Plasmids

To obtain CaMKIa constitutively active and kinase-dead mutants, 20 ng of CaMKI WT pCMV-HA -N terminal plasmid (special gift of Dr. Taku Kaitsuka) were PCR-amplified using Pfu DNA polymerase (©BiotechRabbit, Hennigsdorf Germany). Primers #1 and #2 were used for CaMKIa kinase-dead cloning, while primers #3 and #4 were used for CaMKIa constitutively active cloning. PCR conditions were: 95°C, 5 minutes; (95°C, 30 s; 60°C, 30 s; 68°C, 1 minute + 1 minute each kb) for 18 cycles; 68°C, 7 minutes and 4°C, ∞. PCR products were digested using the DpnI enzyme (Promega) and transformed into TOPTEN cells. Positive colonies were verified by DNA sequencing.

Constitutively active form of CaMKIV was purchased by Addgene (Addgene, https://www.addgene.org/45063/). To obtain CaMKIV constitutively active and kinase-dead mutants, 20 ng of pRSV-CaMKIV-wt (Addgene https://www.addgene.org/45062/) were PCR-amplified using Pfu DNA polymerase (©BiotechRabbit, Hennigsdorf German). Primers #5 and #6 were used for CaMKIV kinase-dead cloning. PCR conditions

were: 95°C, 5 minutes; (95°C, 30 s; 60°C, 30 s; 68°C, 1 minute + 1 minute each kb) for 18 cycles; 68°C, 7 minutes and 4°C, ∞. PCR products were digested using the DpnI enzyme (Promega) and transformed into TOPTEN cells. Positive colonies were verified by DNA sequencing.

For shRNA expression, shRNA sequences were inserted in pLenti.U6-shRNA-pgkGFP-CRE and pLenti.U6-shRNA-pgkGFP-ΔCRE as follow: the GFP-CRE and GFP-ΔCRE cassettes (special gift of Dr. Lorenzo Cingolani) were amplified with primers #7/#8 and #7/#9 and inserted in pLenti.U6-shRNA-pgkCRE (Addgene https://www.addgene.org/24971/) between Sma I and Kpn I sites, than WPRE sequence was amplified from pLenti.U6-shRNA-pgkCRE with primers #10/#11 and cloned in between Kpn I and Pvu II sites. pLenti.U6-shCaMKIV-pgkΔCRE/CRE, pLenti.U6-shCaMKIa-pgkΔCRE/CRE and pLenti.U6-scramble-pgkΔCRE/CRE were cloned as follow: the annealed oligos containing the selected shRNA sequence were inserted in the pLenti U6-shRNA pgkΔCRE/CRE between the XhoI and HpaI sites.

Primers #	**Primers Name**	**Primers Sequence**
1	CaMKI K→A Fw	5'- AAACTGGTGGCCATCGCATGCATTGCCAAGAAG - 3'
2	CaMKI K→A Rv	5'- CTTCTTGGCAATGCATGCGATGGCCACCAGTTT - 3'
3	CaMKI CA Fw	5' – CAGTCAGTGAGTGAGCAGTGAGCGGCCGCGGGGAT - 3'
4	CaMKI CA Rv	5' - ATCCCCGCGGCCGCTCACTGCTCACTCACTGACTG - 3'
5	CaMKIV KD Fw	5' - GCAGTTTCTTCCCAAAGCTTCTTCACGGCTTCA - 3'
6	CaMKIV KD Rv	5' - ATTGTACAAATTCTTGGCTACTCCATGGTACTA - 3'
7	FW CRE/delta	5'- ATCCCCCGGGACCATGGTGAAGCGACCAGC - 3'
8	RV CRE	5'- ATCCGGTACCCTAATCGCCATCTTCCAGCAGG - 3'
9	RV deltaCRE	5'- ATCCGGTACCCTACTTACGGATTCGCCGC - 3'
10	FW WPRE	5'- ATCCGGTACCGCTTATCGATAATCAACCTCTGG - 3'
11	RV WPRE	5'- ATCCCAGCTGCTCCATGTTTTTCTAGGTCTCG - 3'

Table 4.3 List of primers for mutated-CaMKs constructs cloning

4.5 Pharmacological treatment

All the following chemicals used in the pharmacological treatment were from TOCRIS, a biotechne brand, Milano, Italy: W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) 20 µM, 24 h; KN93 (N-[2-[N-(4-Chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt, and KN92 (2-[N-(4-Methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, Phosphate) 10 µM, 24 h; CsA 1 µM, 24 h; Bafilomycin A1 300 nM, 8 h (Cat. No. 1334, TOCRIS a biotechne brand, Milano, Italy); Dimethyl sulfoxide, as a control according to the experimental condition (Cat. No. d2650, Sigma-Aldrich®, Milano, Italy).

4.6 Biochemical Procedures

4.6.1 Protein extraction and Western blotting analysis

Total protein lysates were obtained from cells lysed in RIPA buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 140 mM NaCl) containing protease and phosphatase inhibitor cocktails (Roche, Monza, Italy). The soluble fraction was collected and protein concentration was determined using the BCA Protein Assay Kit (Thermo-Fisher Scientific). For Western blotting, protein lysates were denatured at 99°C in 5X sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol, deionized water) and separated on SDS-polyacrylamide gels (SDS-PAGE). The following antibodies were used: Mouse monoclonal anti-LC3 (#0231, Nanotools, Teningen, Germany, 1 µg/ml), Rabbit anti-REST (Millipore, 07-579, 1 µg/ml), Mouse monoclonal anti-cMyc (Santa Cruz Biotechnology, 9E10 sc-40, 200 ng/ml), Rabbit polyclonal anti-Calnexin (Santa Cruz Biotechnology, sc-11397, 20 ng/ml), Rabbit monoclonal anti-CaMKI (Abcam, ab68234, 200 ng/ml), Mouse anti-CaMKII (Millipore, 07-1496, 500 ng/ml), Mouse monoclonal anti-CaMKIV (BD Biosciences,

610275, 50 ng/ml), Rabbit polyclonal anti-GFP (Invitrogen, a11122, 1 µg/ml) and anti-Hemagglutinin (Santa Cruz, sc-52025, 200 ng/ml).

Horseradish Peroxidase (HRP)-associated secondary antibodies for Western blot analysis were Stabilized Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated (32430; Thermo Scientific, Rockford, IL, USA) and Stabilized Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (32460; Thermo Scientific).

Signal intensities were quantified using the ChemiDoc MP Imaging System (GE Healthcare BioSciences, Buckinghamshire, UK).

4.6.2 Luciferase assay experiments

For luciferase assay experiments, Hek293T cells were co-transfected using Lipofectamine 2000 (Life Technologies) following standard transfection procedures with RE1X3pGL3 promoter vector together with mutated forms for CaMKs vectors. The pRL-TKSV40 vector expressing Renilla luciferase was cotransfected to normalize for transfection efficiency (Promega). Forty-eight hours after transfection, the luciferase activity was measured using a Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Luciferase measurements were performed with a Tecan INFINITE F500 (Tecan, USA).

4.7 Microscopy Techniques

4.7.1 Immunofluorescence staining and confocal imaging

Cells were fixed in phosphate-buffered saline (PBS)/4% paraformaldehyde (PFA) for 15 min at RT. Cells were permeabilized with 1% Triton X-100 for 5 min, blocked with 2% BSA in PBS/Tween 80 0.05% for 30 min at RT and incubated with primary antibodies in the same buffer for 45 min. The primary antibodies used were: Rabbit anti-ATG8 (Rockland, 200-401-H57, 5 µg/ml), Rabbit anti-Lamp1 (Sigma-Aldrich, L1418, 3 µg/ml) and Rabbit anti-βIII Tubulin (Sigma-Aldrich, T2200, 1 µg/ml). After the incubation with primary antibodies and several PBS washes, cells were incubated for 45 min with the

secondary antibodies in blocking buffer solution. Fluorescently conjugated secondary antibodies were from Molecular Probes (Thermo-Fisher Scientific; Alexa Fluor 488 #A11029, Alexa Fluor 568 #A11036, Alexa Fluor 647 #A21450). Samples were mounted in Prolong Gold antifade reagent with DAPI (#P36935, Thermo-Fisher Scientific) on 1.5 mm-thick coverslips. Image acquisition was performed using a confocal laser scanning microscope (SP8, Leica Microsystems GmbH, Wetzlar, Germany) at 63x (1.4 NA) magnification. Each image consisted of a stack of images taken through the z-plane of the cell. For each set of experiments, all images were acquired using identical exposure settings.

For LysoTracker Deep Red (Molecular Probes/Life Technologies) experiments, neurons were incubated with 10 nM LysoTracker for 30 min, at 37C in culture medium, immediately fixed and analysed within 6 h. Image acquisition was performed using a confocal laser scanning microscope (SP8, Leica Microsystems GmbH, Wetzlar, Germany). Settings were kept the same for all acquisitions within each experiment.

4.7.2 Image Analyses and Quantification

4.7.2.1 Synapse quantification

$REST^{GTinv}$ primary cortical neurons were infected with the indicated constructs. To measure excitatory synapses, neurons were labelled with mouse monoclonal anti-Homer 1 (Synaptic System, 160 011, 5 µg/ml), and Guinea pig polyclonal anti-VGluT 1 (Synaptic System, 135 304, 5 µg/ml), antibodies. To measure inhibitory synapses, neurons were labelled with Mouse monoclonal anti-VGAT (Synaptic System, 131 011, 2 µg/ml) and Mouse monoclonal anti-Gephyrin (Synaptic System, 147 011, 5 µg/ml) antibodies. Image acquisition was performed using a confocal laser scanning microscope (SP8, Leica Microsystems GmbH, Wetzlar, Germany) at 40x (0,50 NA) magnification. Each image consisted of a stack of images taken through the z-plane of the cell. Confocal microscope settings were kept the same for all scans in each experiment. The co-localization analysis was performed by evaluating the labelling of the VGLUT1/Homer1 or VGAT/Gephyrin synaptic protein couples. Co-localization puncta with areas of 0.1–2 mm2 were considered bona fide synaptic boutons. Synaptic boutons along neurites were manually counted on 30 µm puncta starting from the cell body.

4.7.2.2 Sholl Analysis.

Flox-REST primary cortical neurons were infected with the indicated constructs as previously described. Cells were fixed for β III-tubulin and GFP. Image acquisition was performed using a confocal laser scanning microscope (SP8, Leica Microsystems GmbH, Wetzlar, Germany) at 20x (0,50 NA) magnification. At least 20 cells were analyzed for each condition, from three independent preparations. Sholl analysis was performed by using the Sholl plugin of ImageJ (starting radius 0 μm, radius step size 10 μm, ending radius 200 μm).

4.7.3 Transmission electron microscopy (TEM) of primary neurons

$REST^{GTinv}$ primary cortical neurons were infected with the indicated lentiviruses at 7 DIV and fixed at 14 DIV with 1.2% glutaraldehyde in 66 mM sodium cacodylate buffer, post-fixed in 1% OsO4, 1.5% K4Fe(CN)6, 0.1 M sodium cacodylate, en bloc stained with 1% uranyl acetate, dehydrated, and flat embedded in epoxy resin (Epon 812, TAAB). After baking for 48 hrs, the glass coverslips were removed from the Epon block by thermal shock and neurons were identified by means of a stereomicroscope. Embedded neurons were excised from the block, and mounted on a cured Epon block for sectioning using an EM UC6 ultramicrotome (Leica Microsystems). Ultrathin sections (60–70 nm thick) were collected on 200-mesh copper grids (EMS) and observed with a JEM-1011 electron microscope (Jeol) operating at 100 kV using an ORIUS SC1000 CCD camera (Gatan, Pleasanton). For each experimental condition, at least 30 images of neuron cell soma were acquired at 10,000x magnification (sampled area per experimental condition: 36 μm2).

4.8 Lentivirus Production and Infection Procedures.

Third-generation lentiviruses were produced by transient four-plasmid cotransfection into HEK293T cells using the calcium phosphate transfection method. Supernatants were collected, passed through a 0.45- μm filter, and purified by ultracentrifugation (20.000 rpm, 2h 4°C). Viral vectors were titrated at concentrations ranging from 1×10^8 to 1×10^9

transducing units (TU)/mL and used at a multiplicity of infection (MOI) of 5–10. The efficiency of infection was estimated to range between 70% and 90% by counting neurons expressing GFP protein with respect to the total number of cells stained with DAPI. Primary cortical neurons were infected at 7 DIV. After 24 h, half of the medium was replaced with fresh medium. Experiments were performed 7 days after infection.

4.9 Electrophysiological Recordings.

All experiments were performed using an EPC-10 amplifier controlled by the PatchMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and an inverted DMI6000 microscope (Leica Microsystems GmbH). Patch electrodes fabricated from thick borosilicate glasses were pulled to a final resistance of 4−5 MΩ. Recordings with the leak current > 100 pA were discarded. All recordings were acquired at 50 kHz. The standard bath saline contained (in mM): 140 NaCl, 4 KCl, 2 MgCl 2, 2 CaCl 2, 10 HEPES, 5 glucose, pH 7.4, with NaOH and osmolarity adjusted to ∼ 315 mOsm with mannitol. The intracellular (pipette) solution was composed of (in mM): 144 KCl, 2 MgCl 2, 5 EGTA, 10 HEPES, pH 7.2 with KOH; osmolarity ∼ 300 mOsm. Experiments were carried out at RT (20–24°C). Recordings of evoked firing activity in current-clamp configuration were performed in Tyrode's extracellular solution in which D-(−)-2-amino-5-phosphonopentanoic acid (AP5, 50 μM), 6-cyano-7 nitroquinoxaline-2,3-dione (CNQX, 10 μM), bicuculline methiodide (BIC, 30 μM), and (2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl) phosphinic acid hydrochloride (CGP, 5 μM) were added to block NMDA, non-NMDA, GABAA, and GABAB receptors, respectively. The internal solution (K-gluconate) was composed of (in mM): 126 K gluconate, 4 NaCl, 1 MgSO 4 , 0.02 CaCl 2 , 0.1 BAPTA, 15 glucose, 5 Hepes, 3 ATP, and 0.1 GTP, pH 7.3. Current-clamp recordings were performed at a holding potential of −70 mV, and action potential firing was induced by injecting current steps of 10 pA lasting 500 ms. All parameters were analyzed using the Fitmaster (HEKA Elektronik,) and Prism6 (GraphPad Software, Inc.) software. Spontaneous miniature excitatory postsynaptic (mEPSCs) currents and spontaneous miniature inhibitory postsynaptic (mIPSCs) currents were recorded in a voltage-clamp configuration in the presence of tetrodotoxin (TTX, 300 nM) in the extracellular solution to block the

generation and propagation of spontaneous action potentials. To isolate mEPSCs currents, 30 μM BIC was added to Tyrode's extracellular solution, in the presence of the internal solution (K-gluconate) described above. To isolate mIPSC currents, 10 μM CNQX was added to Tyrode's extracellular solution, in presence of internal solution composed of (in mM): 120 KGluconate, 4 NaCl, 20 KCl, 1 MgSO 4, 0.1 EGTA, 15 Glucose, 5 HEPES, 3 ATP, 0.1 GTP (pH 7.2 with KOH). mPSCs were acquired at 10 kHz sample frequency, filtered at half the acquisition rate with an 8-pole low-pass Bessel filter, and analyzed by using the Minianalysis program (Synaptosoft, Leonia, NJ, USA). The amplitude, frequency, rise time and decay time of mPSCs were calculated using a peak detector. All reagents were purchased from Sigma Aldrich or Tocris (Tocris, Avonmouth, Bristol, UK)

4.10 Protein kinase prediction

We used three methods including GPS-2.1, PhosphoMotifFinder, and PHOSIDA using the default parameters (Xue et al., 2008; Amanchy et al., 2007; Gnad et al., 2007). We took all the predictions from all three methods.

4.11 Statistical analysis

Data analysis. Results are presented as means ± sem from 4 independent preparations. Normal distribution of data was assessed using the D'Agostino-Pearson's normality test. To compare two normally distributed sample groups, the unpaired Student's t-test was used. To compare more than two normally distributed sample groups, one-way ANOVA was used, followed by the post-hoc tests (Bonferroni's test). A value of $p<0.05$ was considered significant. Statistical analysis was carried out using SigmaStat 13 (Systat Software).

5 RESULTS

5.1 Ca^{2+}/Calmodulin-dependent protein kinases pathway regulates REST protein levels in cortical neurons

To test the hypothesis that CaMK signalling pathway modulates REST protein levels, we firstly evaluated whether calcium signalling may interfere with REST protein stability. To fulfil this goal, cortical neurons were incubated at 13 DIV with CaM inhibitor W-7 in order to pharmacologically inhibit a fundamental second messenger of Ca^{2+} signalling (Fig. 5.1A and B) (Moon et al., 1983). Western blotting analysis revealed that W-7 treatment induced an increase in REST protein level in cortical neurons (Fig. 5.1A), as well as qRT-PCR analysis of its mRNA levels, showed an increase under the same experimental conditions (Fig. 5.1B).

Based on these results, and taking into account that Calmodulin interacts with various protein targets, including both protein kinases and phosphatases, we asked which downstream effectors could be involved. Primary cortical neurons were treated with either Ciclosporin A (CsA) or KN-93, respectively a Calcineurin selective inhibitor and competitive CaMK blockers (Bram et al., 1993; Sumi et al., 1991). Inhibition of calcineurin following CsA treatment did not affect REST expression at protein and mRNA level compared to control condition (Fig 5.1C-D). On the contrary, KN-93 treatment induced an increase of REST protein levels compared to DMSO-treated (CTR) and KN-92 treated neurons, an inactive derivative of KN-93 used as a control compound (Fig. 5.1E). qRT-PCR analysis of REST mRNA levels revealed no change in all three experimental conditions (Fig. 5.1F).

Finally, in order to investigate the cell-specificity of the signalling pathway under investigation, primary astrocytes were treated either with W-7 or KN-93 for 24 h. Western blotting and qRT-PCR analysis revealed that both REST protein level (Fig. 5.1G) and REST mRNA level (Fig. 5.1H) did not show changes. In conclusion, these first experiments suggest that calcium-signalling pathway might have a role in regulating REST protein levels in cortical neurons, probably acting through post-translational events mediated by CaMKs.

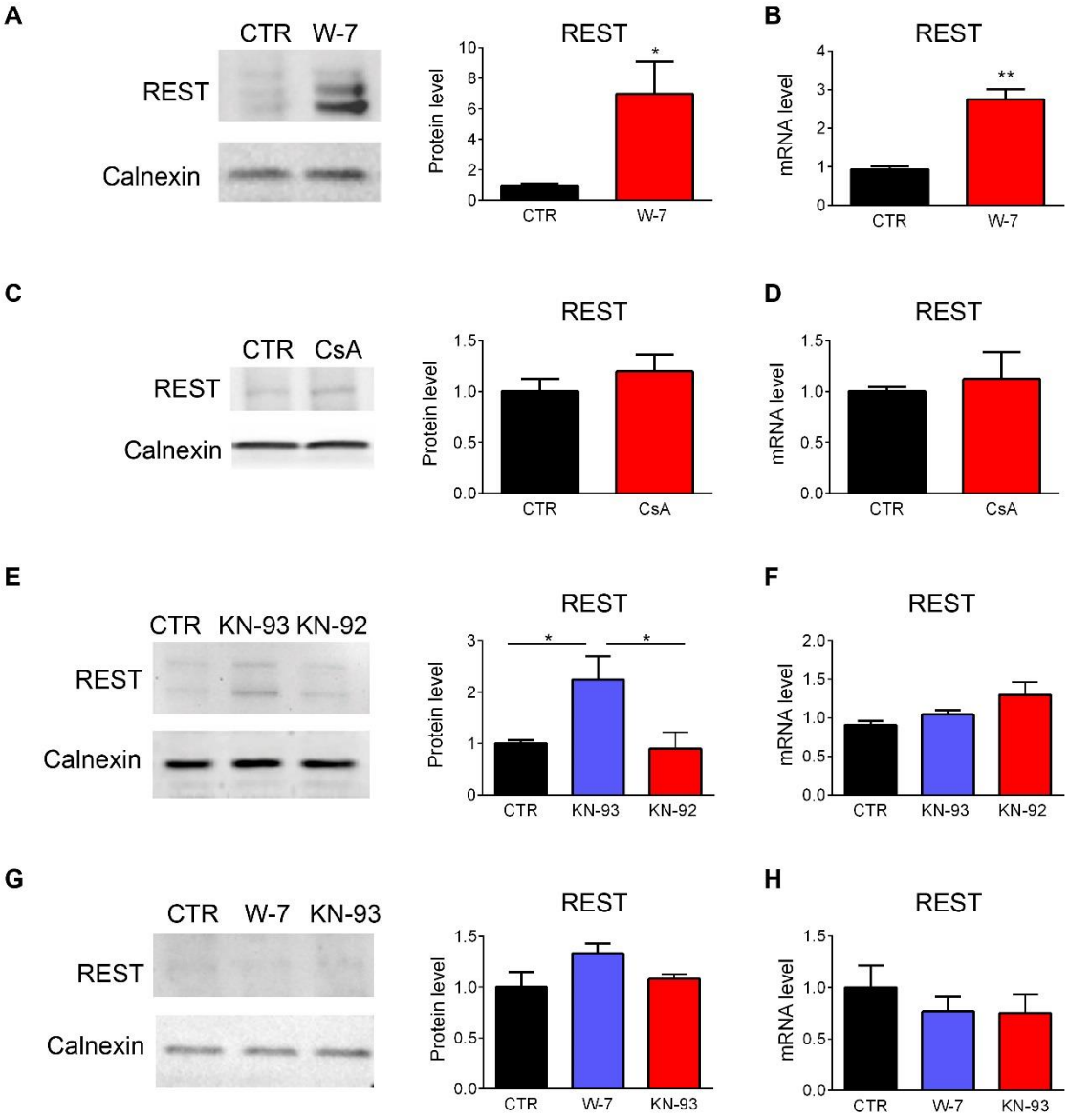

Figure 5.1. CaMK signalling pathway increases REST protein levels in cortical neurons. (A) Western blotting analysis of REST protein level and (B) qRT-PCR analysis of REST mRNA level (B) of primary cortical neurons treated with either vehicle (CTR) or W-7 (20 µM) for 24 h.(C) Western blotting analysis of REST protein level and (D) qRT-PCR analysis of REST mRNA level of primary cortical neurons treated with either vehicle (CTR) or CsA (1 µM) for 24 h. A representative experiment and quantification are shown. Graphs show mean ± sem. (*p < 0.05, **p < 0.01, two-tailed Student's t-test; n = 5-3).(E) Western blotting analysis of REST protein level and (F) qRT-PCR analysis of REST mRNA level of primary cortical neurons treated with either vehicle (CTR), KN-93 (10 µM) and KN-92 (10 µM) for 24 h. (G) Western blotting analysis of REST protein level and (H) qRT-PCR analysis of REST mRNA level of primary astrocytes treated with either vehicle (CTR), W-7 (20 µM) and KN-93 (10 µM) for 24 h. A representative experiment and quantification are shown. In Western blotting analysis, Calnexin was used as a loading control; in qRT-PCR analysis Actin, Gusb and Gapdh were used as reference genes. Graphs show mean ± sem. (*p < 0.05, one way Anova /Bonferroni's test.; n = 6-5).

5.2 CaMKI and CaMKIV phosphorylation activity are required to decrease REST expression and activity in Hek293T cells overexpressing CaMK mutant forms

KN-93 is a broad-spectrum inhibitor with a number of molecular targets, and thus these results do not identify the specific CaMKs that mediate these effects (Enslen et al., 1994; Ledoux et al., 1999; Gao et al., 2006). To address this point we overexpressed different variants of CaMKs in Hek293T cells, a cell line in which REST is endogenously expressed. Wild-type (WT), constitutively active (C.A.) and kinase death (KD) forms of these kinases were employed in order to understand whether and which CaMKs modulate REST stability. In order to evaluate whether the previously seen increasing in REST protein level could affect REST activity, the same experimental approach was employed followed by gene reporter assays using a construct in which luciferase expression was driven by the SV40 promoter fused to a tripleRE1 cis-site.

Hek293T cells were transfected with WT, C.A. and KD forms of CaMKIα and REST protein level were analyzed by Western blotting analysis (Fig. 5.2A). In presence of the C.A. form of CaMKIα REST exhibits a decreased expression level compared to the one in WT and KD overexpressing Hek293T cells. Luciferase assay was performed in the same experimental condition observing an increased luciferase signal in the presence of C.A. form of CAMKIα, indicating reduced REST-mediated repression (Fig. 5.2B).

Following the same experimental procedures WT, C.A. and KD mutant forms of both CaMKIIα and CaMKIV were overexpressed in Hek293T cells. As shown in fig. 5.2C-D, overexpression of mutants CaMKIIα forms did not lead to changes in both REST protein level (Fig. 5.2C) and luciferase signal (Fig. 5.2D) under all the experimental conditions, while overexpression of mutant C.A. of CaMKIV result in a decreased expression of REST protein and increased signalling in luciferase assay, as in C.A. CaMKIα overexpression condition.

These data indicate that among multifunctional CaMK members, CaMKIα and CaMKIV regulate REST protein stability and activity in Hek293T cells.

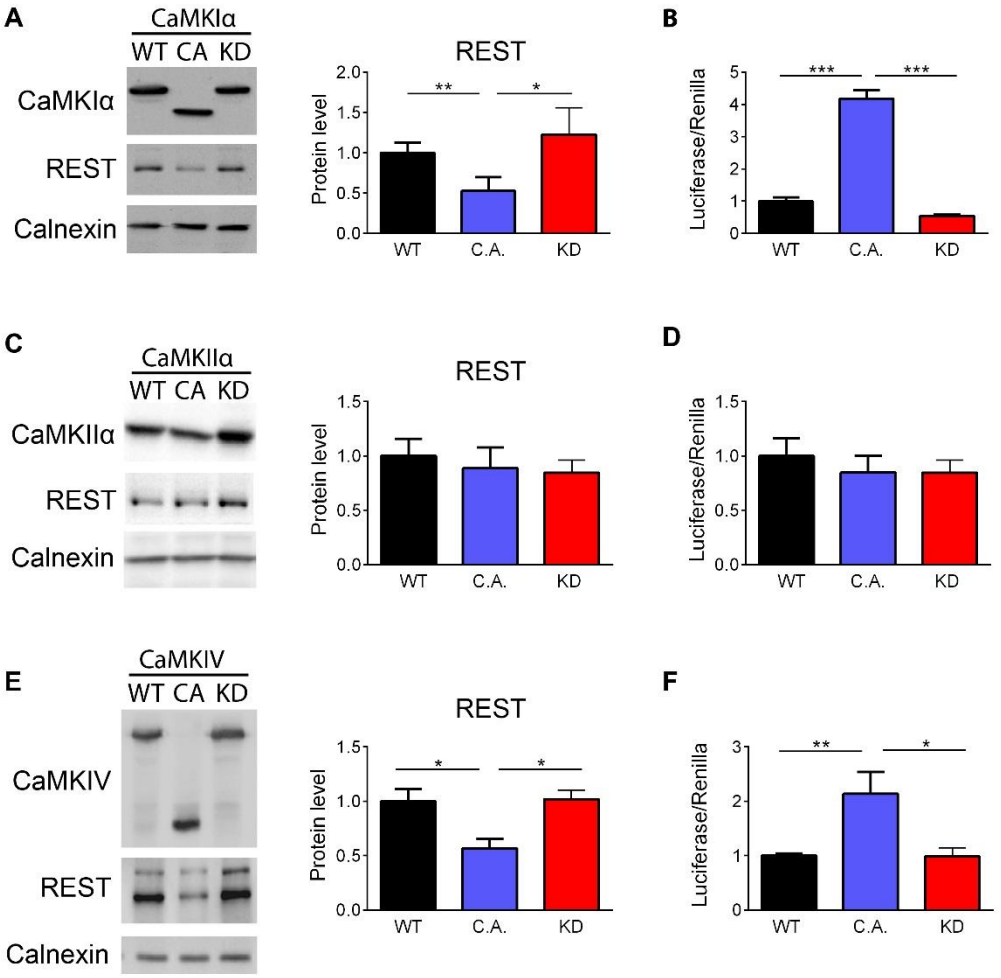

Figure 5.2. CaMK I and CaMKIV decrease REST expression and activity. (A) Western blotting analysis of REST and (B) the relative luciferase activity in HEK293T cells transfected with vectors expressing HA-tagged wild-type (WT), constitutively active (C.A) or Kinase Dead (KD) forms of mouse CaMKIα. (C) Western blotting analysis of REST and (D) the relative luciferase activity in HEK293T cells transfected with vectors expressing GFP-tagged wild-type (WT), constitutively active (C.A) or Kinase Dead (KD) forms of mouse CaMKIIα. (E) Western blotting analysis of REST and (F) the relative luciferase activity in HEK293T cells transfected with vectors expressing wild-type (WT), constitutively active (C.A) or Kinase Dead (KD) forms of mouse CaMKIV. Representative experiments and quantifications are shown. In Western blotting analysis, quantification of REST protein level is shown on the right panel; calnexin was used as loading control. In luciferase gene activity assay, a reporter vector harbouring the RE-1 sequence downstream of a firefly luciferase gene (pGL3-RE1/SV40 reporter vector) were analyzed in the presence of WT, C.A or KD forms of CaMKIα, CaMKIIα or CaMKIV respectively; luciferase activity was measured 48 h after transfection and expressed as the ratio between Firefly and Renilla luciferase signals, and normalized to the expression of wild-type condition. Graphs show mean ± sem. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one way Anova /Bonferroni's test; n = 6-8).

5.3 Silencing of endogenous CaMKIV increase REST expression in cortical neurons

To get more insights into the mechanisms underlying the observed CaMKI/IV-mediated REST regulation, the REST protein level was analyzed in a condition of endogenous CaMKI or CaMKIV silencing (Fig. 5.3). To this aim, primary cortical neurons were infected with lentiviral particles carrying either shCaMKIα or shCaMKIV sequence (Ageta-Ishihara et al., 2009; Takemoto-Kimura et al., 2007). To validate the specificity of the chosen shRNAs, qRT-PCR analysis on RNA samples isolated from cortical neurons were performed and the mRNA levels of CaMKIα, CaMKIIα, CaMKIV, as well as REST, were quantified from either endogenous CaMKIα-silenced (Fig. 5.3A) or endogenous CaMKIV-silenced (Fig. 5.3C) neurons. The chosen shRNA sequences display a proper function, as each sequence efficiently silence the corresponding target mRNA while the mRNAs corresponding to the others CaMKs, along with REST mRNA, remain unaltered compared to control condition (shSCR).

Following the same experimental approach, we asked whether the down-regulation of CaMKIα and/or CaMKIV levels could interfere with REST expression. The Western blotting analysis revealed that interfering with CaMKIα did not lead to changes in REST protein expression under the experimental conditions addressed (Fig. 5.3C). On the contrary, interfering with endogenous CaMKIV alters REST expression inducing an increase of its protein levels compared to neurons treated with shSCR sequence.

In conclusion, these data suggest that endogenous CaMKIV might post-transcriptionally modulates REST protein levels in primary cortical neurons, in line with the preliminary results obtained with the CaMKs inhibitor KN-93 (Fig. 5.1E-F).

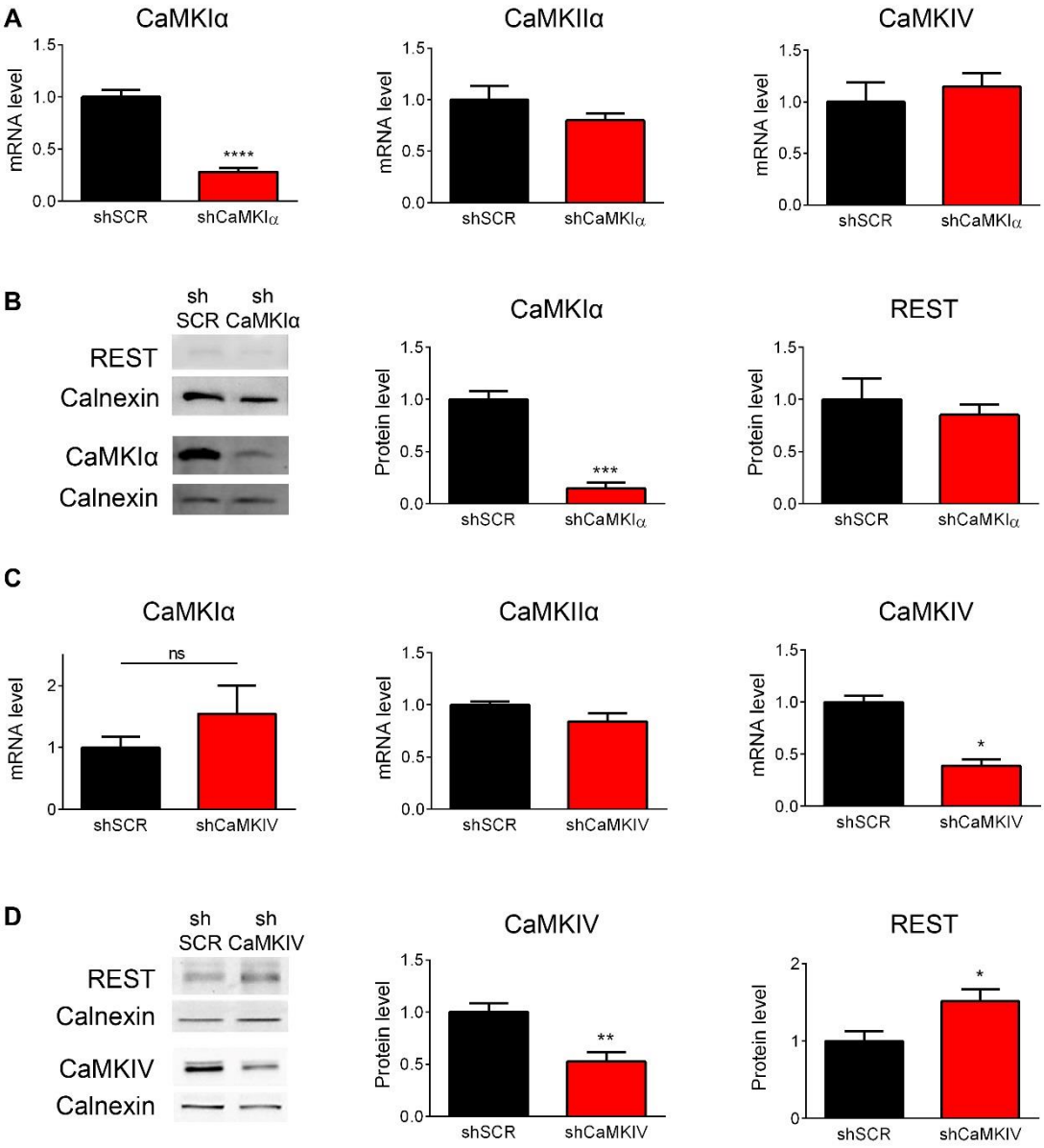

Figure 5.3. Silencing of endogenous CaMKIV increases REST expression in cortical neurons. (**A** and **B**) qRT-PCR analysis of CaMKIα, CaMKIIα, CaMKIV and REST mRNA levels (A) and western blotting analysis of CaMKIα and REST protein levels (B) of primary cortical neurons infected with lentiviral particles carrying either shSCR or shCaMKIα sequence. (**C** and **D**) qRT-PCR analysis of CaMKIα, CaMKIIα, CaMKIV and REST mRNA levels (C) and western blotting analysis of CaMKIV and REST protein levels (D) of primary cortical neurons infected with lentiviral particles carrying either shSCR or shCaMKIV sequence. A representative experiment and quantification are shown. In Western blotting analysis, Calnexin was used as a loading control; in qRT-PCR analysis Actin, Gusb and Gapdh were used as reference genes. Graphs show mean ± sem. (*p < 0.05, **p < 0.01, ***p<0.001, two-tailed Student's t-test; n = 5-3).

5.4 REST activity did not alter early developmental stages but impact synapses maturations in developed primary cortical neurons

To deepen our understanding of the mechanisms of phosphorylation-mediated REST regulation by CaMKIV, we employed a mouse model bearing a conditional gene trap (GT^{inv}) cassette in an intron of the endogenous *Rest* gene (*Rest* GT mice, Nechiporuk et al., 2016). In the above-mentioned animal model, Cre-recombinase action terminates REST transcription prior the initiator codon (Fig. 5.4A), leading to REST downregulation. To evaluate the consistency of the previous animal model, post-natal primary cortical neurons obtained from *Rest* GT mice ($REST^{GTinv}$ cortical neurons) were infected with lentiviral particles carrying either Cre-recombinase (CRE) or ΔCre-recombinase (ΔCRE), an inactive form of Cre-recombinase used as a control, and both Western blotting and qRT-PCR analysis were performed. As shown in figure 4b-c, after Cre-recombinase activity $REST^{GTinv}$ cortical neurons present a significantly reduced expression of both REST protein (Fig. 5.4B) and mRNA (Fig. 5.4C) levels.

In view of REST-mediated neuronal gene regulation during neurogenesis (Ballas et al., 2005), we investigated whether Cre-recombinase- mediated REST silencing might have an effect on *in-vitro* neuronal development. To this aim, $REST^{GTinv}$ cortical neurons were infected with lentiviral particles carrying either Cre-recombinase or ΔCre-recombinase and Sholl analysis were performed to analyzed neurite elongation at both 4 and 7 DIV (Fig. 5.4E-F). The analysis revealed a similar neurite arborization between the conditions under investigation at both considered time, indicating that REST silencing did not affect the outgrowth and branching of neuronal processes during *in-vitro* development.

We then evaluate the impact of REST-silencing on synapse formation in 14 DIV $REST^{GTinv}$ cortical neurons, and both excitatory (Fig. 5.4G) and inhibitory (Fig. 5.4H) synapses were analyzed in the presence (ΔCRE) or absence (CRE) of REST protein. Excitatory synaptic contacts were visualized by double immunostaining with the presynaptic marker VGLUT1 and the postsynaptic marker Homer1 to identify mature excitatory synapses unambiguously, while inhibitory synapses were identified with the presynaptic marker VGAT and the postsynaptic marker Gephyrin. Synapse counting at 30 µm distance from the cell body revealed a significant increase in both excitatory and

inhibitory synaptic connections for Cre-recombinase (CRE) REST^{GTinv} expressing neurons in comparison with the control (ΔCRE) condition.

Altogether, these data suggest that although REST presence did not alter the early *in-vitro* development stages (Fig. 5.4E-F) its presence dramatically impair the formation and maintenance of both excitatory and inhibitory synapses (Fig. 5.4G-H).

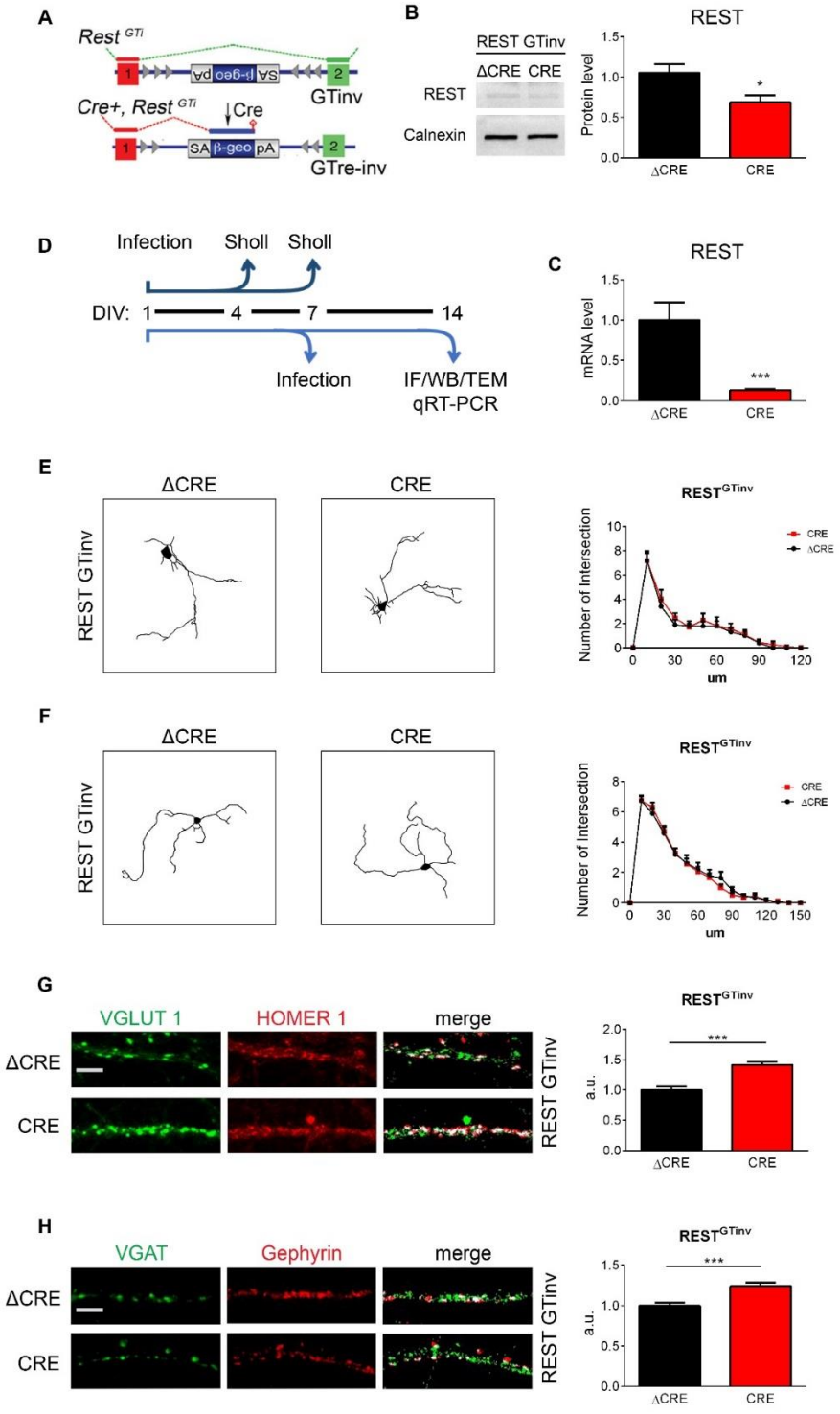

Figure 5.4. REST silencing characterization of REST GTinv cortical neurons. (**A**) Schematic representation of REST GTinv cassette (Rest GTinv). (**B** and **C**) Western blotting analysis of REST protein level (B) and qRT-PCR analysis of REST mRNA level (C) of $REST^{GTinv}$ primary cortical neurons infected with lentiviral particles carrying either ΔCRE cassette (ΔCRE) or CRE cassette (CRE). A representative experiment and quantification are shown. Graphs show mean ± sem. (*p < 0.05, ***p < 0.001, two-tailed Student's t-test; n = 5-3). In Western blotting analysis, Calnexin was used as a loading control; in qRT-PCR analysis Actin, Gusb and Gapdh were used as reference genes. (**D**) Graphical representation of the experimental procedure. (**E** and **F**) *Left*: Representative neurite traces of 4 DIV (E) and 7 DIV (F) $REST^{GTinv}$ primary cortical neurons infected with lentiviral particles carrying either ΔCRE cassette (ΔCRE) or CRE cassette (CRE). *Right*: Sholl analysis of neurite arborization as a function of distance from the soma. A representative experiment and quantification are shown. Graphs show mean ± sem. (two-way Anova/ Bonferroni's test; n = 48-63 neurons for experimental group from four independent preparations). (**G** and **H**) Immunofluorescence analysis of excitatory (G) and inhibitory (H) synapses of $REST^{GTinv}$ primary cortical neurons infected with lentiviral particles carrying either ΔCRE cassette (ΔCRE) or CRE cassette (CRE). *Left*: Synaptic boutons were identified by double immunostaining for VGLUT1 (green) and Homer1 (red) for excitatory synapses and for VGAT (green) and Gephyrin (red) for inhibitory synapses. The co-localization panels (merge) highlight the double-positive puncta (white), corresponding to bona fide synapses along transfected branches. Scale bar = 5 µm. *Right*: Quantitative analysis of synaptic puncta counted on 30-µm branches starting from the cell body. A representative experiment and quantification are shown. Graphs show mean ± sem. (***p < 0.001, two-tailed Student's t-test; n = 27-29 neurons per experimental condition, from 3 independent preparations).

5.5 CaMKIV and REST silencing alters frequency and amplitude of spontaneous excitatory postsynaptic currents in REST GTinv cortical neurons

To better investigate the interplay between CaMKIV and REST from an electrophysiological point of view, $REST^{GTinv}$ cortical neurons were infected with lentiviral particles carrying either shCaMKIV or shSCR sequence in the presence (ΔCRE shCaMKIV or ΔCRE shSCR) or absence (CRE shSCR or CRE shCaMKIV) of REST. We recorded AMPA-mediated miniature EPSCs (mEPSCs) from infected neurons in all experimental conditions. The frequency of mEPSCs was significantly smaller in ΔCRE shCaMKIV -expressing neurons compared to ΔCRE shSCR condition, whereas we found an increase in the amplitude of mEPSCs in the two groups (Fig. 5A-C). As shown in figure 5A-C, REST silencing (CRE shSCR) *per se* induces a significant increase in mEPSCs frequency, in accordance with excitatory synapses increment observe in the same experimental condition (Fig. 5.4G), while mEPSCs amplitude showed a non-statistical tendency to increase compared to control condition (ΔCRE shSCR). Notably,

CaMKIV silencing leads to a remarkable increase of mEPSC amplitude and the effect was abolished in the absence of REST expression. However, the CaMKIV-mediated effect on mEPSC frequency remained unchanged after REST silencing.

Altogether, these results suggest that both CaMKIV and REST activity alter mEPSCs in different levels and that CaMKIV and REST might cooperate in modulating variation in spontaneous excitatory postsynaptic currents.

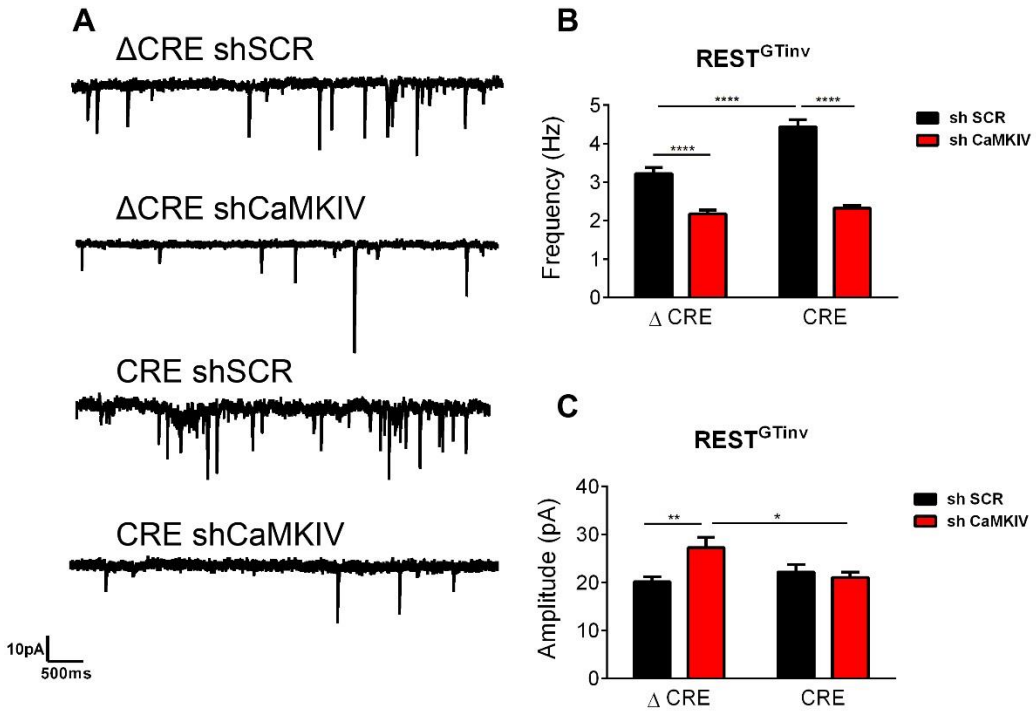

Figure 5.5. CaMKIV silencing affects spontaneous excitatory postsynaptic currents in REST GTinv cortical neurons. (A) Representative traces of mEPSCs recorded at −70 mV in 14 DIV $REST^{GTinv}$ primary cortical neurons infected with lentiviral particles carrying either ΔCRE-shSCR cassette (ΔCRE SCR), ΔCRE-shCaMKIV cassette (ΔCRE shCaMKIV), CRE-shSCR cassette (CRE SCR) or CRE-shCaMKIV cassette (CRE ShCaMKIV). (B) Histograms showing average peak frequency. (C) Histograms showing amplitude. (*p < 0.05, **p < 0.01, ****p < 0.0001, two way followed by Bonferroni's multiple comparison test; n = 23, 15, 22 and 21 neurons from ΔCRE SCR, ΔCRE shCaMKIV, CRE SCR and CRE ShCaMKIV respectively, taken from 4 independent preparations).

5.6 REST and CaMKIV expression are crucial in tuning the autophagic pathway in REST GTinv cortical neurons

CaMKIV activity and more in general calcium signalling has been implicated in a plethora of fundamental cellular mechanisms (Naz et al., 2016). Among the variety of calcium-related processes, we focused our attention on the autophagic pathway. Initiation of autophagy starts with the autophagosome formation, which depends on the lipidation of LC3-I to generate its lipidated counterpart LC3-II. Upon lipidation, LC3-II localizes to the phagophore membrane, enabling elongation of the limiting membrane to enclose the substrate and form mature LC3-II autophagosomes (Klionsky et al., 2016).

CaMKIV has been demonstrated to regulate autophagy in hepatocytes (Evankovich et al., 2012). To test the hypothesis that CaMKIV has also a relevant similar role in neurons, we performed Western blotting analysis of cortical neurons dissected from $REST^{GTinv}$ mouse pups and infected with lentiviral particles carrying either shCaMKIV sequence (shCaMKIV) or shSCR sequence (SCR). As seen in the figure, protein sample analysis and its relative quantification revealed that CaMKIV silencing increased LC3 protein level (Fig. 5.6A).

Considered that in the same experimental conditions we previously showed a correlation between CaMKIV silencing and REST protein increase (Fig. 3D), we wondered whether REST might play a role in controlling autophagy *per se*. We performed Western blotting analysis of cortical neurons dissected from $REST^{GTinv}$ mouse pups and infected with lentiviral particles carrying Cre-recombinase cassette (CRE), that abolished REST protein expression (Fig. 5.6B). As compared to control neurons, infected with the lentiviral particles bearing the ΔCRE inactive recombinase, REST-silenced neurons (CRE) showed increased levels of LC3 protein, highlighting a possible role for REST in tuning the autophagic pathway.

To further validate the hypothesis that both CaMKIV and REST might regulate this process, we also followed LC3 expression by performing immunofluorescence analyses. Neurons, dissected from the cortices of $REST^{GTinv}$ mouse pups, were assayed by indirect immunofluorescence using the specific anti-LC3 antibody further followed by the specific fluorescently-labelled secondary antibody (red), together with the direct GFP-fluorescence (green), as a marker of the CRE/ΔCRE cassettes used for infection. Neurons

were imaged in control conditions, in which both REST and CaMKIV were present (ΔCRE-SCR), or in silenced conditions, in which cells were silenced for either CaMKIV (shCaMKIV-ΔCRE), REST (CRE-SCR) or both the two proteins (shCaMKIV-CRE). As shown in Figure 6C, the silencing of either CaMKIV (shCaMKIV-ΔCRE) or REST (CRE-SCR) correlated with an increase in LC3 mean fluorescence intensity, as compared to control neurons (ΔCRE-SCR). Neurons down-regulated for both CaMKIV and REST (shCaMKIV-CRE) exhibited the same increase on LC3 mean fluorescence intensity, even though at a minor extent as compared to neurons only silenced for CaMKIV (shCaMKIV-ΔCRE) (Fig. 5.6C). In conclusion, these data suggest that both CaMKIV and REST protein expression is necessary for a correct autophagic flux. More, the observation that CaMKIV-silenced neurons (shCaMKIV-ΔCRE) exhibited a higher increase in LC3 expression as compared to neurons silenced for both the two proteins (shCaMKIV-CRE) suggests that the modulation played by CaMKIV on autophagy might be at least partially REST-mediated.

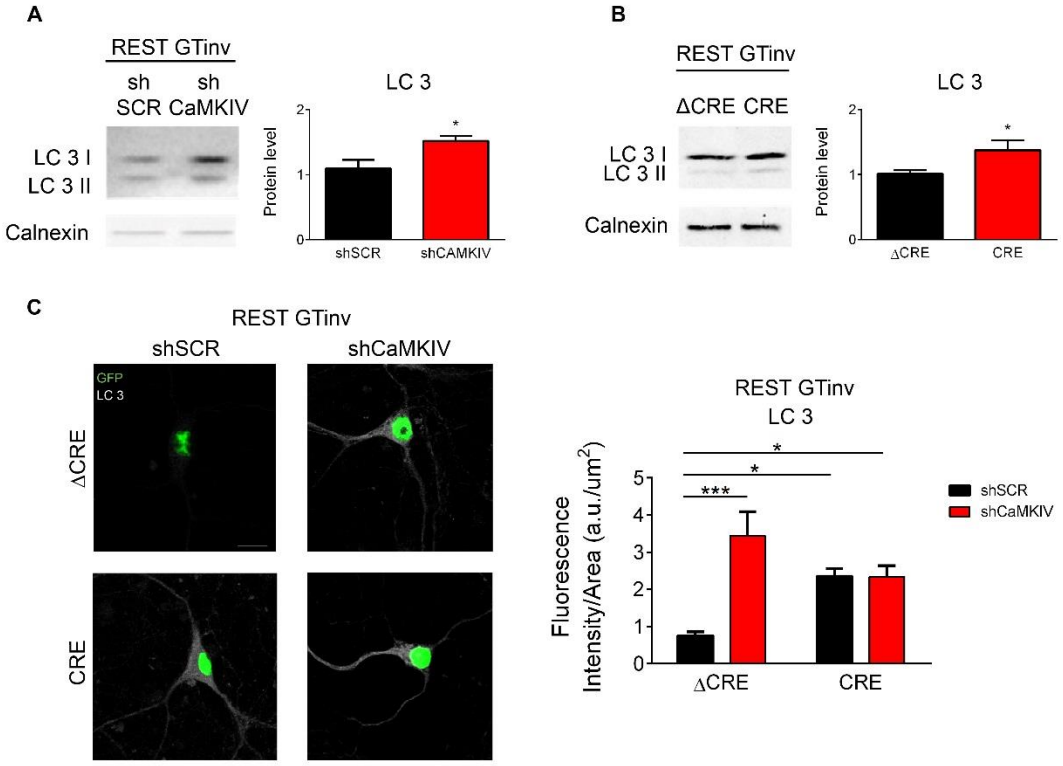

Figure 5.6. Endogenous CaMKIV and REST silencing alter the autophagic pathway. (**A** and **B**) Western blotting analysis of LC3 I and LC3II protein level in REST^{GTinv} primary cortical neurons infected with either lentiviral particles carrying shCaMKIV sequence (shCaMKIV) (A) or Cre-Recombinase (CRE)

(B) in comparison to its relative control (SCR and ΔCRE respectively). A representative experiment and quantification are shown. Graphs show mean ± sem. (*p < 0.05, two-tailed Student's t-test; n = 5-3). (**C**) Representative images of $REST^{GTinv}$ primary cortical neurons infected with either ΔCRE-shSCR cassette (ΔCRE SCR), ΔCRE-shCaMKIV cassette (ΔCRE shCaMKIV), CRE-shSCR cassette (CRE SCR) or CRE-shCaMKIV cassette (CRE ShCaMKIV) all GFP-tagged and immunolabelled with LC3 (white). A representative experiment and quantification are shown. Graphs show mean ± sem. (*p < 0.05, ***p < 0.001, two way Anova followed by Tukey's multiple comparisons test; n = 14, 21, 33, 21 neurons from ΔCRE SCR, ΔCRE shCaMKIV, CRE SCR and CRE ShCaMKIV respectively, taken from 2 independent preparations). Scale bar = 15 μm. In Western blotting analysis, Calnexin was used as a loading control.

5.7 Two aspects of the same argument: opposite roles for REST and CaMKIV in controlling autophagy

Starting from the observation that CaMKIV and REST are able to affect the autophagic pathway (together, as well as independently), we asked ourselves at which point of the pathway they could act. Following the previously experimental approach described in (Fig. 5.6C), we performed immunofluorescence analyses of cortical neurons from $REST^{GTinv}$ mouse pups to assess the lysosomal compartment. We followed the expression of the lysosomal-associated membrane protein 1 (LAMP1), a lysosomal protein required for proper fusion with autophagosomes (Yoshitaka Tanaka et al., 2000).

The quantification of LAMP1 expression in the images analyzed showed that when REST was silenced either alone (CRE-SCR) or together with CaMKIV (shCaMKIV-CRE), a reduction of LAMP1 mean fluorescence intensity was detected (Fig. 5.7A). On the contrary, when neurons were infected with lentiviral particles bearing CaMKIV-targeted shRNA (shCaMKIV-ΔCRE), no difference in LAMP1 expression was observed. Overall, these data suggest that REST silencing might negatively affect the lysosomal compartment.

Considered the previous results, we employed LysoTracker® probe to qualitatively evaluate intracellular acidic organelles in the experimental conditions addressed. Neurons were prepared from the cortices of $REST^{GTinv}$ mouse pups and were infected as in Figure 5.6C. As shown by the quantification of the images, LysoTracker® signal appeared to be increased when REST was silenced alone (CRE-SCR), as well as together with CaMKIV

(shCaMKIV-CRE), while no alteration of LysoTracker® signal was observed when only CaMKIV was down-regulated (shCaMKIV-ΔCRE) (Fig. 5.7B).

Finally, we wanted to address whether REST or CaMKIV might also play a role in lysosomal maturation, a multi-step process which involves a variety of enzymes and proteins. We prepared neurons from the cortices of $REST^{GTinv}$ mouse pups and we infected cells with lentiviral particles carrying either CaMKIV-targeted shRNA (shCaMKIV) or shSCR sequence (SCR) (Fig. 5.7C). We isolated RNA content from these neuronal samples and performed a qRT-PCR analysis to monitor mRNA levels of key players known to have a role in lysosomal maturation and function, such as Cathepsin B, Cathepsin S, Lysosomal protein transmembrane 5 as well as β-hexoaminidase. As seen by the quantification, the down-regulation of CaMKIV correlated with an increase in mRNA expression of all the proteins investigated (Fig. 5.7C). A similar analysis was performed in neurons that were silenced for REST (CRE) and that were compared to control neurons, that were infected with a ΔCRE-recombinase cassette (ΔCRE) (Fig. 7D). In accordance with the previously shown reduction in lysosome number (Fig. 5.7A), the absence of REST was associated with a reduction of lysosomal-related proteins, monitored by qRT-PCR (Fig. 5.7D).

Altogether, the data presented here suggest that both CaMKIV and REST affect the lysosomal compartment and that their role appears to be opposite. Indeed, CaMKIV inhibits lysosomal maturation, while REST promotes lysosomal formation and maturation. In accordance to the latter function proposed for REST, we performed Transmission Electron Microscopy (TEM) on cortical neurons obtained from $REST^{GTinv}$ mouse pups and silenced for REST (CRE), as well as with ΔCRE-recombinase cassette (ΔCRE) as a control. Representative images of samples processed to TEM analysis are shown in (Fig. 5.8A-B). The quantification of our data revealed that the down-regulation of REST (CRE) correlated with an increased number of autophagosomes, with a not yet statistically significant reduction of lysosome number (Fig. 5.8C).

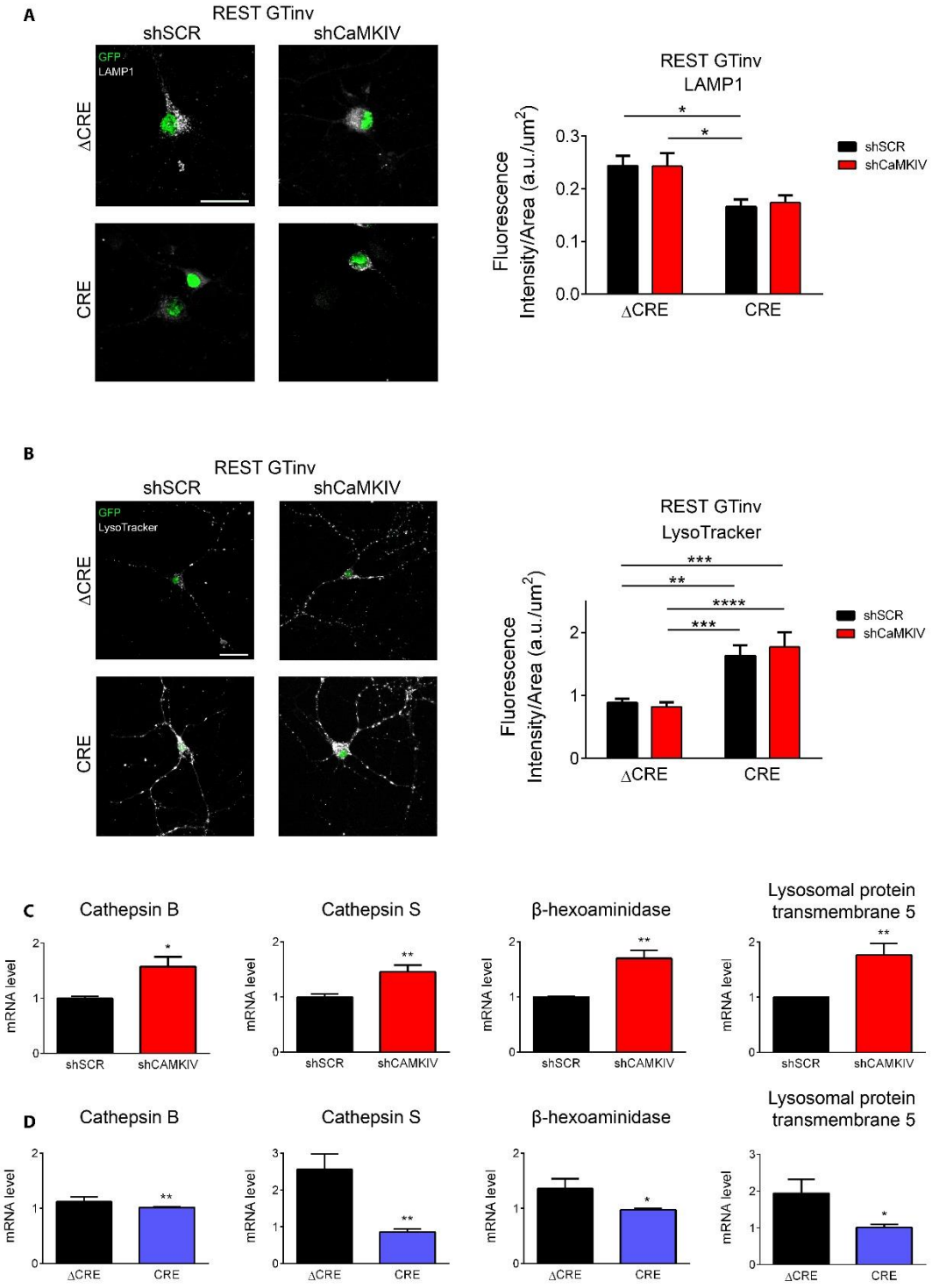

Figure 5.7. Silencing of endogenous CaMKIV and REST differently affect the lysosomal compartment. (**A** and **B**) Representative images of REST^{GTinv} primary cortical neurons infected with either ΔCRE-shSCR cassette (ΔCRE SCR), ΔCRE-shCaMKIV cassette (ΔCRE shCaMKIV), CRE-shSCR cassette (CRE SCR) or CRE-shCaMKIV cassette (CRE ShCaMKIV) all GFP-tagged and immunolabelled with LAMP1 (A) or incubated with LysoTracker® (20 nM, 30 min) (B). A representative experiment and quantification are shown. Graphs show mean ± sem. (A: *p < 0.05, two way Anova followed by Tukey's

multiple comparisons test; n = 35, 36, 33, 31 neurons from ΔCRE SCR, ΔCRE shCaMKIV, CRE SCR and CRE ShCaMKIV respectively, taken from two independent preparations and B: **p < 0.01, ***p < 0.001, ****p < 0.0001, two way Anova followed by Tukey's multiple comparisons test; n = 44, 49, 47, 40 neurons from ΔCRE SCR, ΔCRE shCaMKIV, CRE SCR and CRE ShCaMKIV respectively, taken from three independent preparations). Scale bar = 30 µm. (**C**) qRT-PCR analysis of Cathepsin B, Cathepsin S, Lysosomal protein transmembrane 5 and β-hexoaminidase mRNA level of REST^{GTinv} primary cortical neurons infected with either ΔCRE-shSCR cassette (SCR) or ΔCRE-shCaMKIV cassette (shCaMKIV), and (**D**) ΔCRE-shSCR cassette (ΔCRE) or CRE-shSCR cassette (CRE). Graphs show mean ± sem. (*p < 0.05, **p < 0.01, two way Anova/Bonferroni's test; n = 6). In qRT-PCR analysis Actin, Gusb and Gapdh were used as reference genes.

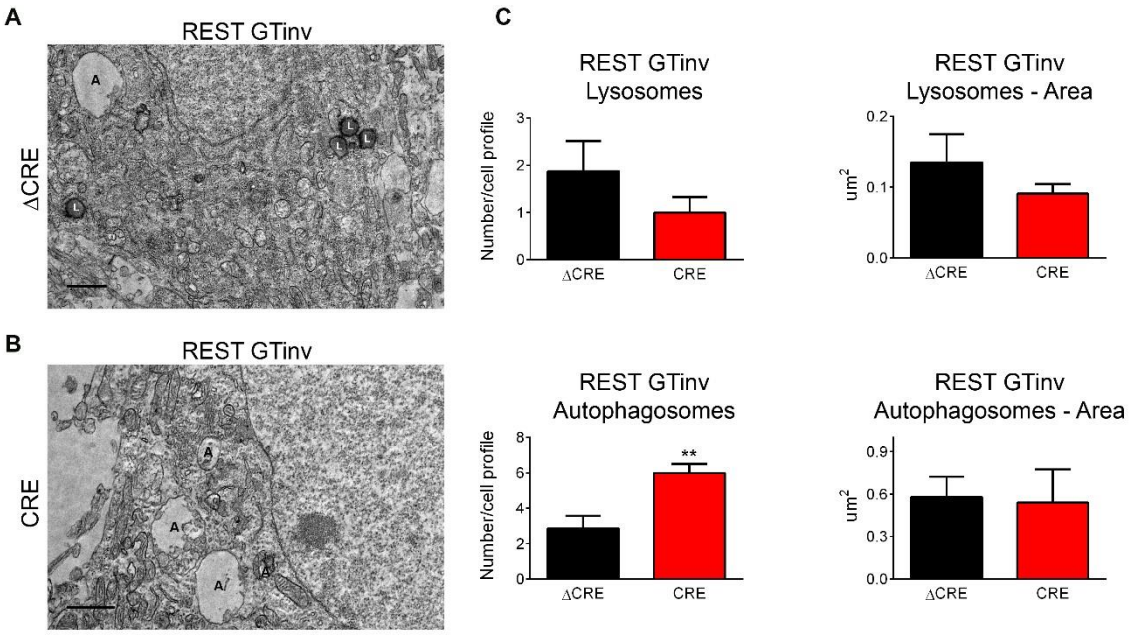

Figure 5.8. REST silencing-induced autophagosome accumulation in REST GTinv cortical neurons. (**A** and **B**) Representative TEM images of cell bodies of REST^{GTinv} primary cortical neurons infected with lentiviral particles carrying either ΔCRE-recombinase cassette (ΔCRE) (A) or CRE-recombinase cassette (CRE) (B). Scale bar = 1 µm. (**C**) Ultrastructural morphometric analysis of lysosomes (L in figures) and autophagosome (A in figures) structures. Graphs show mean ± sem. (**p < 0.01, Mann-Whitney test; n = 7 neuron for each condition).

6 DISCUSSION

REST, that has been identified in 1995 as master regulatory gene of the neuronal phenotype, elicits a pivotal function in neuronal differentiation processes (Chong et al., 1995, Schoenherr and Anderson; Ballas et al., 2005). Consistent to its role, REST protein levels decline in neural progenitors and are maintained low even in differentiated neuronal cells, allowing the transcription of a large panel of genes necessary for the acquisition of the unique phenotype of neural cells (Ballas et al., 2005).

REST dysfunctions are implicated in a various number of diseases and cancers, both in the nervous system and non-nervous tissues (Baldelli & Meldolesi, 2015; Huang & Bao, 2012; Negrini et al., 2013). The final action played by REST on its target genes can be modulated by many different factors, such as the target itself, the cell type in which REST operates, as well as the (dis)functions of REST effectors.

Little is known about REST regulation. Moreover, the possibility that REST transcription might take place indiscriminately in both neuronal and non-neuronal cells raises the attractive opportunity that its gene expression might be defined and tuned at a post-transcriptional/translational level (Kojima et al., 2001).

In 2008 two studies showed that REST protein titration during neural differentiation takes place at the level of protein stability via TrCP-dependent ubiquitin-based proteasomal degradation, in a phosphorylation-dependent manner (Westbrook et al., 2008; Guardavaccaro et al., 2008). These works provided first evidence about the presence of conserved phosphodegron motifs in the aminoacidic sequence of REST, thus highlighting the hypothesis that its phosphorylation status could affect the stability of the protein. Three kinases able to phosphorylate REST have been identified, and among these CK-1 has been identified as one of the main upstream factors regulating REST cellular abundance (Karlin et al., 2014; Nesti et al., 2014).

In order to gain deeper insights into the phosphorylation-mediated regulation of REST protein stability, the principal aim of this study was to investigate whether REST expression may be modulated by calcium-signalling, with special attention to calcium-dependent enzymes.

Calcium is a pivotal, ubiquitously present and evolutionarily-conserved messenger that controls a variety of cellular functions (Berridge et al., 2000; Maier, 2012).

Particularly, calcium-signals have been implicated in cell fate decisions ranging from migration to differentiation, proliferation and cell death (Sammels et al., 2010; Ivanova et al., 2017). Eukaryotic cells rely on a broad range of components for modulating calcium signals and transducing their functional roles. The differential and cell-specific expression of these components allow the maintenance and modulation of a physiological calcium signalling that fits with cellular requirements. When studying calcium-related effects, many are the key aspects that have to be taken into consideration, especially the considerable variation in the frequency, kinetics, amplitude and spatial extent of calcium signals in different cell types, as well as their outcomes.

To address a role for calcium in the regulation of REST protein stability, we performed a detailed analysis of the amino acid sequence of this transcription factor, searching for new additional phosphorylation sites. By a computational bioinformatics analysis, we found the existence of five consensus sites, that are targets for CaMKs.

To investigate the hypothesis that REST can be phosphorylated by CaMKs, we employed different experimental approaches, ranging from pharmacological treatment to cell biology. We performed experiments by applying two different approaches, acting inhibiting either Calcium/calmodulin-regulated enzymes through the calmodulin antagonist W-7 or CaMKs with the specific blocker KN-93 (Moon et al., 1983; Sumi et al., 1991). These data provided the first evidence that in cortical neurons REST protein stability may be regulated by calcium signalling in a CaMK-dependent manner (Fig. 5.1).

Taking advantage of the Hek293T cell model, which is known to express high levels of REST, we performed *in vitro* studies to overexpress different mutated forms of CaMKs, and the results that we obtained drew our attention towards CaMKIα and CaMKIV (Fig. 5.2). Considering that CaMKI and CaMKIV recognize a similar consensus sequence, it was not surprising that they were both able to modulate REST protein levels and activity in Hek293T cells (Lee et al., 1994). Indeed, it is possible to argue that they phosphorylate similar amino acid sequences, leading to a partial overlapping of targets (Lee et al., 1994). The approach that we employed shows some critical aspects that have to consider. First, experiments in which proteins are overexpressed can lead to artefacts due to mislocalization and/or over-function of the overexpressed protein. More, considering that calcium-signalling is overall transient and spatially localized,

overexpression experiments can be considered a good proof of concept, but they need further validation by other approaches.

After the identification of the two putative calcium-related kinases that may regulate REST protein stability, we employed a different cell biology approach to modulate their endogenous expression levels in primary neurons. To achieve this aim, we designed and employed shRNA sequences to specifically down-regulate either CaMKIα or CaMKIV, and we addressed the impact of their down-regulation on REST mRNA and protein levels. Notably, we observed that only the down-regulation of CaMKIV could affect REST protein levels, resulting in its increment (Fig. 5.3). Our results demonstrate for the first time that CaMKIV, a calcium-related kinase, regulates REST protein stability in cortical neurons.

The results obtained in neurons seem to partially contradict data obtained in Hek293T cells, in which the two CaMKs exhibited the same role on REST protein stability. Indeed, CaMKIα and CaMKIV recognize a similar consensus sequence, but they are physiologically enclosed in different subcellular compartments, with CaMKIα enriched in the cytosol and anchored to the plasma membrane and CaMKIV predominantly found in the nucleus (Lee et al., 1994; Kotera et al., 2005). This different localization could explain the fact that the two kinases phosphorylate different targets and affect different processes. Actually, the overexpression of exogenous proteins may lead to non-physiological functions and finally to the phosphorylation of non-specific target proteins. This is what occurs in Hek293T cells transfected with the constitutively active mutated form of CaMKIα, that exhibits a not-physiological nuclear localization upon overexpression. Indeed, the mutation necessary to constitutively active CaMKIα also leads to losing of its nuclear export sequence, allowing CaMKIα nuclear localization and in turn non-specific functions (Stedman et al., 2004). Taking into account all these considerations, we can conclude that CaMKIV exerts a specific regulation of REST protein stability.

In accordance with the previous work showing that REST is an unstable protein targeted for degradation through a phosphodegron-phosphorylation manner, our data demonstrate that upon CaMKIV-dependent phosphorylation, REST protein levels decline (Westbrook et al., 2008; Guardavaccaro et al., 2008). In order to study the relevance of REST in a more physiological context, we decided to employ the $REST^{GTinv}$ animal model

developed by Nechiporuk and colleagues (Nechiporuk et al., 2016) (Fig. 5.4). REST is a negative regulator of a high variety of genes, that are necessary for neuronal differentiation of NSCs to mature neurons. In ES cells, downregulation of REST is sufficient to induce differentiation towards the neuronal lineage, increase the expression of mature neuronal markers, and decrease the expression of the astrocytic marker GFAP (Gupta et al., 2009). Considered its functions, we addressed whether the absence of REST could affect the development of cortical neurons *in vitro*, by following their neuritic arborization (Fig. 5.4E-F). We found that in the experimental time window that we followed, no differences were detected in the development of neurons silenced for REST as compared to control neurons. Thus, we can conclude that our data suggest that REST activity is not fundamental in the process of neurodevelopment, at least in the early stages.

Nevertheless, our previous result could not exclude the possibility that REST elicits important roles in regulating synapse formation and maturation. Indeed, we found that REST-silenced cortical neurons showed an increased number in both excitatory and inhibitory synapses (Fig. 5.4G-H), in accordance with an increased spontaneous excitatory activity (Fig. 5.5). These data are in line with the hypothesis that REST plays a negative control in regulating neuronal genes.

Among the different important cell processes in which calcium is implicated, this ion has been also involved in the autophagic signalling pathway. Evidence for a calcium-dependent autophagy regulation was suggested more than thirty years ago, however, how calcium signalling could regulate autophagy is still under debates (Grinde, 1983).

Autophagy is a cellular process by which dysfunctional cellular components and proteins are degraded inside the cell. Through double-membrane vesicles called autophagosomes, these cytoplasmic components are delivered to the lysosome for degradation. Autophagy has a number of vital roles in physiological as well as in pathological conditions; actually, it has been demonstrated that autophagy has a role in degrading materials, including those causing many neurodegenerative conditions (Stolz et al., 2014).

In line with the hypothesis that calcium signalling can regulate autophagy, neurons silenced for CaMKIV showed an affected activity of the autophagic pathway. CaMKIV silencing leads to an accumulation in LC3 molecules (Fig. 5.6A-C), a protein associated with the autophagic vesicles, a higher amount in lysosomal enzymes (Fig. 5.7C) but no

differences in lysosome protein-marker expression (fig. 5.7A) and acidic organelles (fig. 5.7B). The data presented here support the hypothesis that, in basal conditions, CaMKIV can operate as a factor that negatively controls autophagy, acting at the early stages of the pathway and impairing lysosomal maturation.

Given the above-mentioned interplay between CaMKIV and REST, and given the pivotal role of REST and autophagy in the physio/pathology of the nervous system we silenced REST expression in primary cortical neurons seeking for alterations in the autophagic pathway (Baldelli & Meldolesi, 2015; Menzies et al., 2017). Neurons that have been silenced for REST displayed a reduced number of lysosomes (Fig. 5.7A and Fig. 5.8C) and lysosomal proteins (Fig. 5.7D), and this reduction can be related to the increase in LC3 protein level (Fig. 5.6B-C), autophagosome number (Fig. 5.8C) and the amount of acidic organelles (Fig. 5.7B).

Overall, our data suggest for the first time the intriguing hypothesis that REST acts as a positive regulator of the autophagic pathway, in addition to being a master regulatory gene of the neuronal phenotype. Indeed, the absence of REST may lead to a malfunctioning of the autophagic system, probably impairing the late stages of this process.

In conclusion, the data presented in this work provide evidence that phosphorylation modulates REST protein stability in a calcium-dependent manner and we identified CaMKIV as a novel protein kinase that exerts a post-translational control on REST expression. We also showed that REST may regulate synapse formation and activity, not impacting early stages of neurodevelopment. Finally, our data also demonstrate that both CaMKIV and REST may tune the autophagic pathway, acting in an opposite manner.

Altogether, our data highlight an important interplay between CaMKIV and REST, which can be a crucial control mechanism regulating different aspects relevant in the physiopathology of neurons. As known in literature, REST is a critical factor linking neuronal activity to the activation of intrinsic homeostasis and restoring a physiological level of activity (Pozzi et al., 2013; Pecoraro-Bisogni et al., 2018). Our data demonstrate that REST may take part in this process by also regulating synapse number and functionality, possibly increasing the autophagic pathway. On the other hand, neuronal activity is also linked to CaMKIV activity which in turn facilitate REST protein turn over

and impair lysosome maturation, possible mechanisms aimed to counterbalance REST-mediated intrinsic homeostasis function.

A schematic model, summarizing the most relevant finding of this work, is reported in Figure 6.1.

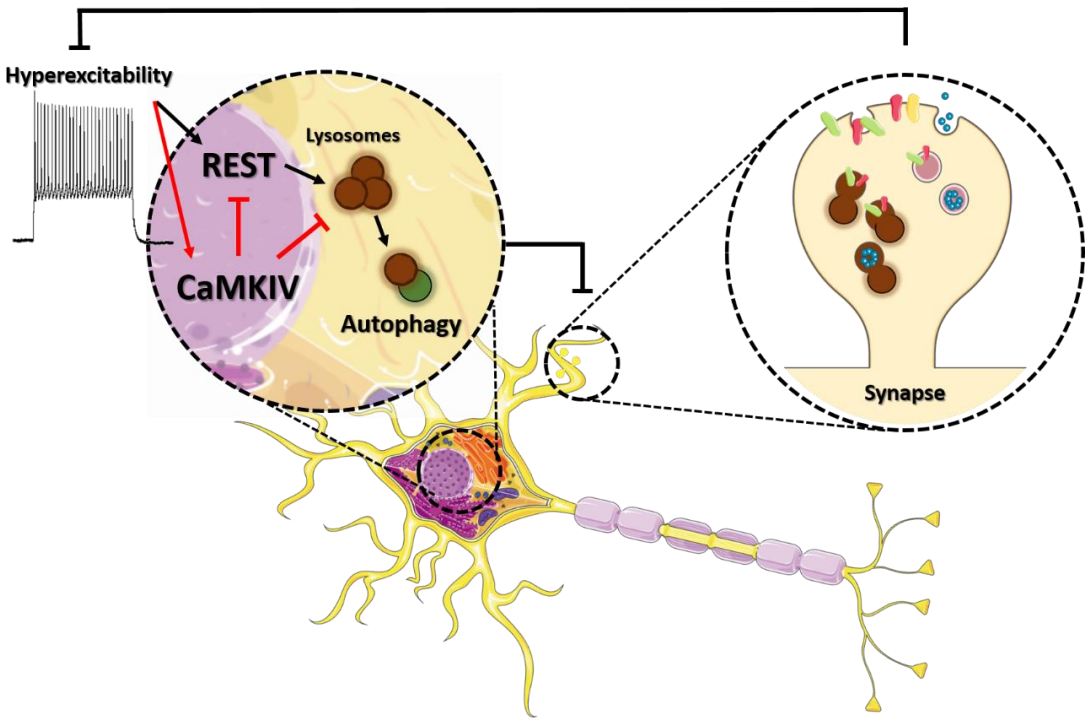

Fig. 6.1 Schematic representation of REST and CaMKIV interplay.

7 FUTURE PERSPECTIVES

Overall, the results presented in this thesis provide the proof of principle for a new mechanism of modulation of REST protein stability played by a calcium-dependent kinase in neurons and for the relevance of their interplay on the autophagic pathway.

Future experiments are needed to better characterize the relationship between REST and CaMKIV. To this aim, we plan to employ immunoprecipitation assays to isolate REST in cells overexpressing the constitutively active form of CaMKIV and perform a high-throughput screening based on mass spectrometry approach to identify phosphorylated residues modified by CaMKIV.The procedure we want to follow will allow us to get better insights into the complete overview of REST/CaMKIV interaction. Considered that one of the main interests of our laboratory is the investigation of different aspects of synaptic transmission, we also intend to elucidate how REST and CaMKIV can act together to affect neuronal homeostatic plasticity. Indeed, we will focus our attention on the effects played by CaMKIV on REST protein stability, especially in a condition of neuronal hyper-excitability, where REST is known to exert a homeostatic role. In details, we will silence REST and/or CaMKIV in cortical neurons and we will reproduce features of hyper-excitability *in vitro*, addressing whether the silencing of either one or both of the two proteins could alter any of the homeostatic responses.

Finally, considering that we ascertained a previously undiscovered role of REST on the autophagic pathway, we will plan to better investigate the impact of this function and of the putative modulatory role played by CaMKIV. Indeed, we will employ our tools to modulate the expression levels of either REST and/or CaMKIV in cortical neurons eventually exposed to a set of molecules able to interfere with different steps of the autophagic pathway. This approach will allow us to ascertain at which point REST and/or CaMKIV play their functions in this scenario. More, we will also plan to complete the preliminary electron microscopy characterization that is already ongoing, in order to better confirm the results that we obtained by our biochemical and immunofluorescence data. These new approaches will help us to better unravel the new mechanistic role(s) of REST and CaMKIV in the autophagic pathway.

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10 APPENDIX

Article published by Emanuele Carminati during the PhD course:

Regulation of neural gene transcription by optogenetic inhibition of the RE1-silencing transcription factor.

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Abstract

Optogenetics provides new ways to activate gene transcription; however, no attempts have been made as yet to modulate mammalian transcription factors. We report the light-mediated regulation of the repressor element 1 (RE1)-silencing transcription factor (REST), a master regulator of neural genes. To tune REST activity, we selected two protein domains that impair REST-DNA binding or recruitment of the cofactor mSin3a. Computational modelling guided the fusion of the inhibitory domains to the light-sensitive Avena sativa light-oxygen-voltage-sensing (LOV) 2-phototrophin 1 (AsLOV2). By expressing AsLOV2 chimaeras in Neuro2a cells, we achieved light-dependent modulation of REST target genes that was associated with an improved neural differentiation. In primary neurons, light-mediated REST inhibition increased Na (+)-channel 1.2 and brain-derived neurotrophic factor transcription and boosted Na (+) currents and neuronal firing. This optogenetic approach allows the coordinated expression of a cluster of genes impinging on neuronal activity, providing a tool for studying neuronal physiology and correcting gene expression changes taking place in brain diseases.