Anche un viaggio di mille miglia inizia con un singolo passo…

a Te, Nonna, che ci sei sempre stata e che ora mi guardi e mi sorridi da lassù

INDEX

Abstract

The development and maturation of the nervous system imply a precise temporal and spatial modulation of gene expression, coordinated by transcriptional enhancers and repressors. In this context, the key role of repressor element 1-silencing transcription factor (REST) is largely known. REST regulates neurogenesis and neuronal identity through cell-specific gene repression, allowing expression of its targets in mature neurons. During neuronal development REST levels are reduced and REST is quiescent in mature neurons, which are able to modulate its expression in response to pathological stimuli. Such dysregulation has been implicated in several neurodegenerative disorders, including Alzheimer's and Huntington's diseases, tumors of the central nervous system (CNS) and epilepsy. Our pilot study had indicated that REST is significantly over-expressed in murine chronic experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis (MS), at acute early phase. MS is a CNS autoimmune-mediated disease characterized by chronic inflammation and demyelination in the white and grey matter leading to an impairment of synaptic transmission and network connectivity, with both neuronal and axonal loss. Chronic inflammatory processes that continuously disturb neuro-axonal homeostasis drive neurodegeneration, so the clinical outcome is likely to depend on the balance between inflammation and the remaining capacity for neuronal self-protection and repair. Hence, therapeutic approaches halting neurodegeneration and promoting tissue repair are sorely needed. On this matter, specific targeting of REST-dependent transcription and/or molecular pathways could be an appealing strategy towards therapy in MS. In this project, we have addressed REST expression throughout disease to characterize the role of its unbalance in EAE, assess its potential as biomarker for disease progression, and understand if modulation of REST leads to rescue of EAE phenotype. Starting from these observations, we have first analyzed the expression of REST and its target genes in vivo, during EAE, and in vitro, in the context of neuroinflammation investigating the differential contribution of REST transcripts to REST up-regulation and how this affects its targets. Moreover, in order to study the cellular and molecular pathways involved in the

processes of neuroinflammation, we have analyzed REST activity in cell culture, under inflammatory conditions mimicking the EAE microenvironment. Our results support the involvement of REST in the pathological process of EAE, suggesting a possible mechanism of action of REST in the control of its target gene expression in response to neuroinflammation, and demonstrating the involvement of signal pathways regulating REST expression, under these conditions.

Introduction

Modification of molecular pathways involved in neuronal development is an appealing strategy towards therapy for neurodegenerative diseases such as multiple sclerosis. Mechanisms leading to neurodegeneration include microglia activation, oxidative and mitochondrial stress that continuously disturb neuro-axonal homeostasis, leading to neuronal death. Thus the clinical outcome is likely to depend on the balance between inflammation and the remaining capacity of neurons for self-protection and repair. Therapeutic approaches halting neurodegeneration and promoting tissue repair are sorely needed. A possible therapeutic approach targets pathways involved in neuronal development, one of which is based on the repressor element 1-silencing transcription factor (REST), which regulates neurogenesis and neuronal function and is known to be dysregulated in several neurodegenerative diseases. We have speculated that dysregulation of REST could also be important in the pathogenic process of MS, and we have studied this hypothesis in its most relevant animal model, experimental autoimmune encephalomyelitis (EAE), associated with neuronal damage and myelin loss, such as occurs in MS.

1. REST: a regulator of neurogenesis

The development and maturation of the nervous system imply a precise temporal and spatial modulation of gene expression, coordinated by transcriptional enhancers and repressors that bind specific sequences to gene promoters. In this context, the key role of the transcriptional factor, REST, is largely known. REST, also known as neuron-restrictive silencer factor, is a zinc finger protein that binds a conserved motif of 21 base pairs (bp) known as repressor element-1 (RE-1), in a large number of genes encoding neuronal traits, such as ion channels, synaptic vesicle proteins and neurotransmitter receptors. In 1990, two different groups identified an upstream cis-acting silencer element responsible for down-regulating two neuronal genes: SCG10 and type II sodium channel (NaII) in non-neuronal cells [1, 2]. Two years later, the sequence of this element was

characterized and is thereafter referred to as repressor element-1 (RE1). In 1995, the same labs isolated the protein that binds to RE1 site, a negative transcriptional factor, originally described as a transcriptional repressor of neuron-specific genes in non-neuronal cells. This protein, REST, binds to gene promoters that contain an RE-1 consensus site and mediates a cell-specific gene repression [3, 4], through the recruitment of two co-repressors, mSin3a at the N-terminus and Co-REST at the C-terminus [5]. The assembly of the co-repressor complex, in turn, recruits other chromatin remodeling factors that highly package genomic material for gene repression (**Fig. 1**).

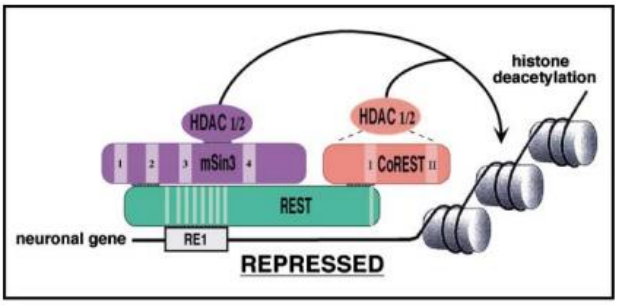


Fig. 1 RE-1-dependent REST repressor complex. REST recruits mSin3 and CoREST to RE-1 sequence through its repressor domains. CoREST and HDAC1/2 are shown in the same color to indicate that they are tightly associated as a core complex interacting with DNA binding proteins other than REST. mSin3 has an HDAC interaction domain for mediating the transcriptional repression exerted by REST. The ZFs in REST are represented as light-green vertical stripes. The ZF at the C-terminus is required for interaction with CoREST and for repression Taken from [5].

Several studies have shown through chromatin immunoprecipitation sequencing analysis that REST binding is not limited to the canonical RE1 sequence, but binds multiple variations of it across the genome. Since then, multiple RE1 sites have been discovered in promoter regions of various neuron-specific genes [6, 7].

1.1 Role of REST during development

REST contributes to the regulation of neuronal gene expression during the transition from embryonic stem cells (ESCs) to mature neurons. Experiments performed both in vitro and in vivo have suggested that REST is expressed in both nuclear and cytoplasmic compartments of mouse ESCs (mESCs) before differentiation and continues to be expressed also in derived neural stem

cells (**Fig. 2**).

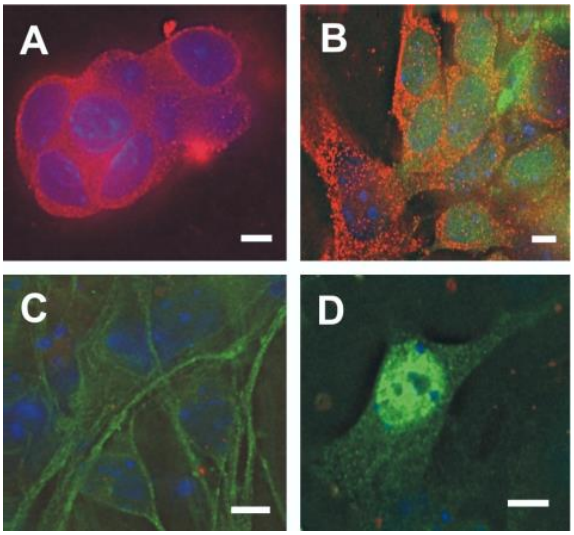


Fig. 2 REST expression during differentiation from embryonic stem cells into neurons. (A) REST in red is expressed in both nuclei and cytoplasm of ES before differentiation. (B) REST continues to be expressed in ES-derived neural stem cells. (C-D) REST is downregulated in differentiated early (C) and mature neurons (D). Scale bar, 10 μm. Taken from [8].

Moreover, it has been shown that REST is also required for the maintenance of ESC pluripotency and self-renewal, by preventing microRNA-mediated down-regulation of the stem cells factors Oct4, Sox2, Nanog, and c-Myc [8]. During neuronal differentiation from ESCs, in both early differentiated and mature neurons, REST levels are reduced allowing expression of neuron-specific genes [9, 10]. Since neuron-specific genes are lowly expressed in non-neuronal cells where REST levels are high, and vice-versa in neuronal cells, REST has been considered a key regulator of the neuronal phenotype and function. In fact, during neuronal development and differentiation, epigenetic transcriptional changes occur across the genome that modulate gene network in specific cell types. In support of this idea, REST knockout (KO) mouse embryos showed forebrain malformation at the beginning of neurogenesis, i.e. at embryonic day 9, and embryonic lethality with 100% penetrance [11, 12]. Surprisingly, only two of REST target genes analyzed were de-repressed in REST KO mouse embryos, suggesting that REST down-regulation is only one of the factors contributing to neuronal commitment. these data indicated that the gene-specific repression of REST is important in early phase during embryonic development, but is also

required at a later stage for preventing neuronal differentiation in non-neuronal cells [12] . Hence, REST function is not restricted to embryonic development, but is fundamental also for the maintenance of key properties in mature neurons. Now it is largely known that REST is an epigenetic regulator that affects cellular genome, in response to environmental cues, through chromatin remodeling in a dynamic and context-dependent manner, in both neuronal and non-neuronal cells [13]. The context-dependent function of REST is influenced by different mechanisms that regulate its expression including proteasome degradation, nuclear translocation and transcript splicing [14, 15]. REST exerts its multiple and broad regulatory functions in neuronal differentiation and development [16-19], such as fine-tuning neural gene expression [19], modulating synaptic plasticity [20], and maintaining the self-renewal capacity of neural stem cells [17] (**Fig. 3**).

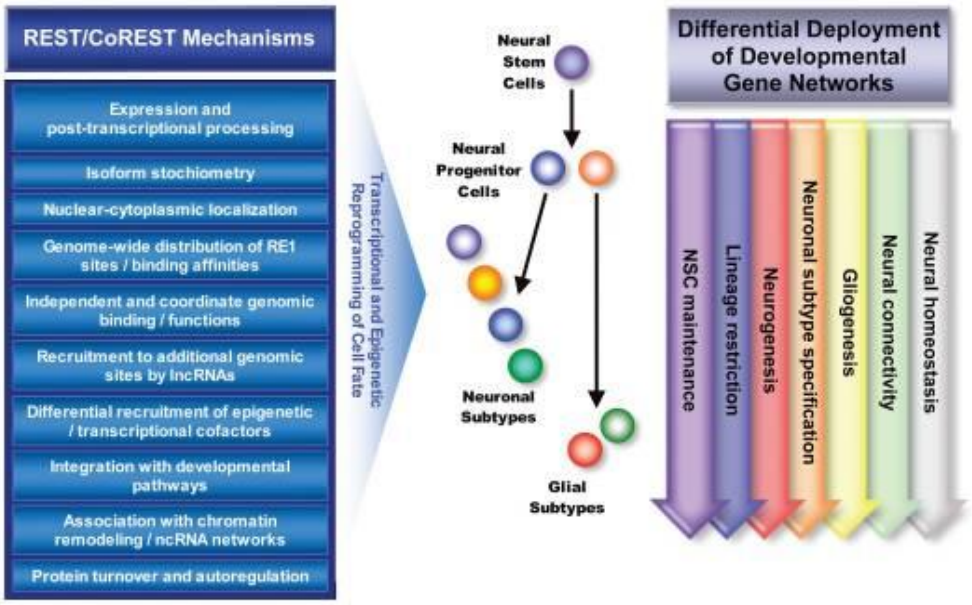


Fig. 3 Scheme of REST-mediated regulation of gene networks during development. The figure shows the mechanisms by which REST and CoREST are responsible for the transcriptional and epigenetic regulation of gene networks involved in neural cell fate decisions during development. Taken from [19].

1.2 REST gene and its mode of action

REST gene consists of two repressor domains separated by a DNA binding domain (DBD) composed of eight zinc finger motifs (ZF), a lysine- and proline-rich region between the DBD and

the C-terminal repressor domain [21, 22] (**Fig. 4**). The nuclear localization sequence (NLS) of REST was originally believed to be located between the eighth and ninth ZFs at residues 512-522, an idea supported by immunofluorescence analysis in COS-1 cells [23] that showed the failure of nuclear translocation of REST due to a mutation in this region. However, recent mutation experiments in various individual ZFs or groups thereof have identified the ZF domain 5 as necessary and sufficient for nuclear targeting [24, 25].

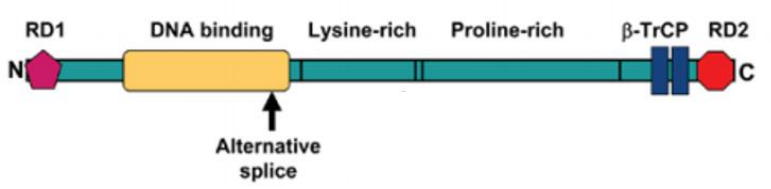


Fig. 4 Schematic representation of the functional domains of REST. REST has N- and C- terminal repressor domains (RD1 and RD2) that serve as scaffolds for distinct gene repressor/silencing complexes. The DNA binding domain is followed by lysine- and proline-rich domains and two β-TrCP binding sites. Alternative splicing leads to truncated forms of REST that terminate in the region indicated by the arrow. Taken from [22].

The two independent repressor domains of REST, located at the amino-(N) and carboxy-(C) termini, interact with several complementary chromatin-modifying enzymes. The N-terminus repressor domain interacts with mSin3, a corepressor found in all eukaryotes that recruits histone deacetylases (HDAC1 and HDAC2) [23, 26]. The C-terminal repression domain interacts with CoREST, and, similarly to mSin3, establishes complexes with HDACs for a more stable silencing of neural genes [5, 27]. A well-defined mechanism for repression of neuronal genesis has been described. HDACs remove acetyl groups on the N-terminal tails of histones H3 and H4, highly packaging genomic material and thereby inhibiting transcription activity. Additionally, following REST recruitment, other silencing proteins were found to bind CoREST including the methyl CpG-binding protein 2, the suppressor of variegation 3-9 homolog 1 and the heterochromatin protein [28]. CoREST can form complexes not only with HDACs, but also with the histone H3 lysine 9 (H3–K9) methyltransferase G9a and the histone H lysine 4 (H3–K4) demethylase LSD1 [29]. . Similar to as occurs with the acetylation process with HDACs, H3-K9 methyltransferase

G9a recruited by CoREST methylates nucleosomes on histone tails resulting in chromosome condensation, to mediate epigenetic silencing in non-neuronal cells. The silencing proteins recruited by REST alter chromatin structure and regulate transcription through several mechanisms including histone deacetylation, chromatin remodelling and methylation. Methylated H3-K9 can recruit the heterochromatic protein HP1 through its chromodomain, resulting in chromatin compaction of genomic material [30]. The recruitment of silencing machinery by REST–CoREST results in the propagation of silencing complex along a large chromosomal trait containing several neuronal genes that do not have their own REST binding sites [28], suggesting a relationship between higher order chromatin structure and patterns of gene expression.

1.3 REST alternative splicing

The REST gene is present and highly conserved in all vertebrate genomes [31], and comprises three untranslated first exons in the 5'UTR, three coding exons, and a 28 bp alternatively spliced exon [32, 33]. REST undergoes alternative splicing, a post-transcriptional event that produces multiple and different transcripts predicted to encode protein variants with different numbers of ZF motifs, which differ in their DNA binding capacity and their repressor activity [34, 35] Alternative splicing of REST transcript produces numerous predicted coding and non-coding mRNAs with species and cell-type or tissue-specific expression [36], but only a limited number of splicing variants have been experimentally demonstrated at mRNA and/or protein level. The splicing variants can result from a complete or partial skipping of exons, allowing for at least 45 predicted mRNAs variants and potential corresponding protein isoforms [36]. Two mRNA variants are generated by alternative in-splicing of a neuron specific exon (exon N) located between exons V and VI [31], which introduces 2 possible stop codons. Thus, these transcripts are characterized by the insertion of 16- or 28-bp (N16 and N28) in the region encoding the spacer between zinc fingers 5 and 6 of REST protein (**Fig. 5**).

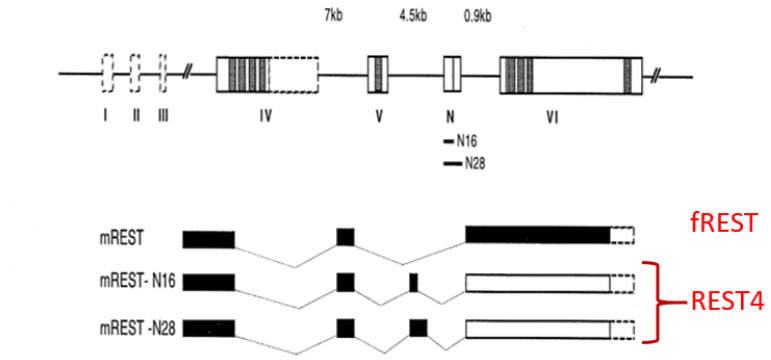


Fig. 5 Mouse REST (mREST) gene and transcripts. Exons are shown as boxes and introns as lines. The neuron-specific exon located between exons V and VI are indicated as N. Taken from [31].

The incorporation of this exon into the REST mRNA is dependent on a splicing regulator, which is selectively expressed in neurons and hair cells [32, 37]. The resulting encoded proteins, experimentally demonstrated [38] and predicted, are C-terminal-truncated isoforms with five ZF motifs. One of these, termed REST4, lacks the repressor domain as a consequence of the insertion of the first 16 bp of exon N, which is spliced out in the REST full-length (fREST) isoform. Is now largely recognized that the ZF-5 is fundamental for REST nuclear targeting [25] and dysregulation of nuclear REST is involved in many neurodegenerative disorders [34]. In humans the open reading frame of REST is comprised between exon II and exon IV. Exon-3 and can be skipped totally or partially, resulting in the loss of NLS signal needed for REST nuclear translocation [39].

1.4 REST4: role and its regulation

The C-terminal truncated variant, known as REST4, has been well documented [34, 35]. It was first described in rats as a variant characterized by the inclusion of 16 nucleotides of exon N, followed by an inframe stop codon that introduces a premature stop codon [40, 41]. This transcript is a truncated form which terminates prematurely retaining only the first five zinc fingers, needed for the transport of REST4 into the nucleus [24]. The biological function of REST4 is controversial. Although REST4 itself does not bind to the RE-1, it acts as a dominant negative by inhibiting the binding of REST to the RE-1 sequence. Thus, REST4 itself does not act as a transcriptional repressor but rather regulates the repressor activity of REST [14]. Although REST4

was found to be neuron specific, recent evidence has shown that REST4 could be expressed also in non-neuronal cell populations, especially in glial cells, influencing mRNA levels of REST [42]. As it lacks the C-terminal repression domain of REST, it is believed that this isoform is unable to interact with CoREST [13, 43], and partially suppresses the silencing function of fREST [14, 44, 45], promoting neural gene expression and neurogenesis [38, 46]. The alternative splicing of REST that produces the REST4 isoform is directly regulated by the neural-specific Ser/Arg repeat-related protein of 100 kDa (nSR100/SRRM4). The expression of nSR100 correlates with high levels of REST4 and low fREST expression. In turn, overexpression of REST causes repression of nSR100, containing RE1-element controlled by REST, and thereby of REST4 (Fig. 6).

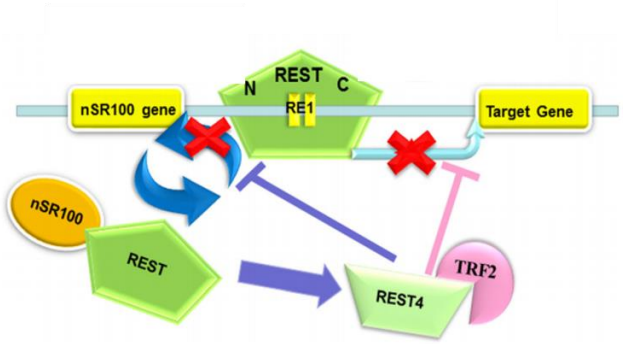


Fig. 6 The feedback loop between REST and REST4 regulating REST expression is mediated, in part, by nSR100. nSR100 induces alternative splicing of REST to the truncated isoform, REST4. High levels of REST in turn cause repression of nSR100 and lowered expression of REST 4, TRF2 interacts with REST4 for its protection from ubiquitin mediated degradation by the proteasome for positively regulating neural progenitor formation and maintenance. Taken from [45].

The repression of nSR100 in non-neural cells is needed to prevent the activation of neuron-specific splicing events, suggesting that interactions between a splicing activator and a transcriptional repressor represent a mechanism in the regulation of gene expression during neurogenesis [46]. But the role and the function of REST4 in brain has to be clarified. In the pre-frontal cortex (PFC) during the early postnatal period, REST4 overexpression was associated to an enhanced transcription of some RE1 containing genes [38]. However, the expression of other REST targets was not affected, suggesting that the expression of REST is not only under the control of REST4 [38]. The balance between REST and REST4 plays a role in neurological disorder, including

epilepsy [47], Parkinson's disease [48], mood disorders [49], and fetal alcohol syndrome [50]. Moreover, REST4 isoform has been involved also in some kind of tumors, such as small cell lung [51] and breast [52] cancers, imparting a neuroendocrine phenotype on the cells. Thus, REST4 is overexpressed in small cell lung cancer, causing the de-repression of many neuronal genes, which are targets of REST. Moreover, upon REST4 inhibition, re-expression of REST leads to cell apoptosis, suggesting that REST4 could promote cell survival [51] . Similarly, in breast cancer, there is an increase of REST4 levels associated with a loss of REST [52]. Taken together, the balance of REST and REST4 is important not only for neural differentiation and neuronal precursor cell maintenance, but also for the regulation of gene expression, representing a new possible target that could be affected in neurological disorders and possibly in cancer.

1.5 REST protein

REST full length transcript has a predicted molecular weight of ~120-kDa based on its amino acid sequence, but often is not observed at the expected size on electrophoresis, spanning from 116 to 200 kDa, due to different post-translational modifications. The availability of REST is reduced in pluripotent stem cells, neural progenitors and mature neurons, whilst the co-repressor complexes differ between these cells [53], leading to different affinity of REST with RE1 containing genes and consequently difference in their expression. The first class of genes with lower affinity to RE1, is automatically expressed upon loss of REST at neuronal maturation, whilst the second class exhibits low expression due to the presence of corepressors at methylated DNA, even in the absence of REST. It has been demonstrated that the regulation of REST sub-cellular localization is needed for controlling the availability of REST to RE1 sites in the nucleus, thereby modulating the expression of target genes. REST is localized to the inside and outside of the nucleus in several cell lines including human embryonic stem cells, human neuroblastoma cells, and hippocampal primary neurons [8]. However, the observation of REST in the cytoplasm of multiple cell lines raises questions about its function in this cellular compartment beyond sequestration from the

nucleus. The synthesized protein is shuttled into the nucleus, where it exerts its transcriptional activity, by its NLS and through the binding with RILP, a LIM (Lin11, Isl-1 & Mec-3) domain protein [54] (**Fig. 8**).

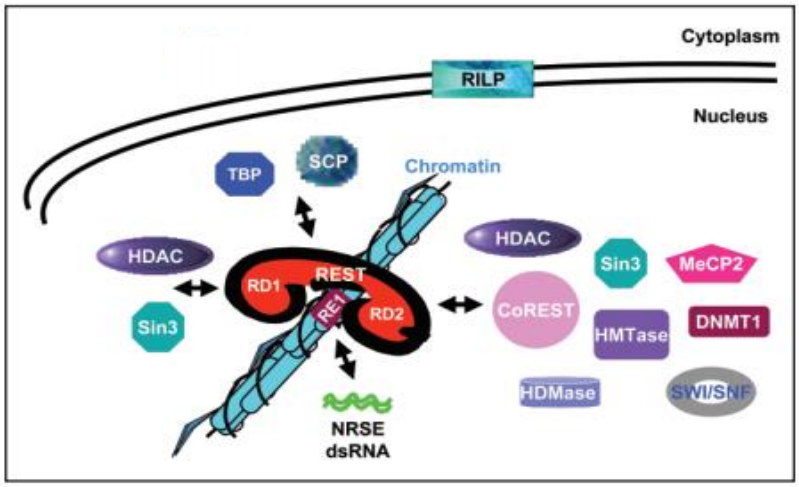


Fig. 8 REST can be translocated into nucleus by binding to REST-interacting LIM domain protein **(RILP)**. In the nucleus, RD1 (repressor domain 1) can interact with mSin3A, the HDAC complex, and N-CoR, whereas RD2 can interact with CoREST, mSin3A/HDAC complex, histone H3-K9 methyltransferase G9a (HMTase), histone H3-K4 demethylase LSD1 (HDMase). Taken from [54].

Both REST and REST4 can interact with this domain for nuclear translocation [55], confirming that the region surrounding the ZF domain 5 is the only one effective for the functional signal needed for translocation into nucleus. It has been known that REST exerts different and opposite functions, depending on the context and cell types. The first function consists in blocking the expression of neuronal genes through the recruitment of silencers and repressors in terminally differentiated non-neuronal cells, where its expression is high. On the other hand, REST is expressed at low, albeit detectable, levels in neurons, presumably to allow dynamic regulation of neuronal genes. The low levels of REST protein during the transition from stem cells to mature neurons are maintained by the recognition of a degradation signal sequence, located near the C-terminus of REST, via β transducin repeats-containing protein (β-TrCP), an E3 ubiquitin ligase. This system directs the protein for proteasome-mediated degradation for proteasome-mediated degradation [56].

2. Function of REST: modulation of neuronal activity and synaptic plasticity

Epigenetic modifications recently emerged as a crucial event to rapidly adapt the neuronal transcriptional response to developmental and environmental hints [57]. In this context, REST plays a central role in the determination of the neuronal fate, as well as in the modulation of neuronal activity and plasticity [58]. Increased REST levels in mature neurons, induced after the administration of kainic acid (KA, a glutamatergic agonist) or after ischemia, are followed by a decrease in the expression of neuron-specific genes and neurodegeneration. It is therefore not unexpected that increased REST levels have been often considered as harmful in mature neurons. In contrast, other works sustain the beneficial role of REST increase associated with an intrinsic homeostatic response during hyperexcitability states. Homeostatic plasticity is a phenomenon that occurs in neurons after a perturbation of neuronal network activity and is characterized by adjusting both the synaptic strength (synaptic homeostasis) and the firing properties (intrinsic homeostasis). Intrinsic homeostasis leads to the establishment of neuronal activity levels through the modulation of voltage-gated conductance. Different forms of intrinsic homeostasis have been reported, but the one mostly investigated is based on the modulation of membrane excitability during prolonged silencing of neuronal activity. It results in an elevated neuronal excitability through adjustments in voltage-dependent conductance. The opposite form of intrinsic homeostasis occurs when hippocampal cultures are exposed to chronic hyperexcitation and it is still poorly understood. REST was shown to exert a protective mechanism in governing the inhibitory control of intrinsic excitability after the prolonged treatment with the stimulatory agent 4-aminopyridine (4AP, a potassium channel blocker [59]. The 4AP-induced hyperactivity increases the expression of REST, that is accompanied by the homeostatic down-regulation of voltage-gated sodium (Na^+) channels and neuronal Na^+ current density, readjusting the network firing activity at a physiological set point. Conversely, interfering with REST expression impaired this homeostatic response.

3. REST in neurodegenerative diseases

REST controls many cellular processes that occur in both physiological and pathological conditions. It has been shown that different kinds of pathological stimuli might trigger an activation of REST associated with increased transcript levels. Not surprisingly, dysfunction of REST and the repercussion on the regulation and expression of its target genes are closely related to neurological diseases, especially in neurodegeneration [45]. The role of REST in the onset of pathologies is really complex and controversial, acting under some circumstances as an oncogene [60, 61], and under other conditions as a promoter of insult-induced neuronal death (brain ischemia, Huntington's disease) [62] or dysfunction (epilepsy) [63]. REST levels are relatively low in mature neurons, to release gene repression and promoting the expression of specific genes required in neurons. Glial cells, such as astrocytes [64], oligodendrocytes [65] and microglia [66], where neuronal-specific genes are constitutively repressed, express higher levels of REST. In mature neurons, however, REST expression is not completely absent. In fact, it is becoming clear that neurons are able to modulate REST expression in response to several environmental stimuli and pathological insults. As a result, it has been suggested that, by its mechanism of target gene repression, REST is actively involved in controlling gene expression in the adult brain as well and is essential for the determination of neuronal fate.

3.1 Huntington disease

As previously reported, the sub-cellular localization of REST can modulate target gene expression, controlling the availability of REST to RE1 sites in the nucleus. Huntington's disease (HD) is a neurodegenerative disease, characterized by the expansion of a mutated huntingtin protein, which leads to death of neuronal cells in some brain districts. Wild-type huntingtin (wHtt) protein interacts with REST within the cytoplasm to relieve repression of RE1-regulated genes, including brain derived neurotrophic factor (BDNF), a survival factor for neurons that die in Huntington’s disease. The corepressor complex does not form at the RE-1 site and gene expression can proceed in neurons. Thus, wHtt acts as a positive transcriptional regulator for REST target genes involved

in the maintenance of the neuronal phenotype. Expansion of mutated huntingtin protein disrupts its binding with REST, affecting its cytosolic sequestration. As a consequence, REST accumulates into the nucleus for repressing neuronal genes, important for the maintenance and function of specific neurons, and contributing to neurodegeneration [67]. It has been shown that dysregulation of nuclear REST is involved in many neurodegenerative disorders [34], suggesting that the modulation of REST activity could represent a possible therapeutic strategy for HD. The skipping of exon-3, accompanied by the loss of NLS signal, eliminates ZF5 needed for REST nuclear translocation. Treatment with antisense oligos, which targets the splice sites of exon-3, and induces skipping of that exon in vitro, resulting in the reduction of nuclear REST and the rescue of specific neuronal genes, is being considered as a possible approach to therapy of HD [39].

3.2 Alzheimer disease

A hypothesis that support the beneficial role of REST in the context of neurological disorders, is the correlation between REST levels and cognitive preservation and longevity during aging. REST expression was shown to be significantly increased in the ageing human prefrontal cortex and hippocampus with a striking induction of REST in the nucleus, while lower levels of REST were detected in microglial cells and astrocytes. These observations demonstrated that increased levels of REST are a common trait of normal ageing, appearing essential for the maintenance of neuronal viability [68]. The same study showed that REST is lost in mild cognitive impairment and Alzheimer's disease (AD). In fact, it was observed that REST represses genes that promote cell death and AD pathology and induces the expression of stress response genes, protecting neurons from oxidative stress and beta-amyloid toxicity through the coordination of a neuroprotective response in the aging brain. However, a role of REST in AD-associated neurodegeneration has also been demonstrated. In fact, increased levels of REST were found in the cortex and hippocampus of 3xTg-AD mice (a transgenic mouse model of AD), associated to a reduction of choline acetyltransferase expression in the same areas [69].

3.3 Parkinson disease

REST was also significantly lost in frontotemporal dementia and dementia with Lewy bodies, where it has been found to appear in autophagosomes together with pathological misfolded proteins [70]. This may represent a common pathogenic mechanism that links altered proteostasis to aberrant gene expression. It was demonstrated that the expression of some REST-target genes, like BDNF and tyrosine hydroxylase, is decreased in human dopaminergic cell line after the exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[48, 71]. Moreover, REST neuronal deficient mice are more vulnerable to MPTP toxicity. Experiments performed in vivo, in conditional REST knock-out mice, have demonstrated that these mice developed a significant decline in locomotor behavior compared to wild-type mice and the loss of dopaminergic neurons in the substantia nigra is more severe [72]. This study suggests that REST exerts its protective function also in a model of Parkinson's disease as a mediator for neuroprotection [71]. In contrast, another study demonstrated a reduction of (SCF) ubiquitin ligase activity, which leads REST degradation [56], in PD pathology, that could be involved in neuronal cell death in both the MPTP model and PD patients [73]. It has been show that exposure of human dopaminergic cells to MPTP induced an increase expression of REST and its isoform, REST4 at both transcript and protein levels. In particular, the SCF activity is correlated to an increase expression of REST and consequently with the down-regulation of its target genes. These data indicate that an abnormal expression of REST and REST4 could be involved in the pathological process of PD [48].

3.4 Epilepsy

Several papers have shown an up-regulation of REST after seizure activity, even if its involvement in epileptogenesis remains controversial. It was demonstrated that the conditional knock-out of REST in excitatory neurons leads to an increase in seizure progression and duration and sprouting of mossy fibers in kainate-induced epileptogenesis [74]. As a consequence of REST deletion, the levels of fibroblast growth factor 14 and BDNF were up-regulated in kainic acid-induced status epilepticus, indicating that REST might act on genes involved in the induction of epileptogenesis.

The opposite role of REST in the pathological process was demonstrated in a study where blockade of REST function was associated with an attenuation of the epileptic status, even if the mechanism that induces increased expression of REST, which promotes phenotypic changes in vulnerable neurons, remains to be clarified [63].

3.5 Global ischemia and brain tumors

REST dysregulation could represent both a beneficial compensatory response or a pathological mechanism contributing to neuronal damage. This conflicting role has also been shown in cancer, where REST could act as a suppressor in non-neuronal tumors, or as an oncogene in neuronal tumors, such as neuroblastomas, medulloblastomas, and pheochromocytomas [75]. REST activation can also occur during development in differentiated neurons and in adult neurons in response to pathological stimuli such as seizures and ischemia, but the mechanisms involved in REST expression under these conditions are still unknown. Casein Kinase 1 (CK1), an upstream effector that regulates REST abundance and stability, promoting its proteasomal degradation through phosphorylation has been identified, whose reduction during global ischemia is accompanied by an increase in REST in neurons destined to die, suggesting that loss of CK1 and REST up-regulation are related to neuronal death [76]. Similarly, another study has shown an increase in REST expression associated with CA1 pyramidal neuron cell death [77]. Hence, REST dysfunction appears to be involved in the pathogenesis of several neurodegenerative disorders [78], where the activation state of REST might be related to neuroprotection or neurodegeneration (**Fig. 9**).

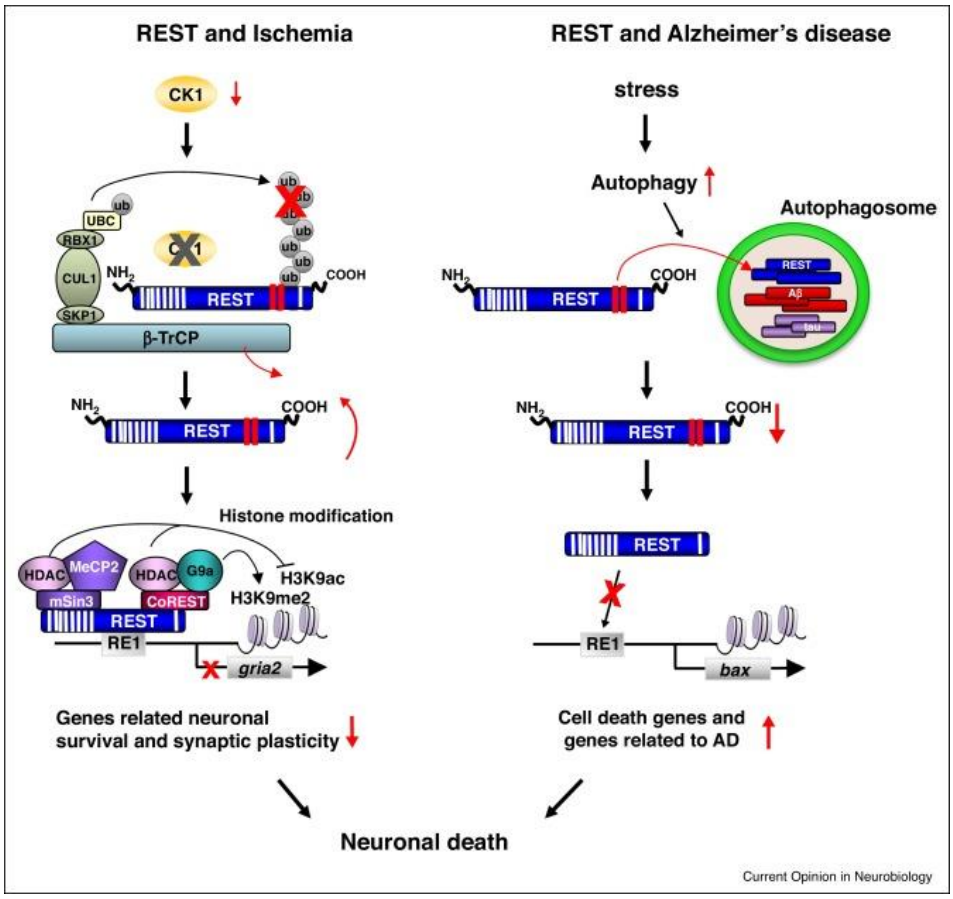


Fig. 9 Regulation of REST in global ischemia and AD. *G*lobal ischemia reduces abundance of CK1 and E3 ligase β-TrCP, resulting in increased REST expression in the hippocampus and repression of gene transcription. In AD REST is engulfed in autophagosomes, together with misfolded proteins Taken from [78].

3.6 Multiple sclerosis

Very recently, REST dysregulation was found, for the first time, in a mouse model of MS. In the disease the continuous attack of infiltrating and resident immune cells in the CNS leads to demyelination and axonal injury, which represents the main contribution for neurological disability. A novel role of the cell adhesion molecule L1 in the crosstalk between the immune and nervous systems was recently reported. Its hypothesis is that the down-regulation of neuronal L1, which involves the transcription repressor REST and whose expression was demonstrated on axons of MS patients and human T cells, is an adaptive mechanism for promoting neuronal self-protection during neuroinflammation [79]. In EAE, CD4+T cells were found associated with degenerating axons in the spinal cord, both expressing L1. In this model, neuronal L1 expression in the spinal cord was reduced, coinciding with up-regulation of REST known to repress L1

transcription. Similar results were found in vitro, where activated CD4+T cells caused contact-dependent down-regulation of L1, up-regulation of its repressor REST and axonal injury in co-cultured neurons [79].

4. Multiple sclerosis

MS is an inflammatory, demyelinating and neurodegenerative disease that attacks the CNS. Evidence from animal models and the early presence of T cells in the typical lesions suggest that MS is caused by an autoimmune attack of both adaptive and innate immune systems on myelin, the fatty substance that surrounds and protects the nerve fibers in the CNS. Astrocytes form multiple sclerotic glial scars in white matter lesions, which gives the disease its name. An early event in the development of disease is believed to be represented by autoreactive lymphocytes that mount aberrant responses against CNS auto-antigens, even if the precise nature of them, however, remains enigmatic.

4.1 Epidemiology

MS affects about 100:100,000 subjects in developed countries, and attacks the CNS. Clinically, most patients exert an initial phase characterized by relapses and remissions (RR) with sub-acute episodes of deficit in CNS functions, usually followed by complete or partial recovery. This phase of disease may evolve into a secondary progressive (SP) course, which is characterized by the establishment of irreversible and slowly progressive disability and a decline in relapse frequency. The progressive form may also occur with these features directly from the onset, but its frequency is low (about 10% of patients) [80]. MS patients typically show the first symptoms between the ages of 20–40 years, and, in particular, women are more affected compared to men in a ratio 2:1. The variety of symptoms, that occur during the disease, are related to the disruption or damage of myelin sheath and nerve fibers, that interrupt the nerve impulses traveling to and from the brain and spinal cord. The heterogeneous pattern of symptoms include sensory and visual defects, motor impairments, fatigue, pain and cognitive deficits [81, 82].

4.2 Aetiology of MS

Several hypotheses about MS aetiology have been proposed, including infectious, vascular or metabolic and they involve both genetic and environmental factors as well as immune dysregulation. It is believed that in individuals with genetic predisposition, the immune dysregulation could be an important factor in developing disease [83-85]. Autoreactive B cells and T cells can be activated in the periphery to become aggressive effector cells by molecular mimicry, novel autoantigen presentation, recognition of sequestered CNS antigen released into the periphery or bystander activation. Naive T cells primed by antigen-presenting cells (APC) such as dendritic cells (DC) can differentiate into T regulatory-1 (Tr1)/T helper type 3 (Th3), Th1, Th2 or Th17 cells depending upon the cytokine environment [86] (**Fig. 10**).

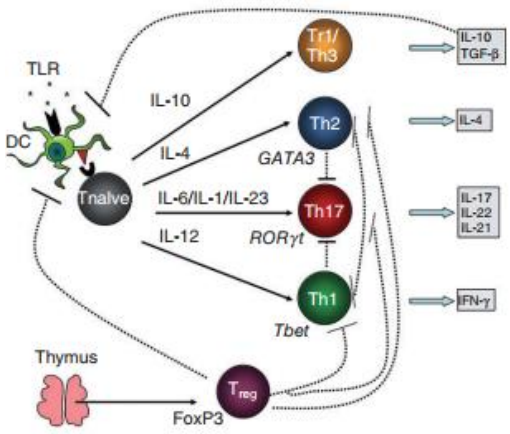


Fig. 10 Naive T cells primed by APC such as DC can differentiate into T regulatory-1 (Tr1)/T helper type 3 (Th3), Th1, Th2 or Th17 cells depending upon the cytokine environment [86].

Once activated, $CD8^+$ T cells, differentiated $CD4^+$ T helper 1 (Th1) and Th17 cells, B cells and innate immune cells can infiltrate the CNS, leading to inflammation and tissue damage.

4.3 Neuropathology

The featured hallmarks of the disease are the inflammatory plaques in the white and grey matter of the brain and spinal cord, with complete or partial loss of myelin, perivascular lymphocyte cuffs, and cell infiltration in the CNS, with a subsequent accumulation of macrophages and astroglial activation. Inflammation, gliosis and axonal injury are additional prominent neuropathological

characteristics, that become evident as the clinical evolution, from intermittent and slow attacks, pass through the progressive form characterized by the worsening of symptoms. So, it has been largely accepted that disease is mediated by pathogenic T cell responses against myelin antigens, followed by a broader neurodegenerative process [86]. The auto-reactive T cells migrate across the BBB in the CNS for mediating neuronal damage against myelin sheaths, in particular, to the axons. The immune cell infiltration promotes inflammation, demyelination, gliosis and neuroaxonal degeneration, leading to disruption of neuronal signaling [87]. Resident inflammatory components in the CNS are involved in the recruitment of innate immune cells and contribute to axonal damage and loss of myelin-producing oligodendrocytes [88-90]. The CNS resident inflammatory cells have been found, predominantly, in demyelinated lesions, where it seems that the number correlates with tissue damage [91]. Although inflammation is constantly present throughout disease, it is more pronounced in acute phase. In the recovery phase, a resolution of the inflammation and oedema in the CNS, can occur, leading to a partial restoration of CNS conduction due to glial response and remyelination. Axons and neurons are partially preserved in early phase of MS, but with ongoing disease, gradual neuroaxonal loss occurs, correlating with disability, brain atrophy accompanied by ventricular enlargement. Axonal loss is irreversible and it is the most important cause for neurological dysfunctions in chronic MS. Early lesions are characterized by invading peripheral immune cells and breakdown of the BBB, with macrophages as the most predominant cells in infiltrates, followed by $CD8^+$ T cells and few $CD4^+$ T cells, B cells and plasma cells. [92]. With disease progression, it is possible to observe a switch in cellular composition of infiltrates with inflammatory T cells and B cells, both microglia and astrocyte activation, and diffuse myelin reduction and axonal injury. Microglia and macrophages remain in a chronic state of activation throughout the disease [93]. All these events are responsible for pronounced atrophy of the grey and white matter [92]. In the progressive phase of disease, it is possible to observe the formation of tertiary lymphoid structures in the meninges, made by

inflammatory aggregates that contribute to cortical demyelination and tissue injury in the later stages [94].

4.4 Immunopathology

Several mechanisms have been proposed as triggering events in the disease, including inflammatory processes, CNS viral infection, genetic risk loci involved in MS susceptibility [human leukocyte antigen (HLA)-DR2 and HLA-DR4 are the most commonly affected] and dysregulation in immune cell response [95]. In particular, several theories support the role of an unbalance in tolerance mechanisms, which reflects a disturbance in the balance between cells contributing to tissue damage (activated effector T-cells) that become resistant to suppressive mechanisms, and cells suppressing the self-reactive T-cells (regulatory T cells). The crosstalk between the innate and adaptive immune systems is involved in immune dysregulation in MS. In fact, immune cells such as dendritic cells (DCs) act also as antigen-presenting cells (APCs), to naïve $CD4^+$ T cells, inducing their proliferation. This process is responsible for the adaptive immune response through the production of cytokines by DCs and lymphocytes [96]. Depending on the extracellular milieu, APC can secrete differentiating cytokines that lead naive $CD4^+$ T cells to follow different pathways. After the differentiation from naïve T cells into pathogenic $CD4^+$ T helper 1 (Th1) cells and Th17 cells in lymph nodes, these cells enter the circulation, and after crossing the BBB, invade the parenchyma (perivascular area) of CNS, where they can exert their effector functions within the CNS [81]. The breakdown of BBB leads also to the migration of other peripheral immune cells (macrophages, B cells) that, in turn, secrete pro-inflammatory cytokines and chemokines [97] which propagate inflammation, lesions formation, demyelination and neurodegeneration [98].

4.5 Neuroinflammation

Infiltrating immune cells, interacting with activated CNS-resident microglia and astrocytes, promote demyelination, oligodendrocyte and neuro-axonal injury, through both direct mechanisms

via cell-cell contact and through the release of soluble inflammatory and neurotoxic mediators, including reactive oxygen species (ROS), glutamate, cytokines and chemokines. ROS and reactive nitrogen species were found in MS and EAE lesions and their oxidation products correlate with inflammation [99-101]. These mediators are produced by endothelial cells, peripheral infiltrating immune cells and by resident glial cells (microglia and astrocytes). In the last years, growing evidence has emphasized the role of microglial and astrocyte activation and proliferation as an important contribution of resident CNS cells in disease progression [102] with oligodendrocyte injury and axonal degeneration [103]. Several mechanisms have been proposed for the contribution of astrocytes to EAE pathology; indeed, astrocytes are a source of cytotoxic factors, and, through the formation of a glial scar, they are active mediators in promoting demyelination, impairing axonal regeneration and contributing to axonal mitochondrial dysfunction [102, 104]. BBB disruption in EAE, followed by T-cell invasion of the CNS parenchyma, is linked also to astrocyte activation [102], representing an early event in lesion development [105]. Moreover, astrocytes represent the main source of the chemokine CCL2 in EAE [106] that act as chemoattractant for monocytes and T cells and has a role in disease onset. On the other side, astrocytes might limit the detrimental effects of pro-inflammatory factors, promoting protection of oligodendrocytes and neurons [107, 108]. In this pathological context, microglia are also activated, proliferating and migrating to the damaged area of the neuronal parenchyma, where it exerts both beneficial and detrimental functions during myelin damage and repair. In fact, microglia cells can release different kinds of mediators, including both neurotoxic (reactive oxygen and nitrogen species and glutamate) and neurotrophic molecules, pro- and anti-inflammatory cytokines or chemokines, and act as local APC [95]. Thus, microglia can regulate different aspects of inflammation, such as repair, cytotoxicity, regeneration, and immunosuppression [109]. In healthy conditions, microglia has a key role in immune response, maintaining the homeostasis and normal function of the CNS, playing an important role in tissue

repair and infection control [88]. Microglia is also involved in the clearance of debris and in synaptic plasticity [110], regulating neuronal activities. The existence of a dual function of microglia cells highlights the different involvement and the opposite role of this cell population in demyelination.

4.6 Demyelination and neurodegeneration

Demyelination is a hallmark of MS, where the myelin sheath is destroyed by the inflammatory process. A widely accepted theory is that MS initiate in brain and spinal cord through T-cell mediated inflammation, followed by demyelination and progressive neurodegeneration driven by both adaptive and innate infiltrating and CNS resident cells [111]. This autoimmune response induces many neuroinflammatory events, leading to the attack of myelin-producing oligodendrocytes and culminating in demyelination followed by axonal damage [112, 113]. It has been shown demyelinating lesions are characterized by the presence of T-cell infiltration and macrophage/microglia inflammation [95]. In fact, microglia express CD74, CD40, CD86, CD80, and the chemokine receptor CCR7 at high levels [114], and are considered harmful, contributing to oligodendrocytes damage and releasing neurotoxic factors. Oxidative stress, mitochondrial injury and subsequent ion channel dysfunction, caused by ongoing chronic neuroinflammation seem to have a constant impact on neurons and axons, amplifying demyelination and neurodegeneration through energy deficiency and hypoxia [115, 116]. In particular, ion channel dysregulation seems to play a pivotal role in neuronal and axonal injury during MS. Ion channels change their distribution in neurons and relocalize from the somata and dendrites in healthy individuals to axons in MS and EAE as a compensatory response to the perpetuation of inflammatory stimulus to counteract altered conductance and loss of axonal integrity [117]. This process eventually becomes a maladaptive response contributing to neuro-axonal injury (**Fig. 12**). With disease progression, the intensity of inflammation is reduced, but oxidative injury and

mitochondrial damage are aggravated by additional factors, ultimately leading to neuronal death [118].

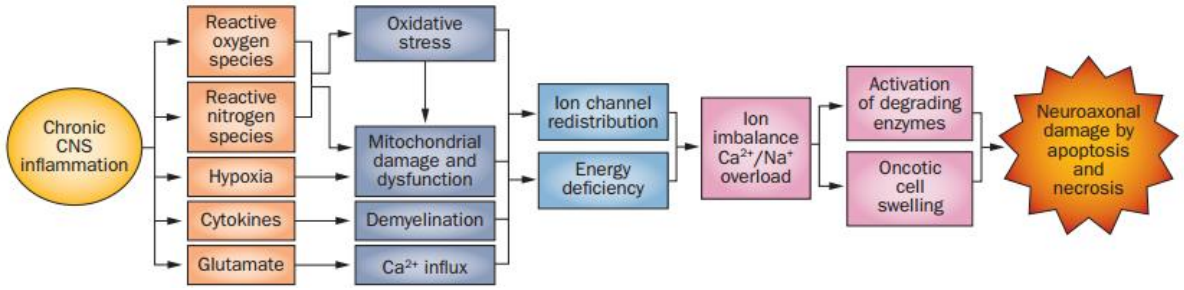


Fig. 12. Cascades leading to inflammation-induced neuroaxonal injury. The scheme illustrates the hypothetical sequence of events eventually leading to neuroaxonal degeneration in multiple sclerosis. Chronic CNS inflammation lies at the root of dysregulation of neuronal and axonal metabolism. The cascade culminates in the hallmarks of inflammation-induced neurodegeneration. Taken from [117].

In fact, although these alterations could exert a transient beneficial role, prolonged changes seem to be deleterious and accelerate neuronal degeneration, that is probably mediated by the initiation of apoptosis and Wallerian degeneration. Neuronal and axonal degeneration in MS is a slow process initiated by acute lymphocytic inflammation, and subsequently driven by chronically smoldering, diffuse parenchymal myeloid and meningeal lymphocytic inflammation. There are several stressors that control inflammation including electrons, protons and oxidants, whose effect disturb neuro-axonal metabolic pathways [119], with a central role for mitochondrial damage and glutamate metabolism. These events lead to energy deficits and Ca^{2+} overload in the neurons. The accumulation of Ca^{2+} initiate a vicious cycle that activates degrading enzymes, disturbs mitochondrial function with a consequent reduction of energy and compromises the axonal transport. The perpetuation of these phenomenon finally leads to death by apoptosis or necrosis of neuronal cells [120].

4.7 Animal model of MS: Experimental autoimmune encephalomyelitis (EAE)

EAE is a demyelinating disease of the CNS that shares clinical and pathological features with MS and is an useful model for the human disease [86]. A milestone to confirm the central role of

immune system in the pathological process and demonstrate that it is a T-cell mediated disease was achieved by Ben-Nun, Cohen and Wekerle who demonstrated that the adoptive transfer of MBP-specific T-cell lines induced EAE in naıve syngeneic recipients [121]. EAE is induced in susceptible animals by immunization with one of a number of myelin antigens emulsified in complete Freund's adjuvant (CFA) [122] that drives a myelin-specific T cell response in the periphery. After EAE induction, DC are activated in the lymph nodes by Toll-like receptor (TLR) agonists within the mycobacterium tuberculosis component of CFA, and present myelin antigen to naive T cells. The activated auto-reactive T cells home to the CNS, and egress into the parenchyma through a disrupted BBB [123, 124]. Upon entering the CNS, the T cells are reactivated by local microglia and infiltrating activated APC which present major histocompatibility complex (MHC) class II-associated peptides (**Fig. 13**).

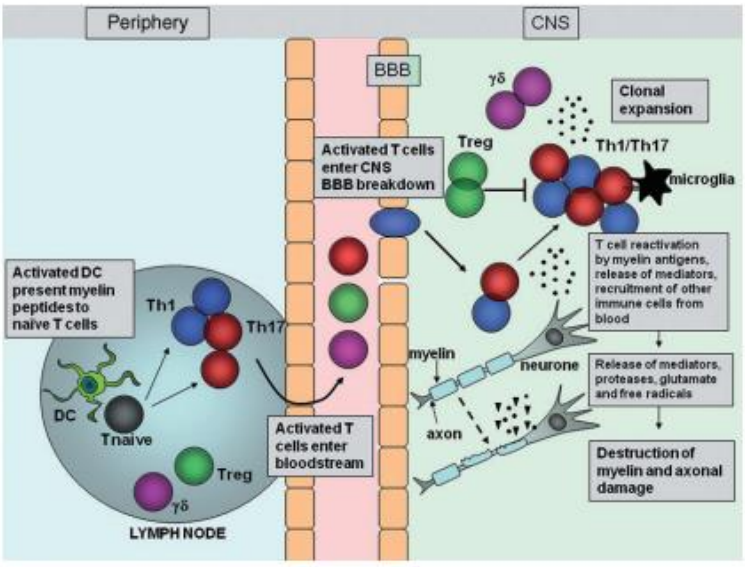


Fig. 13 The activated myelin-specific T cells enter the bloodstream and traffic to and enter the CNS. Breakdown of the BBB occurs, allowing recruitment of other inflammatory cells into the CNS. T cells entering the CNS encounter their cognate myelin antigens and become reactivated by local APC. T cells expand and release inflammatory mediators which help recruit other immune cells to the site of inflammation, resulting in subsequent inflammatory processes and eventually in demyelination and axonal damage. Taken from [86].

Activation of local microglial cells and infiltrating cells results in production of proteases, glutamate, reactive oxygen species and other cytotoxic agents, which promote myelin breakdown.

Neuroinflammation has an impact on synaptic excitability and consequently on neuronal functions, since pro-inflammatory and anti-inflammatory cytokines are involved in the cross-talk between neurons and glial cells within the CNS [125-127]. Since glial cells act as sensors of the CNS environment and secrete cytokines, growth factors and/or neurotransmitters, they might contribute to synaptic dysfunction in the development of disability in MS. In fact, synaptic dysfunction is due in part to axonal or neuronal damage, but also to chronic inflammation. Such dysfunction reflects alteration of, and unbalance between, both glutamatergic and GABAergic transmission in the brain and spinal cord, which is detrimental for both motor and cognitive functions. The parallel increase in glutamate transmission and the reduction in GABAergic signaling results in synaptic hyperexcitation and, possibly neurodegeneration [128]. Depending on the animal model used, damage to the myelin sheath surrounding axons can be associated with axonal damage [86]. At the clinical level, EAE presents as ascending paralysis. The caudo-rostral paralysis is often accompanied by body weight loss 2-3 days before disease onset around day 11 after encephalitogenic challenge. Motor deficits are scored on a scale from 0-5 based on the degree of ascending paralysis and correspond to the following deficits: 0 = no symptom; 1 = flaccid tail; 2 = ataxia; 3 = hindlimb paralysis; 4 = hindlimb and forelimb paralysis; 5 =moribund [129-131]. Disease expression at both clinical and neuropathological levels is influenced by the selected autoantigen, species and the genetic background. Depending upon the immunization protocol and background of mice used, EAE can take an acute, chronic progressive or relapsing-remitting course [132]. EAE can also be induced by adoptive transfer of activated myelin-specific CD4+ T cells from mice with EAE into naive recipient mice (**Table 1**) [133, 134].

Model	Similarities to human disease	Differences from human disease	Further comments
Lewis rat Active EAE (CNS myelin, MBP, MOG, PLP)	T-cell inflammation and weak antibody response	Monophasic, little demyelination	Reliable model, commonly used for therapy studies. With guinea-pig MBP little demyelination
Adoptive-transfer EAE (MBP, S-100, MOG, GFAP)	Marked T-cell inflammation. Topography of lesions	Monophasic, little demyelination	Homogeneous course, rapid onset. Differential recruitment of T cells/macrophages depending on autoantigen
Active EAE or AT-EAE + co-transfer of anti-MOG antibodies	T-cell inflammation and demyelination	Only transient demyelination	Basic evidence for role of antibodies in demyelination
Congenic Lewis, DA, BN strains Active EAE (recombinant MOG aa 1–125)	Relapsing–remitting disorders, may completely mimic histopathology of multiple sclerosis and subtypes	No spontaneous disease	Chronic disease course, affection of the optic nerve, also axonal damage similar to multiple sclerosis
Murine EAE (SJL, C57BL/6, PL/J, Biozzi ABH) Active EAE (MBP, MOG, PLP and peptides)	Relapsing–remitting (SJL, Biozzi) and chronic-progressive (C57BL/6) disease courses with demyelination and axonal damage	No spontaneous disease	Pertussis (toxin) required for many strains, whilst it is often not needed for SJL and some Biozzi EAE models. Higher variability of disease incidence and course, often cytotoxic demyelination in C57BL/6. With rat MBP inflammatory vasculitis with little demyelination
Murine EAE in transgenic mice or knockout mice (mostly C57BL/6 background)	Specifically addresses role of defined immune molecules/neurotrophic cytokines/ neuroanatomical tracts	Most results obtained with artificial permanent transgenic or knockouts	Extensive backcrossing (>10 times) on C57BL/6 background required. Future work with conditional (cre/loxP) or inducible (e.g. Tet-on) mutants

Table 1. Rodent and primate models of EAE. Taken from reference [134].

EAE can be induced in different species, although rodents are the most common animals used for this model, representing a useful tool for understanding the mechanisms potentially involved in MS pathology [135]. Although there are many differences between EAE models and MS that highlight limitations of animal models when applied to human disease [134, 136-138], it should be noted that EAE has been crucial in the development of new approaches to therapy for MS, including Glatiramer Acetate [139] and Natalizumab [140]. Thus, the usefulness of EAE is dependent on the use of the appropriate models for answering the specific questions that are being addressed. The most commonly used mouse EAE models include relapsing-remitting EAE induced with PLP139-151 (Proteolipid Protein) peptide in SJL mice and chronic EAE induced with MOG35-55 (Myelin Oligodendrocyte Glycoprotein) peptide in C57/BL6 mice, which is associated with axonal damage [141]. **Table 1** presents a recapitulation, albeit not exclusive, of murine EAE models. These studies suggest that there is a pool of CNS autoantigens that can initiate an encephalitogenic T-cell response in EAE. In MS, T cells that recognize epitopes within these encephalitogenic myelin antigens have been demonstrated [142], suggesting that these myelin proteins could be processed within the CNS to provide epitopes that can be presented by the host's

class II MHC molecules. The importance of axonal injury as a component of the MS lesion was rediscovered in the late 1990s having been first described almost a century earlier [143-145]. Experimental studies soon showed parallel findings in EAE [146, 147] and identified specific molecular abnormalities such as redistribution of ion channels on chronically demyelinated axons that may play an important role in the axonal pathology of MS [148]. Changes in intra-axonal ionic homeostasis due to altered or aberrantly expressed ion channels may indeed result in axonal damage in EAE, as axonal loss is significantly reduced by pharmacological agents that reduce sodium and calcium transport/entry across the axolemma [149, 150]. However, axonal loss, demyelination and inflammation in MS are intimately inter-related and therapeutic strategies must take this into consideration. Inflammatory mediators such as nitric oxide are per sé deleterious to axonal function, and this is compromised further by demyelination that not only results in acute electrophysiological dysfunction but also increases susceptibility to inflammatory mediators and reduces long-term axonal survival by disrupting axonal/glial interactions. EAE provides an important tool to investigate how the interplay of these neurobiological and immune-mediated mechanisms results in axonal injury and ultimately in degeneration. Activated macrophages and microglia cells are the obvious suspects responsible for mediating this pathology, both producing a large number of deleterious soluble factors, which can induce functional blockade and/or structural damage in axons in vitro. Nitric oxide, possibly in combination with reactive oxygen species, is one important candidate [151], but other molecules including excitotoxins [152] and proteases [153] may play equally important roles. These molecules are all involved in disease pathogenesis but it should not be forgotten that inflammatory cells including macrophages also produce neurotrophic factors such as BDNF that may provide a neuroprotective function [154]. The clusters of microglia are associated with oligodendrocyte stress and to a lesser extent with myelin damage, leukocyte infiltration and BBB leakage [155]. These pre-active lesions represent the first stage of lesion formation, even if not all of them develop directly into active demyelinating

lesions [155]. Microglia cells present in pre-active lesions express cytokines such as tumor necrosis factor (TNF), IL-10, and IL-23 [87, 156]. However, preactive lesions are not associated with BBB disruption, suggesting that an intrinsic trigger represented by activated microglia induces lesion formation, rather than infiltrating immune cells [87, 155]. EAE remains an essential tool for preclinical studies and for the understanding of mechanisms underlying T-cell mediated immune damage of the CNS and the associated effector cascade of innate immunity. This animal model has greatly contributed to current knowledge of mechanisms involved in inflammation-induced neurodegeneration and immune response in MS [135]. An EAE model most frequently used is induced in mice by immunization with $MOG_{35–55}$ peptide in CFA [157], resulting in an acute or chronic inflammatory disease in the spinal cord and brain with axonal injury, depending on the genetic background of the animals. Moreover, MOG-induced EAE is also used for studying molecular mechanisms involved in neuroinflammation crucial for pathogenesis. Therefore, I have used MOG-induced EAE in my PhD project to investigate REST dysregulation in the context of neuroinflammation occurring during EAE and monitoring the underlying signaling pathways involved in its regulation.

5. Rationale and aims

The transcriptional repressor REST has a key role in the development and maturation of the CNS through a coordinated program of gene expression modulation for ensuring the specific neuronal phenotype and function. While in health conditions, REST is expressed at low levels and is quiescent in mature neurons, pathological stimuli can lead to REST dysregulation with relevant impact on the expression on its target genes, as occurring in several neurodegenerative disorders. Hence, modification of molecular pathways involved in neuronal development could represent an appealing strategy towards therapy for neurodegenerative diseases such as MS. We have hypothesized that dysregulation of REST and the repercussion on its neuronal targets, can occur also in EAE. In a pilot study, we have found that REST is significantly over-expressed in the spinal cord of EAE mice, one day after disease onset, indicating that REST-dependent network is markedly dysregulated in CNS of EAE mice, a response possibly resulting from neuronal over-expression. Such dysregulation could be considered as a causative factor for the disease or, conversely, the expression of a protective tissue reaction. In the former case, a therapeutic approach would be to inhibit REST expression/function, while in the latter REST function would need to be strengthened. In both cases, REST appears to be a very promising target for interfering with neuronal fate during neurodegeneration. Our overall goal is to assess the role of REST dysregulation in neurodegeneration occurring in CNS of EAE mice and attempt to modulate its expression and activity in order to halt neuronal loss and to foster tissue repair. Accordingly, we aimed at:

i) characterize the role of REST unbalance in EAE, as a pathogenic mechanism directly contributing to neurodegeneration, or an endogenous neuroprotective response. We will characterize cell-specific REST over-expression in EAE mice and the repercussion on the expression of its target genes in the context of EAE, and decipher underlying pathways.

ii) evaluate the potential of REST as a biomarker for monitoring disease progression. We will evaluate the temporal profile of REST over-expression to understand in which disease phase it is dysregulated and which CNS area(s) is/are affected.

ii) analyze the signaling transduction pathways that trigger REST over-expression and are involved in its regulation. We will use a neuronal-like cell line for studying the involvement of these pathways in vitro under inflammatory conditions mimicking EAE microenvironment.

These studies will establish whether REST dysregulation and its repercussion on neural excitability and synaptic transmission are involved in the pathological process of EAE with the perspective to consider it as a potential molecular target for neurodegenerative diseases therapy, potentially for MS.

6. Material and methods

6.1 Mice

Wild-type C57Bl/6J mice were purchased from Charles River (Calco, Italy). All mice were housed in pathogen-free conditions with food and water ad libitum. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Decreto Legislativo 4 marzo 2014, n. 26, legislative transposition of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). The research protocol was approved by the Italian Ministry of Health (Approval No. 384).

6.2 EAE induction

Chronic EAE was induced in female mice (6–8 weeks of age, weighing 18.5 ± 1.5 g) by subcutaneous injection at two different sites in the right and left flanks with an emulsion (300 µl total) containing 200 µg myelin oligodendrocyte glycoprotein peptide spanning amino acids 35–55 (MOG35–55) (Espikem) in incomplete Freund's adjuvant (Sigma-Aldrich) supplemented with 1200 µg *Mycobacterium tuberculosis* (strain H37RA; Difco). Mice were injected in the tail vein with 400 ng pertussis toxin (Sigma-Aldrich) in 100 µl of phosphate buffer saline solution (PBS, pH 7.6) immediately, and 48 h after the immunization. The mice were scored daily for clinical manifestations of EAE on a scale of 0–5 [130]. Body weight and clinical score (0, healthy; 1, limp tail; 2, ataxia and/or paresis of hindlimbs; 3, paralysis of hindlimbs and/or paresis of forelimbs; 4, tetraparalysis; 5, moribund or death) were recorded daily.

6.3 Cell culture and treatments

Murine neuroN2a (N2a) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), and antibiotics (penicillin and streptomycin, 10 000U/ml), in a humidified 5% CO2 atmosphere at 37 °C. All reagents for in vitro experiments were provided by Gibco. N2a cells were seeded in 24-well plates (3×10^5) or 6-well plates (1×10^6) in 1 or 2 ml of DMEM complete. As pro-inflammatory stimulus we used the supernatant of T cells stimulated or not with antiCD3/CD28 for 48 hours, which is known to result

in a Th1 phenotype. The stimulus was added in cell culture at a ratio 1:3 for different times. For differentiation, N2a cells were seeded at 60% confluence, and culture medium was replaced after 24 h with DMEM + 1% FBS + 20 μM retinoic acid (RA). Differentiating medium was replaced every 24h, for 3 days.

6.4 Preparation of activated T-cell supernatant

For the preparation of activated T-cell supernatant, mice were sacrificed for aseptically removing spleens or lymph nodes from donor. Splenocyte suspensions were prepared by mechanically teasing the spleen of naive mice with the blunt end of a 10 ml plastic syringe plunger in a Petri dish, then passing suspensions through a BD Falcon 70 μm cell strainer (Falcon) to remove debris, and finally suspending the cells in 10% FBS, Roswell Park Memorial Institute 1640 (RPMI, Gibco), glutamine (2mM), antibiotics (penicillin and streptomycin, 10 000U/ml), and 50 μM β-mercaptoethanol. Cell suspension was prepared from lymph node isolated from MOG35-55-immunized mice at 9 days post-immunization with the same procedure mentioned above for splenocytes. The cells were seeded (2×10^5cells/well or 1×10^6cells/well) in 96-well flat-bottom or 24-well plates respectively, in the presence or absence of anti-CD3/CD28 (Biolegend) at a concentration of 1mg/ml for 48 hours and supernatants were collected and stored frozen at -20°C until use.

6.5 ELISA

Cytokines were evaluated in supernatants from T cells stimulated or not with anti-CD3/CD28 for 48h by ELISA Standard Set kits (IL2, IL-4, IL-10, IL-17, TNFα and IFN-γ ELISA MAX, Biolegend). Briefly, 96-well ELISA plates coated overnight with appropriate capture antibodies at 4 C were washed with PBS supplemented with 0.1 % Tween 20 and blocked with PBS containing 5 % FBS for 1 h at room temperature. Supernatants of cell cultures (100 μl) were added and the plates were incubated for 2 h at room temperature. Plates were washed and incubated with the relevant horseradish peroxidase-coupled detection antibodies for 1 h at room temperature. The

plates were washed, substrate (3,3′,5,5′tetramethylbenzidine, Sigma-Aldrich) was added, and the plates were developed for 20–30 min. The reaction was stopped with 2 N H_2SO_4 and plates read at optical density (OD) 450 nm on a Multilabel Victor3 reader (Perkin Elmer).

6.6 RNA extraction and Real-time PCR

Total RNA was isolated from N2a cells and from EAE and naive mouse CNS tissues using Qiazol reagent (Qiagen) according to the manufacturer's instructions, and quantified by Nanodrop. First Strand cDNA were generated from 500 ng or 1 µg RNA using the QuantiTect Rev. Transcription kit (Qiagen). Real-time PCR was performed using a LightCycler 480 (Roche) with a final reaction volume of 20 µl containing 25 ng cDNA, 1 µl of each primer pair (10 µM), and 10 µl of LightCycler FastStart Essential Green Master Mix (Roche). Thermal cycling conditions comprised an initial step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 5 seconds, 60°C annealing/extension temperature for 10 sec. All samples were run in triplicates or duplicates. The specificity of the PCR reactions was confirmed by melting curve analysis. The threshold cycle (CT), defined as the fractional PCR cycle number at which the fluorescence reaches 10 times the baseline standard deviation, was compared for the expression. The ΔCT method was used to evaluate the relative expression ratio for all genes compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used for normalizing the expression data. The primer pairs for the indicated genes were synthetized by Tib Molbiol (Sequences are shown in **Table 2**).

GAPDH
for: 5'- AAT CTC CAC TTT GCC ACT GC
rev: 5'- ATG GTG AAG GTC GGT GTG A
REST
for: 5' - TTC ACA TTT ATA CGG GCG TTC
rev: 5' - CCT GCA GCA AGT GCA ACT AC
REST full-length
for: 5'-ACCACTGGAGGAAACACCTG-3'
rev:5'-ATGGCTTCTCACCTGAATGAGTC-3'
REST4
for: 5' - ACG CCC GTA TAA ATG TGA ACT T
rev: 5'- ACC CAG CTA GAT CAC ACT CTG
Nav1.2

for: 5’ - GGC TCT GCT GCT ATT GTT GGT A

rev: 5’ - GAA GGC TAG GTG AGT ACA TCC C

Syn

for: 5’ - ATC TTC CTC CAA CCT CCA

rev: 5’ - TTT GCT TCC CGA CTC TTC

SST

for: 5’ - CTG AAG GAG ACG CTA CCG AA

rev: 5’ - GTA CTT GGC CAG TTC CTG TTT

NMDArI

for: 5’ - AAA CCA GGC CAA TAA GCG AC

rev: 5’ - GCG TAG ACC TGG CTA GAG AT

Table 2. Primer pairs for PCR analysis of selected genes

6.7 Nanostring analysis

nCounter® gene expression assay (NanoString Technology) was used for transcriptional analysis of REST network, A total of 80 genes were selected, including REST-target (RE1-containing) and non-target (RE1-negative) genes. The complete list of the selected genes, including accession numbers and targeted sequences, is shown in Table 1. We analyzed total mRNA extracted from spinal cord and striatum of EAE (4 dpo) and naïve mice. For each gene, we expressed the results as fold change EAE/naïve in a color-coded fashion, where blue/red colors correspond to genes that are respectively less/more expressed in EAE samples compared to naïve. Optimal sequences were designed on the chosen genes by Nanostring Technologies. **Table 3** shows the following for each gene: (**i**) the gene name; (**ii**) the accession number; (**iii**) whether it is a REST-target gene (YES/NO) or a housekeeping (HK) gene; the position (**iv**) and the sequence (**v**) of the targeted region.

Gene Name	Accession #	REST-Target	Pos ition	Target sequence
Map2k2	NM_023138.4	NO	2268-2367	CACATTCCTAAATACTAGGAAGGCTGAGTCGGGAAAACGACAGGTTTTGGGCCACTGTGGGCTACCTAGTGAGAATG TCTTACATCATGGAAATGGTGCA
Gtf3c1	NM_207239.1	NO	4587-4686	ACCAACGGCATGCTAGACCAGCCTGATCATTTTTCTTTCAAGGACCTGGATAGCAGTGACCCTTCAAATGACCTGGTG GCATTTTCTTTGGACAGCCCTG
Tuba1a	NM_011653.2	NO	1379-1478	GGGAGGAAGAAGGAGAGGAATACTAAATTAAATGTCACAAGGTGCTGCTTCCACAGGGATGTTTATTGTGTTCCAACA CAGAAAGTTGTGGTCTGATCAG
Camkk2	NM_145358.2	NO	1321-1420	AAAGGCGTTGGATGTTTGGGCCATGGGTGTGACGCTGTACTGCTTTGTCTTTGGCCAGTGCCCTTTCATGGATGAAC GAATCATGTGTTTGCACAGTAAG
Syt17	NM_138649.1	NO	1391-1490	AACTGGAAAACGCCAGCCTAGTATTCACAGTGTTCGGCCACAACATGAAAAGCAGCAATGACTTCATCGGGAGGATC GTCATCGGCCAGTATTCCTCCGG
Ubc	NM_019639.4	NO	22-121	GCGGAGTCGCCCGAGGTCACAGCCCTGCCCTCCCACACAAAGCCCCTCAATCTCTGGACGCCACCGTGAAACAACT CCGTGAGAGAGACGATGCAGATCT
Gpi1	NM_008155.3	NO	2676-2775	CAGGGATGAACTCGATTATACCCTTCAAAAGACAGTTTTAGCAGGGTGTCATTACTGTCACATTCATATCCTGGCTATC AGTGAGTCCTTGTGAGACTGA
Map3k5	NM_008580.4	NO	641-740	CATCATCCTCTACTGCGATACTAATTCCGATTCACTCCAGTCCCTGAAGGAAATTATTTGCCAGAAGAATACTGTGTGC ACCGGGAACTACACCTTCATC
Rcan1	NM_001081549.1	NO	1061-1160	ACACAAGGACACTGGGGACATCCTGAGAAAACTGATAGTTCTTGTAATTGCTCATTTCTAGGTTCTGTTTTTGGCAAG GACAGGTTGACTGGTGGCCCAG
Tbx21	NM_019507.1	NO	626-725	CACTAAGCAAGGACGGCGAATGTTCCCATTCCTGTCCTTCACCGTGGCTGGGCTGGAGCCCACAAGCCATTACAGG ATGTTTGTGGATGTGGTCTTGGTG
Tdo2	NM_019911.2	NO	496-595	AGAGTCTACAGTTCCGGCTGCTGGAAAATAAGATTGGTGTTCTTCAGAGCTTGAGAGTCCCTTACAACAGGAAACACT ATCGTGATAACTTTGGAGGAGA
Gapdh	NM_001001303.1	HK	891-990	AGGTTGTCTCCTGCGACTTCAACAGCAACTCCCACTCTTCCACCTTCGATGCCGGGGCTGGCATTGCTCTCAATGAC AACTTTGTCAAGCTCATTTCCTG
Hdac3	NM_010411.2	HK	1026-1125	GGCCATTAGTGAGGAACTTCCCTATAGTGAATACTTCGAGTACTTTGCCCCAGATTTCACACTCCATCCAGATGTCAG CACCCGCATCGAGAATCAGAAC
Hprt	NM_013556.2	HK	31-130	TGCTGAGGCGGCGAGGGAGAGCGTTGGGCTTACCTCACTGCTTTCCGGAGCGGTAGCACCTCCTCCGCCGGCTTC CTCCTCAGACCGCTTTTTGCCGCGA
Pgk1	NM_008828.2	HK	37-136	CCGGCATTCTGCACGCTTCAAAAGCGCACGTCTGCCGCGCTGTTCTCCTCTTCCTCATCTCCGGGCCTTTCGACCTC ACGGTGTTGCCAAAATGTCGCTT
Ppia	NM_008907.1	HK	391-490	CCAAGACTGAATGGCTGGATGGCAAGCATGTGGTCTTTGGGAAGGTGAAAGAAGGCATGAACATTGTGGAAGCCAT GGAGCGTTTTGGGTCCAGGAATGG

Gene Name	Accession #	REST-Target	Position	Target sequence
Stat1	NM_009283.3	YES	1591-1690	ACGCTGGGAACAGAACTAATGAGGGGCCTCTCATTGTCACCGAAGAACTTCACTCTCTTAGCTTTGAAACCCAGTT GTGCCAGCCAGGCTTGGTGATTGA
Nfasc	NM_001160316.1	YES	4936-5035	CTCTTCGTATGCCTTATACAGCTCGGATCTAACCCTGCGGTTCCCAGATCCCTAGCCCTATTCTGCAAGTCTTGATG CCTCCAGTGATGTGTGTCTCGGG
Nrxn1	NM_177284.2	YES	721-820	AGATGCCCTCACTTAAACATCATCGGATGCAAATGGATCAGTGATCGCTCTTGAGCCTCGGTGGCCCTCTTTTTCA GAACGTTGCCTCCAAAGTGTATCC
Grin2b	NM_008171.3	YES	6341-6440	GGGAAAGCTCTTCGTATAAGGCTTTGTGAAAGAGCCATTACAGTAGGGTGAGAGAGGGGGATGTTTTTAGTCATTA ACGGTAGGGTTAGTGAGAAAGGGG
Cacna1h	NM_021415.4	YES	3213-3312	ATCGTCACTGTGTTTCAGATCTTGACACAGGAAGACTGGAATGTGGTTCTTTACAACGGCATGGCTTCCACCTCGTC CTGGGCTGCCCTTTACTTTGTGG
Syp	NM_009305.2	YES	733-832	TGTTTGGCTTCCTGAACCTGGTGCTCTGGGTTGGCAACCTATGGTTCGTGTTCAAGGAGACAGGCTGGGCCGCCC CATTCATGCGCGCACCTCCAGGCGC
Grin1	NM_008169.2	YES	493-592	ACAGATGGCCCTGTCAGTGTGTGAGGACCTCATCTCTAGCCAGGTCTACGCTATCCTAGTTAGTCACCCGCCTACT CCCAACGACCACTTCACTCCCACC
Cacna1i	NM_001044308.2	YES	9301-9400	TGCCCTGGCTTATTCCTGTCTTCGGCTCTGGTCCTGGCTTTCCCTCAGAGGAGGATGAACGAATCATGAGCAGTAC TTACCTGTCTGCTTGTCCCTAATA
Calb1	NM_009788.4	YES	344-443	ATGGAAAAATAGGAATTGTAGAGTTGGCTCACGTCTTACCCACAGAAGAGAATTTCTTGCTGCTCTTTCGATGCCAG CAACTGAAGTCCTGCGAGGAATT
Aplp1	NM_007467.3	YES	2301-2400	CTGGGTCCCAGGTATGTATGTCACTCCCTGGAATTCACCATCCCACGTTTCTTCACTAACATCCCAATAAAGTCCTC TTTCCCACCCGGCCAAAAAAAAA
Syt4	NM_009308.3	YES	1121-1220	TCTGGTCTCTCTCTGTTATCAGTCCACTACAAACACGCTCACTGTGGTGGTCTTAAAAGCGCGGCACCTACCGAAAT CTGATGTGTCTGGACTTTCAGAT
L1cam	NM_008478.3	YES	3561-3660	TGAAGACTAATGGAACTGGCCCTGTGCGAGTTTCTACTACAGGTAGCTTTGCCTCCGAGGGCTGGTTCATCGCCTT TGTCAGCGCTATCATTCTCTTGCT
Sst	NM_009215.1	YES	47-146	CCTGCGACTAGACTGACCCACCGCGCTCCAGCTTGGCTGCCTGAGGCAAGGAAGATGCTGTCCTGCCGTCTCCAG TGCGCCCTGGCTGCGCTCTGCATCG
Neurod1	NM_010894.2	YES	565-664	CCTGCGCTCAGGCAAAAGCCCTGATCTGGTCTCCTTCGTACAGACGCTCTGCAAAGGTTTGTCCCAGCCCACTACC AATTTGGTCGCCGGCTGCCTGCAG
Bdnf	NM_007540.4	YES	3261-3360	AGTCCCGTCTGTACTTTACCCTTTGGGGTTAGAAGTCAAGTTGGAAGCCTGAATGAATGGACCCAATGAGAACTAG TGTTAAGCCCATTTCCCTAGTCAG
Penk	NM_001002927.2	YES	1011-1110	CGCTTTGCTGAGTCTCTGCCCTCCGATGAAGAAGGCGAAAATTACTCGAAAGAAGTTCCTGAGATAGAGAAAAGAT ACGGGGGCTTTATGCGGTTCTGAA
Glra3	NM_080438.2	YES	757-856	GGACTTGCGGTACTGCACTAAACACTACAATACAGGAAAGTTTACATGCATAGAAGTGCGATTCCATCTTGAGCGTC AGATGGGCTATTACTTGATCCAG
Gabrg2	NM_177408.5	YES	1614-1713	TTAGAACAAGAGCTGTTACACTGAGCAAGATACCTTTGAGCAACAGCAATGAAAACAGTGGAAGCTGGGAGGGTTT AAAGTGGCATTATCAGTCTTTGAC
Oprm1	NM_001039652.1	YES	1197-1296	GATTCCAGAAACCACTTTCCAGACTGTTTCCTGGCACTTCTGCATTGCCTTGGGTTACACAAACAGCTGCCTGAACC CAGTTCTTTATGCGTTCCTGGAT
Cacng2	NM_007583.2	YES	645-744	GACGCGGACTACGAAGCTGACACCGCAGAGTATTTCCTCCGGGCCGTGAGGGCCTCGAGTATCTTCCCGATCCTG AGTGTGATCCTGCTTTTCATGGGTG
Nrxn3	NM_001198587.1	YES	6111-6210	CTCAAAATCTACCATGGCATTCACTCCATGTAGCAGGTTGTGGTGTCTCTAGAACCAATTGTTAGTTTCCTAATGCTT TGGTGAACCCATGTGGGATGAC

Table 3: Sequences of RE1 and non-RE1 genes analyzed by NanoString

6.8 Confocal microscopy analysis

N2a cells were seeded onto RNase-free, Poly-D-lysine-coated chamber slides **(**Millicell® EZ SLIDES Merck-Millipore). The cells were differentiated with retinoic acid, replacing the differentiation medium every 24 hours for three days. Supernatant from unstimulated naïve splenocytes or MOG35-55-primed lymph node cells stimulated with anti-CD3/CD28 was added for different time points in a humidified 5% CO2 atmosphere at 37 °C. Then, the supernatant was removed and the cells were washed with PBS. Differentiating N2a cells were fixed with 4% formaldehyde and permeabilized using 0.5% Triton X-100. Multiple immunostainings were carried out with the following primary antibodies: anti-Casein Kinase 1 δ (CK1 δ) (1:250, ThermoFisher, PA5-32129), anti-beta Catenin (1:1000, ThermoFisher, PA5-19469), anti-Phospho p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:250, Cell Signaling, #9101), anti-Neurofilament 200KDa clone N52, a marker of high molecular weight neurofilaments (1:500, Merck-Millipore, MAB-5266). The cells were then stained with secondary species-specific antibodies conjugated to

Alexa-488 or Alexa-594 (Invitrogen, Waltham, MA), and counterstained with DAPI to reveal nuclei. After washes, they were mounted with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Images were acquired at an SP8 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with 63x objective. Images were visualized and processed by using the Leica LAS X, ImageJ software programs.

6.9 Western blotting on tissue lysates

Dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed according to standard procedures. Spinal cord tissue was lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate) supplemented with proteases and phosphatases inhibitors (complete EDTA-free protease inhibitors, Roche Diagnostic; serine/threonine phosphatase inhibitor and tyrosine phosphatase inhibitor, Sigma) and equal amounts of proteins were loaded, as determined by BCA assay (Thermo Scientific). Samples were separated on 7-10% SDS polyacrylamide gels and proteins transferred to a nitrocellulose membrane with 0.2 μm pore size (GE Healthcare). Membranes were washed in TBS containing 0.1% Tween (TBST) and blocked with 5% BSA in TBST buffer for one hour at room temperature (RT). The membranes were incubated overnight at 4°C in a humidified chamber with primary antibodies diluted in blocking solution. Primary antibodies used: anti-REST 1:1000 (#07-579, Millipore), anti-REST4 1:1000 (homemade, kindly gifted by Dr. Uchida, Yamaguchi University Graduate School of Medicine); anti-Calnexin 1:5000 (#ADI-SPA-860, Enzo Life Sciences). Membranes were washed 3 times in TBST. Appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies were diluted in blocking solution and incubated for one hour at RT. Membranes were washed 3 times in TBST and antigen/antibody reactions were detected using the ECL™ Western Blotting Detection Reagents (GE Healthcare). Images were acquired via the ChemiDoc MP System (BioRad).

6.10 Western blotting on cell lysates

N2a cells were lysed in RIPA buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein samples (30 μg) were electrophoresed on a 4–12 % gradient polyacrylamide pre-cast gel (Life Technologies), using Bolt® Mini Gel Tank (Life Technologies) and transferred to nitrocellulose membrane (BioRad) using XCell II™ Blot Module (Life Technologies). Membranes blocked with 5 % bovine serum albumin (BSA) in TBS/0.1 % Tween 20 were incubated with primary antibodies overnight, washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Merck-Millipore, Cat. # 12-348) for 1 h, and visualized using ECL Plus (Thermo Fisher Scientific). Quantification of relative protein amounts was performed by densitometric analysis using ImageJ software (NIH), normalized to a loading control protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or calnexin (CXN). Primary rabbit antibodies used were: anti-Casein Kinase 1 δ (CK1 δ) (1:1000, ThermoFisher, PA5-32129), anti-Serine/Arginine Repetitive Matrix 4 (SRRM4) (1:1000, ThermoFisher, PA5-45083), anti-beta Catenin (1:2000, ThermoFisher, PA5-19469), anti-Phospho-beta Catenin (Ser33, Ser 37, Thr41) (1:1000, ThermoFisher, PA5-67518), anti-p44/42 MAPK (ERK1/2) (1:1000, Cell Signaling, #9102), anti-Phospho p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1000, Cell Signaling, #9101), anti-Calnexin (1:2000, ThermoFisher, PA5-34754) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000, Cell Signaling, #2118) antibodies.

6.11 Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). The difference in means between two groups was assessed by Kolmogorov-Smirnoff *t* test. Statistical analysis was performed using Prism 6 (GraphPad Software). *P* values were considered significant at $P < 0.05$ and highly significant at $P < 0.01$ and $P < 0.001$.

7. Results

7.1 *Pilot study showing overexpression of REST in EAE*

Our pilot study has demonstrated that REST is overexpressed in the lower part of spinal cord in EAE-affected mice 24 hours after disease onset. We sought to investigate the mRNA levels of REST and its target genes in EAE. We extracted mRNA from the spinal cord of EAE-affected mice one day after disease onset and subjected it to qRT-PCR. Interestingly, the levels of the REST transcript were dramatically increased (almost three-fold) in EAE mice compared to age-matched healthy controls. Next, we investigated whether the major gene targets of REST, the voltage-dependent Na+ channel Nav1.2, the neurotrophin BDNF and synaptic protein SNAP-25, were affected by the elevated REST expression. Interestingly, Nav1.2 was significantly down-regulated, confirming the transcriptional repression by the high levels of REST and suggesting the primary neuronal origin of the REST dysregulation (**Fig. 14A**).

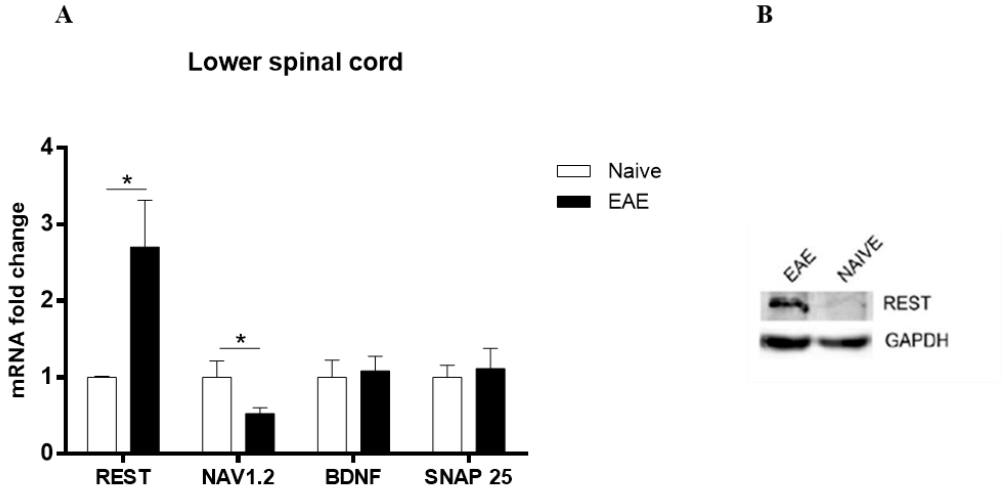


Fig. 14 REST is overexpressed in lower spinal cord of EAE-affected mice 24 hours after disease onset and its up-regulation is accompanied by the down-regulation of the neuronal sodium channel Nav1.2. (**A)** mRNA was extracted from brain and spinal cord of naïve (n = 3) and of EAE-affected mice 24 hours after disease onset (n = 3) and analyzed by qRT-PCR using GAPDH as housekeeping gene. Data are shown as mean fold change ± SEM. * $P <$ 0.05; Kolmogorov-Smirnov test of EAE vs naive mice. **(B)** Representative immunoblot from EAE-affected and naïve mouse spinal cord samples (n=2 mice per group). GAPDH expression was assessed to verify equal sample loading and permit normalization of data.

On the other hand, the transcription of BDNF and SNAP-25 was not markedly affected, indicating that REST over-expression may exert a differential regulation of the various target genes in different cell types. To verify that the increased mRNA levels were accompanied by increased REST protein, spinal cord samples from the very same animals were extracted and subjected to SDS-PAGE and immunoblotting with specific anti-REST antibodies. The results show that also the protein levels of REST showed a parallel increase, albeit to a smaller extent than those of mRNA levels (**Fig. 14B**). These data suggest, for the first time, the existence of transcriptional changes in EAE brain tissue mediated by REST up-regulation, and potentially offer a molecular mechanism for the defects in neuronal excitability observed in EAE mice. Our preliminary results indicate that REST is markedly dysregulated in CNS of mice with EAE, a response possibly resulting from neuronal over-expression. Whether elevated REST levels are pathogenic or the expression of an endogenous protective response to disease is unclear.

7.2 Temporal profile of REST mRNA overexpression has revealed that it is dysregulated at the acute early phase of EAE

To understand the temporality and localization of REST dysregulation, we have measured the levels of REST mRNA expression at different relevant stages of EAE. Thus, REST mRNA was quantified at the asymptomatic phase (7 days post-immunization (dpi)), around 4 days before disease onset, at the acute inflammatory phase (4 days post-onset (dpo)), and at the chronic phase (14 dpo), in various CNS regions (cortex, cerebellum, striatum, and lower and upper spinal cord segments). Before disease onset (7 dpi), we observed an increase of REST expression in the cortex, whereas its expression did not change in the other brain areas studied at this time point (**Fig. 15A**), suggesting that REST dysregulation occurs from before disease onset. At the acute phase (4 dpo), REST was over-expressed by around 2 folds in the spinal cord and striatum of EAE-affected mice (**Fig. 15A**). At the chronic phase (14 dpo), a slight increase was observed in the hippocampus.

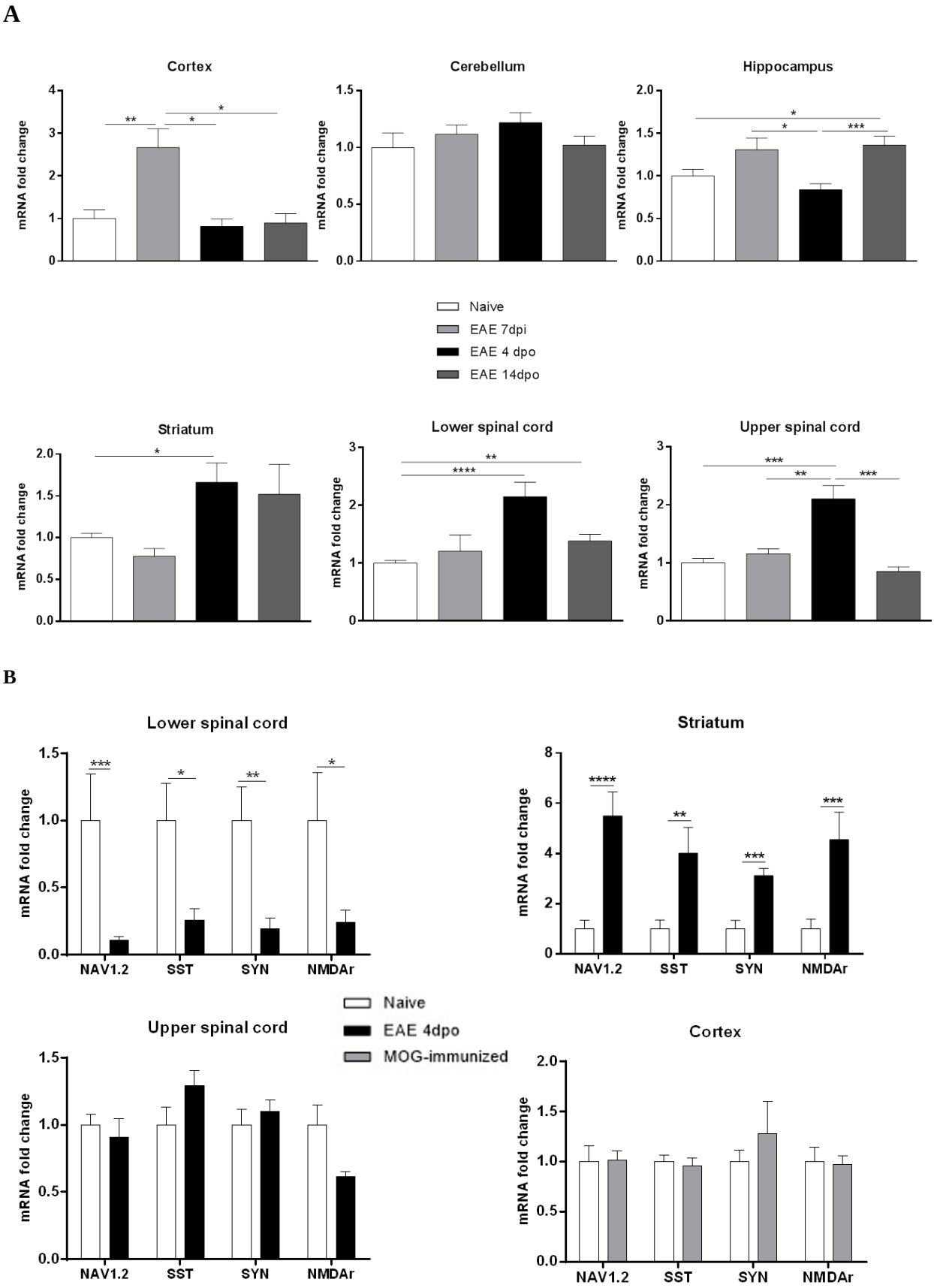


Fig. 15. REST mRNA over-expression in spinal cord and striatum at early phase of EAE is accompanied by dysregulation of relevant target genes. (A) REST mRNA expression is increased in spinal cord and striatum of

EAE-affected mice. mRNA was extracted from brain and spinal cord of naïve (n = 10) and of EAE-affected mice before disease onset (7 dpi n = 10) and at early (4 dpo n = 13) and chronic (14 dpo n = 4) phases and analyzed by qRT-PCR using GAPDH as housekeeping gene. Data are shown as mean fold change ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; Kolmogorov-Smirnov test of EAE vs naive mice. **(B)** REST target gene expression in CNS areas displaying REST up-regulation. REST overexpression in lower spinal cord at acute phase is associated with down-regulation of its target genes, while these are up-regulated in the striatum (upper panel). In upper spinal cord at EAE acute phase (4 dpo) and in cortex of MOG-immunized mice (7 dpi), REST overexpression is not accompanied by dysregulation of relevant target genes (lower panel). mRNA from spinal cord, striatum and cortex of naïve (n = 5) and MOG-immunized mice (n = 9) and at 4 dpo (n = 7) was extracted and processed for qRT-PCR analysis. NAV1.2, sodium channel, voltage-gated, type II, alpha subunit; SST, somatostatin; SYN, synapsin; NMDA, *N*-methyl-D-aspartate receptor.

Analysis of REST target genes at 4 dpo, time when REST is overexpressed in EAE, indicated that up-regulation of REST was associated with down-regulation of relevant targets in the lower spinal cord, as expected. Unexpectedly, in the striatum, the same genes are instead up-regulated. (**Fig. 15B**, upper panel). In upper spinal cord of EAE-affected mice at acute phase, and in cortex of MOG-immunized mice before EAE onset, the increase in REST mRNA expression was not accompanied by dysregulation of the target genes analyzed **(Fig. 15B**, lower panel). These data suggested that, even if REST is an important factor in the regulation of expression of its target genes, other possible mechanisms are also involved. In mice, the REST gene could be expressed as a full-length (fREST) or a splicing variant REST4, lacking the repressor domain and competing with fREST to derepress its targets. REST4 protein is believed to act by de-repression, competing with full-length REST for association with Sin3 corepressor complex, thereby permitting the expression of REST target genes (see **Fig. 1)**. Accordingly, we have speculated that the lack of repressor effect of REST over-expression on its targets, which are up-regulated in striatum of EAE-affected mice at acute phase, could be due at least in part, to an up-regulation of REST4. Whether an unbalance in the expression of REST and REST4 affects the anomalous expression of REST in EAE at specific stages and in particular CNS areas remains to be established. In order to investigate this issue, we have designed qRT-PCR primer pairs, specific for the full-length form of REST or for REST4, as shown by the amplification curves obtained using fREST- and REST4-specific

primers on plasmids coding for REST4 (**Fig. 16A**) and full-length REST (**Fig. 16B**). These new tools allow us to better characterize the role of REST in the EAE model, and to distinguish between REST- and REST4 dependent-effect.

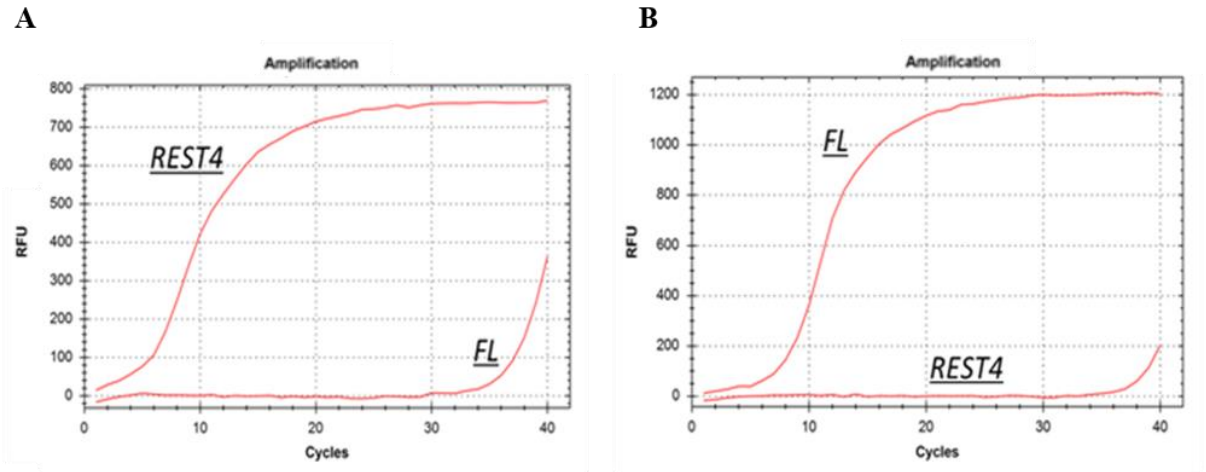


Fig. 16. REST full length-specific primers do not recognize REST4. qRT-PCR amplification curves obtained with REST4- and REST-specific primers, using as template a vector containing the REST4 sequence (**A**) or a vector containing the full-length REST sequence (**B**). FL: primers specific for full-length REST; REST4: primers specific for REST4.

Analysis of the different REST transcripts in EAE-affected mice at 4 dpo points to an up-regulation of fREST in lower spinal cord, which is consistent with the down-regulation of its target genes. Conversely, in striatum the up-regulation of REST target genes that we observed at acute phase, was associated with a reduction of fREST and a concomitant increase in REST4 mRNA expression (**Fig. 17,** upper panel). In upper spinal cord of EAE-affected mice at acute phase and in cortex of MOG-immunized mice (7 dpi), where REST up-regulation is not accompanied by dysregulation of its targets, analysis of REST transcripts indicates a balance in expression of the two isoforms in the spinal cord and a reduction of fREST in cortex (**Fig. 17** lower panel). These results indicate that, even if REST is an important factor in modulating gene expression, other possible mechanisms are also involved.

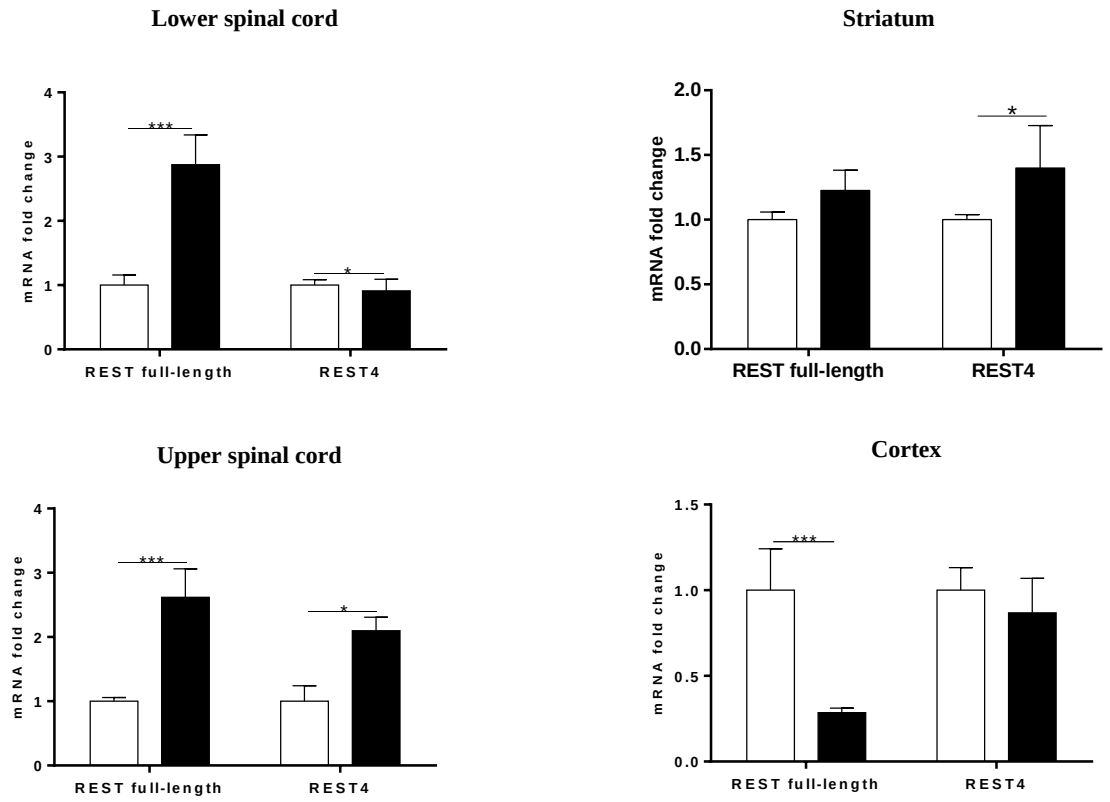


Fig. 17. Evaluation of mRNA expression of REST splicing isoforms in CNS areas. REST full length and REST4 mRNA were analyzed by qRT-PCR of striatum, spinal cord and cortex samples of naïve (n =10) and EAE-affected mice at acute phase (n = 13) and before EAE-onset (n = 10). Data are shown as mean fold change ± SEM. * $P < 0.05$; *** $P < 0.001$; Kolmogorov-Smirnov test of EAE vs naive mice.

7.3 Transcriptional analysis of CNS samples through the Nanostring nCounter system confirms the down-regulation of REST targets in the spinal cord of EAE-affected mice

To achieve a more comprehensive picture of the changes in gene expression taking place in EAE mice, we employed the nCounter® gene expression assay (NanoString Technology), which provides a sensitive method for detecting and quantifying cellular mRNAs through molecular color-coded probes, without the use of reverse transcription or amplification, removing possible bias. A total of 80 genes were selected, including REST-target (RE1-containing) and non-target (RE1-negative) genes. We performed two independent experiments for transcriptional analysis of CNS samples using Nanostring technology. We analyzed total mRNA extracted from spinal cord and striatum of EAE (4 dpo) and naïve mice. For each gene, we expressed the results as fold change EAE-affected/naïve and reported such values in a color-coded fashion, where blue/red colors correspond to genes that are respectively less/more expressed in EAE samples compared

to naïve (**Fig. 18**). Transcriptional analysis was repeated on CNS samples from two additional EAE-affected and naïve mice, to increase the number of independent replicates from the first experiment previously reported. The fold change EAE-affected / naïve is reported for the total 80 genes selected, including REST-target (RE1-containing) and non-REST target (non RE1) genes, and keeping the results of the first and second experiment separate to appreciate the inter-experimental variability. Interestingly, these data show a trend for RE-1-containing genes to be down-regulated both in spinal cord and striatum, consistent with what is expected from an up-regulation of REST in these regions. On the other side, the expression of most 'non RE-1' genes is very similar between EAE-affected and naïve mice, with some genes (particularly those belonging to cytokine signaling pathways) being clearly up-regulated in samples from EAE-affected mice. The data from the second experiment are in line with the first, with a trend for RE-1 genes to be down-regulated both in spinal cord and striatum. Most of RE-1-negative genes were similarly expressed in EAE and naïve mice, while some were clearly up-regulated in EAE samples. The down-regulation of REST target genes in the spinal cord, is consistent with what we observed by RT-PCR analysis, while in the striatum, only synapsin expression is increased.

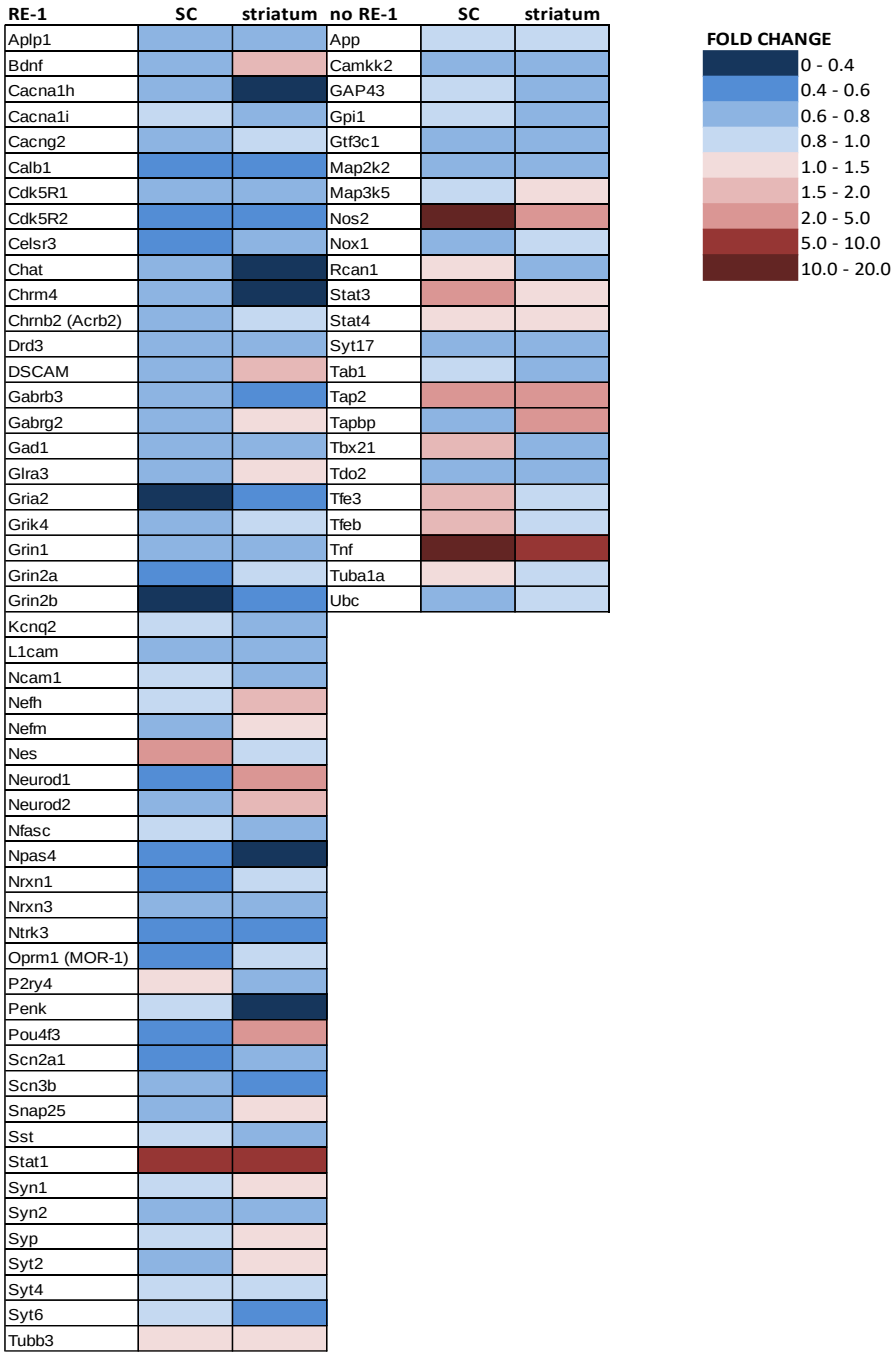


Fig. 18 Transcriptional analysis of CNS samples from EAE-affected mice using the Nanostring nCounter technology. mRNAs from lower spinal cord (SC) and striatum samples of EAE (4 dpo) and naïve animals was analyzed (n=4 animals per group). A total of 80 REST-target (RE1-containing) and non-target (RE1-negative) genes were analyzed and the values were normalized against 5 housekeeping genes and expressed as fold change EAE/naïve. Blue / red colors correspond to genes that are respectively less / more expressed in EAE samples than in naïve samples. Data show a trend for down-regulation of RE-1-containing genes both in spinal cord and striatum, in line with the observed REST up-regulation, in EAE-affected mice. The results are the summary of two independent experiments (2 mice per group in each experiment).

7.4 Optimization of protocol for REST detection by Western Blotting

To further understand the mode of action and consequences of REST dysregulation, we need to evaluate the expression of REST also at the protein level. While REST was initially demonstrated as a 116 kDa protein, a molecular weight commensurate with its amino acid sequence, several molecular weights, spanning from 116 to 200 kDa, have been reported in the literature upon SDS-PAGE and immunoblotting, probably due to different post-translational modifications of the protein, that include glycosylation, which is notorious in modifying the apparent molecular weight of glycoproteins upon electrophoresis. Because of the very low expression of REST in the adult brain, we have optimized our Western blotting experiments for REST using brain slices from KA-treated mice. Indeed, KA treatment has been reported to increase REST levels in cortex and hippocampus in rodents [41, 74]. Mice were treated with saline or KA for 24 and 48 h. We observed that, after a 20 h KA treatment, an appreciable increase in the intensity of a double band around 180 kDa occurs in mouse cortical neurons, which corresponds to a phosphorylated state of the REST protein. Various CNS areas (cerebellum, cortex, hippocampus, striatum, lower and upper spinal cord) were isolated from vehicle- and KA-treated (**Fig. 19**) mice and analyzed by western blotting, using Millipore #07-579 anti-REST and anti-calnexin (CXN) antibodies, and a protein molecular weight ladder that identified more clearly the range between 150 and 250 kDa was run in parallel.

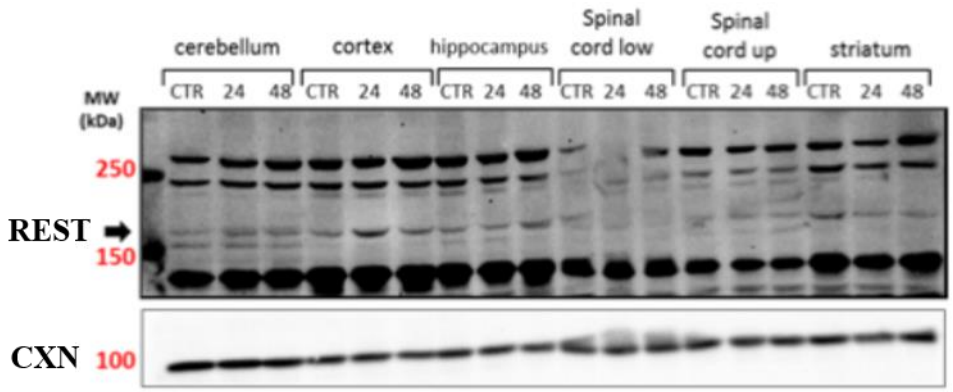


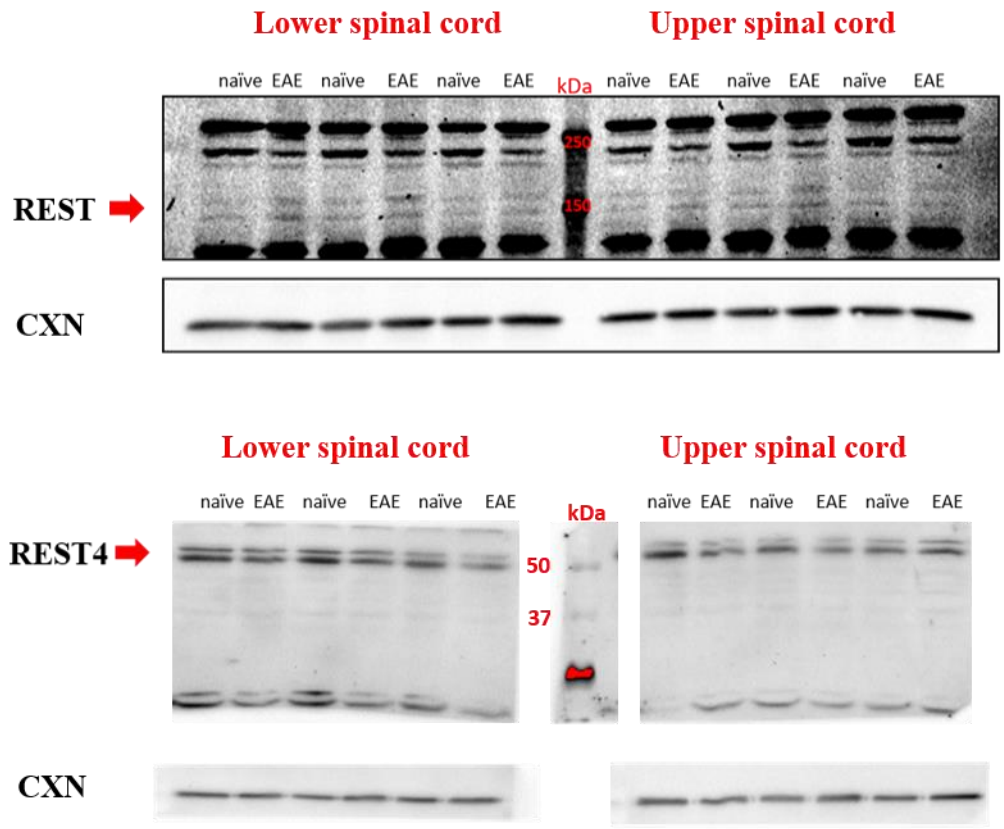
Fig. 19 REST protein levels are up-regulated in the hippocampus and in cortex of KA-treated mice, Representative immunoblot of different tissue samples from mice treated with KA, for the indicated time points.

Quantification of the immunoreactive band intensities indicated that REST increased in the hippocampus and cortex of the mice treated with KA (**Fig. 19**, as previously reported [47, 74]. This optimized protocol was used thereafter to analyze REST protein expression in EAE.

7.4.1 REST is overexpressed in EAE also at protein level

Once the REST immunoreactive band had been correctly identified, lower and upper spinal cord samples extracted from naïve and EAE-affected mice at 4 days post onset (4dpo) were subjected to Western blotting as above. The results showed a significant up-regulation of REST protein levels in the lower spinal cord, while in the upper spinal cord REST levels were comparable between EAE-affected and naïve mice (**Fig. 20**).

A



B

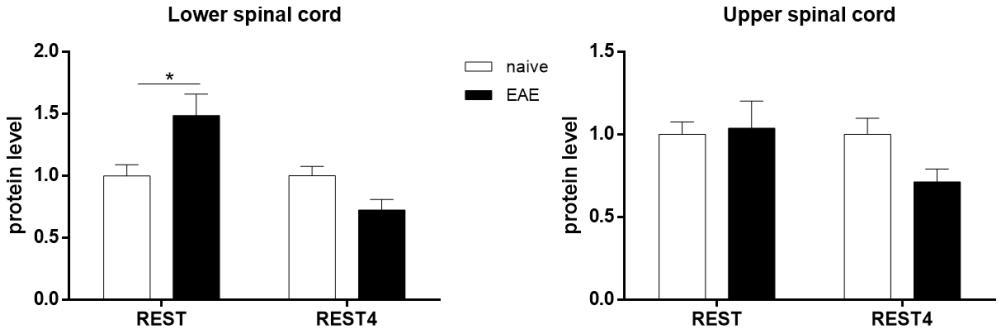


Fig. 20. REST and REST4 protein expression in spinal cord of EAE-affected mice at 4 dpo. (A) Representative immunoblot and **(B)** quantification of EAE-affected and naïve mouse spinal cord samples (*P < 0.05, two-tailed Student's t-test of EAE-affected vs naïve mice; n = 3 or 5 animals per each experimental group).

Moreover, for having a better understanding of the impact of REST up-regulation on the regulation of its target genes, we have analyzed also the expression of REST4 at protein level. In both lower and upper spinal cord of EAE-affected mice at disease peak, the levels of REST4 are similar compared to naïve mice. These data fully confirm the results previously obtained at the transcriptional level, where the down-regulation of REST target genes in the lower spinal cord, is associated to an up-regulation of REST, but not of REST4. In addition, in the upper spinal cord, the balance in the expression of the two isoforms, also at protein level, support the results of no change in REST target genes expression that we observed in this area. These results, have enabled the definition of the most relevant time points of REST dysregulation in EAE, as well as the CNS areas where this dysregulation mainly occurs. While the cellular localization of such dysregulation is still not clear, immunofluorescence analysis will enable us to map REST up-regulation unequivocally.

7.5 Analysis of signal transduction pathways controlling REST overexpression

To analyze signal transduction pathways triggering REST overexpression in neurons under inflammatory conditions similar to what would be expected in EAE, we have used the differentiated neuroblastoma cell line (N2a) cultured in the presence of supernatant of T cells stimulated or not with anti-CD3/CD28 antibodies for 48 hours, a condition which results in the

induction of a pro-inflammatory Th1 phenotype, with production of inflammatory cytokines as we confirmed by ELISA assay [**Fig. 21**; anti-inflammatory cytokines IL-4 and IL-10 in the T-cell supernatant were below detection levels (data not shown)].

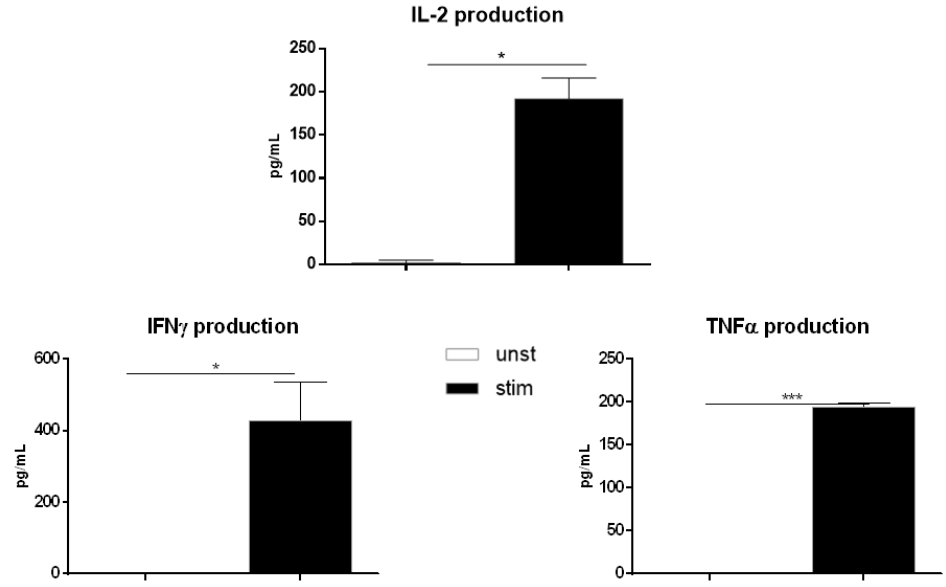


Fig. 21. T-cells activated with anti-CD3/CD28 antibodies produce mostly inflammatory cytokines. Supernatant of T cells stimulated with anti-CD3/CD28 antibodies for 48 hours was collected and analyzed by ELISA (BioLegend kits: Mouse IL-2 ELISA Max Standard; Mouse IFN-γ ELISA Max Standard; Mouse TNF-α ELISA Max Standard).

We observed an increase in both fREST and REST4 mRNA expression in N2a cells exposed to stimulated T-cell supernatant, albeit to a greater extent for REST4 (**Fig. 22**, left panel). Analysis of mRNA expression of some REST relevant target genes involved in synaptic transmission, which is altered during neuroinflammation, indicated that these genes were significantly up-regulated under inflammatory conditions (**Fig. 22**, right panel), a data seemingly commensurate with the increased extent of REST4 overexpression in relation to fREST.

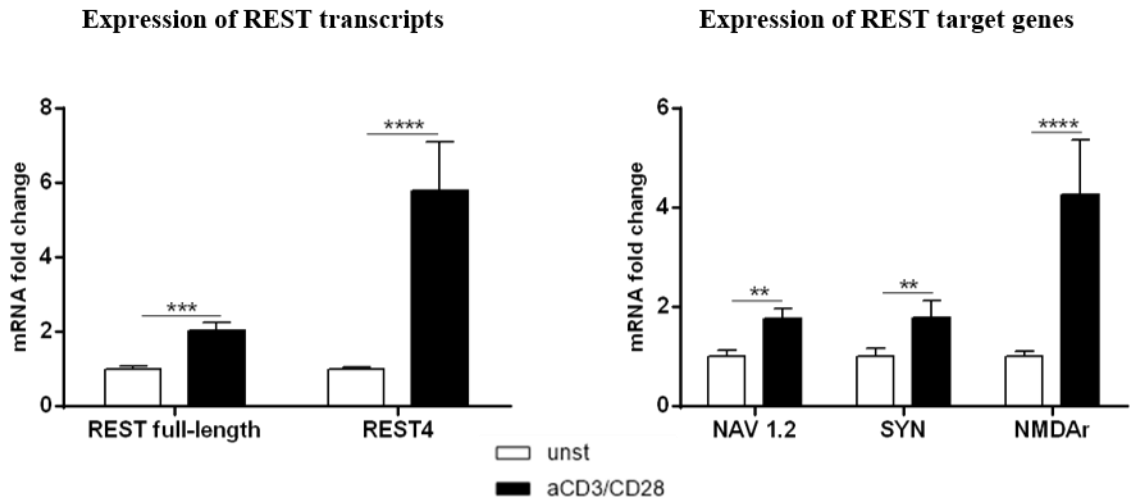


Fig. 22. Analysis of mRNA expression of REST isoforms and relevant target genes in N2a cells under inflammatory conditions. mRNA from N2a cells stimulated or not with anti-CD3/CD28 antibodies for 48 hours was extracted and processed for qRT-PCR analysis. Data from three independent experiments are shown as mean fold change ± SEM. ** $P < 0.01$; **** $P < 0.0001$; Kolmogorov-Smirnov test of N2a cells exposed to not activated or activated T cells supernatant. Unst: N2a cells exposed to supernatant from non-stimulated T cells; aCD3/CD28: N2a cells exposed to supernatant from T cells stimulated with anti-CD3/CD28 antibodies.

7.5.1 *Wnt signal controls β-catenin translocation into nucleus which is associated to an increased transcription of REST*

Since REST regulates neuronal cell differentiation and this process is orchestrated by several signaling pathways, including Wnt, we have started to analyze these pathways in N2a cells in the context of how inflammatory conditions affect REST expression and subsequent activity. The formation of the complex responsible for β-catenin phosphorylation, including casein kinase 1 (CK1), is prevented by the presence of Wnt ligands leaving β -catenin free to enter the nucleus and activate Wnt responsive genes, including REST. CK1 is also directly responsible for REST phosphorylation, promoting its degradation in physiological conditions (Fig. 23).

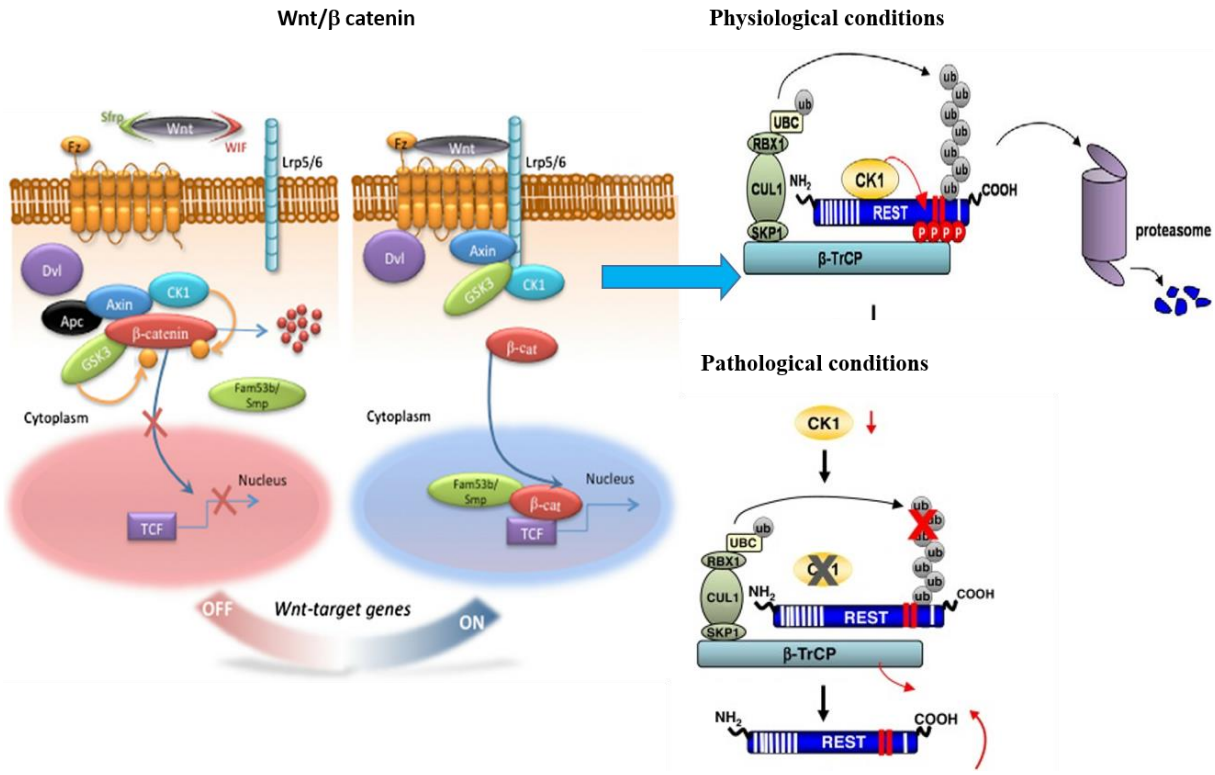


Fig. 23. Wnt/β catenin and CK1 signaling regulate REST activity and expression differentially. In the presence of Wnt ligands, the complex responsible for β-catenin phosphorylation, including CK1, does not form and β-catenin can translocate into the nucleus to activate the transcription of Wnt target genes, which include REST. CK1 is also directly involved in REST phosphorylation, promoting its degradation via proteasome. Taken from [76, 158].

Accordingly, we have assessed the levels of β-catenin, in N2a cells exposed to supernatant of unstimulated or stimulated T cells by confocal microscopy analysis. We observed an increase in total-β-catenin fluorescence at both 6 and 24 hours of exposure of N2a cells to activated T-cell supernatant (**Fig. 24A**), accompanied by a concomitant increase in nuclear-β-catenin. While the levels of total-β-catenin did not seem to differ between the 6- and 24-hour time points after the addition of T-cell activated supernatant, the levels of nuclear-β-catenin were higher at 24 hours, indicating a greater translocation into the nucleus, as expected, according to REST up-regulation (**Fig. 24B**). We have repeated the same experiments also with differentiated N2a cells, after 6 hours of exposure of pro-inflammatory supernatant, and we confirmed the increase in β-catenin expression. Indeed, β-catenin translocation into nucleus is associated with activation of the

transcription of Wnt target genes, including REST, which is consistent with REST mRNA data in N2a cells exposed to activated T-cell supernatant.

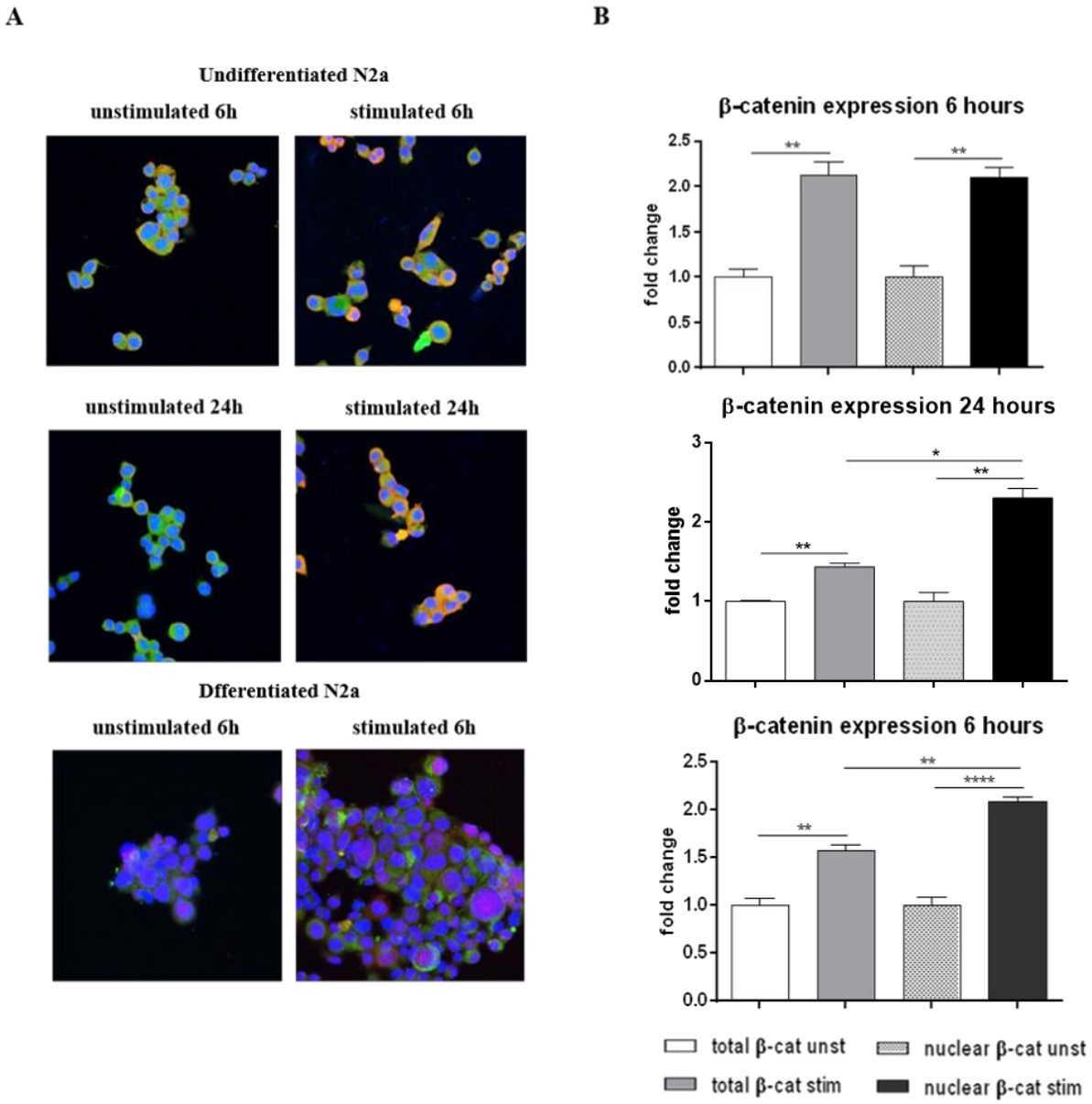


Fig. 24 Increase in nuclear β–catenin levels measured by confocal microscopy analysis support the involvement of Wnt signaling in REST up-regulation upon neuroinflammation. (A) Analysis of β−catenin fluorescence intensity in differentiated or not N2a cells exposed for 6 and 24 hours to supernatant from T cells unstimulated (unst) or stimulated with anti-CD3/CD28 antibodies (stim). Nuclei are stained in blue with DAPI; N52, marker of high molecular weight neurofilaments, in green used as a housekeeping protein; β−catenin in red. **(B)** Quantification of β−catenin data from (A). Data are shown as ratio ± SEM. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; unpaired Student's t-test of N2a cells exposed to supernatant from unstimulated vs stimulated T cells.

7.5.2 The up-regulation observed in REST in stimulated N2a cells is associated with a decrease in ERK phosphorylation

C-terminal domain small phosphatase 1 (CTDSP1) maintains REST activity by dephosphorylation on a specific regulatory site in non-neuronal cells (**Fig. 25**, left panel). Conversely, during neuronal differentiation, growth factors (e.g., EGF) increase the activation of kinases like Ras and ERK that, in turn, decrease REST activity via β TrCP through phosphorylation-dependent degradation (**Fig. 25**, right panel).

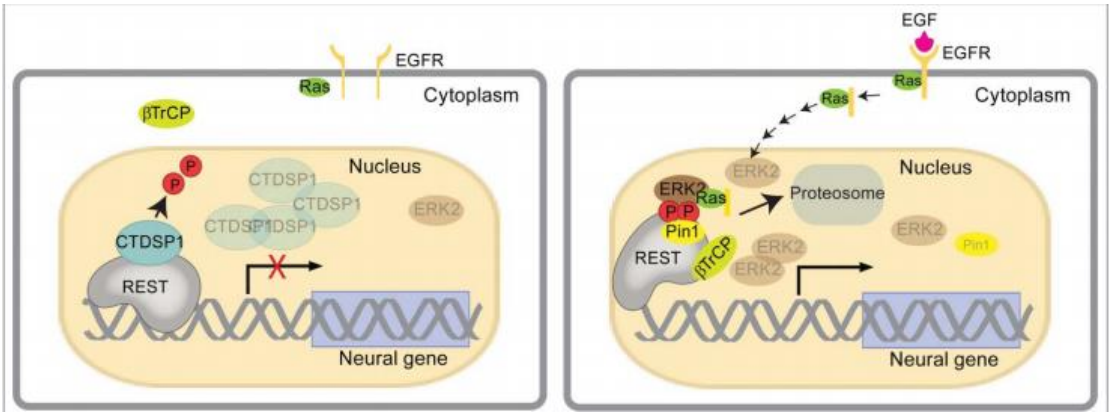


Fig. 25 REST is regulated by post-translational modifications at a precise regulatory site. Two opposite signals regulate REST activity: a small phosphatase, CTDSP1, stabilizes REST through dephoshorylation, while EGF-Ras-ERK signaling phosphorylates it at the same regulatory site, decreasing REST activity via ubiquitin-proteasome pathway Taken from [159].

These two opposite signals reciprocally regulate REST activity at the same regulatory site and are responsible for temporal regulation of REST in neuronal cells. Confocal microscopy analysis of phospho-ERK levels (**Fig. 26A**) and quantification of N52/phospho-ERK ratio (**Fig. 26B**) indicated that these are reduced after 30 minutes and 1 hour of exposure of undifferentiated N2a cells with the same supernatant. In differentiated N2a cells, we analyzed the levels of phospho-ERK after 30 minutes of exposure to activated T-cell supernatant by Western blotting analysis; as can be seen in **Fig. 26C**, there was also a decrease in ERK phosphorylation.

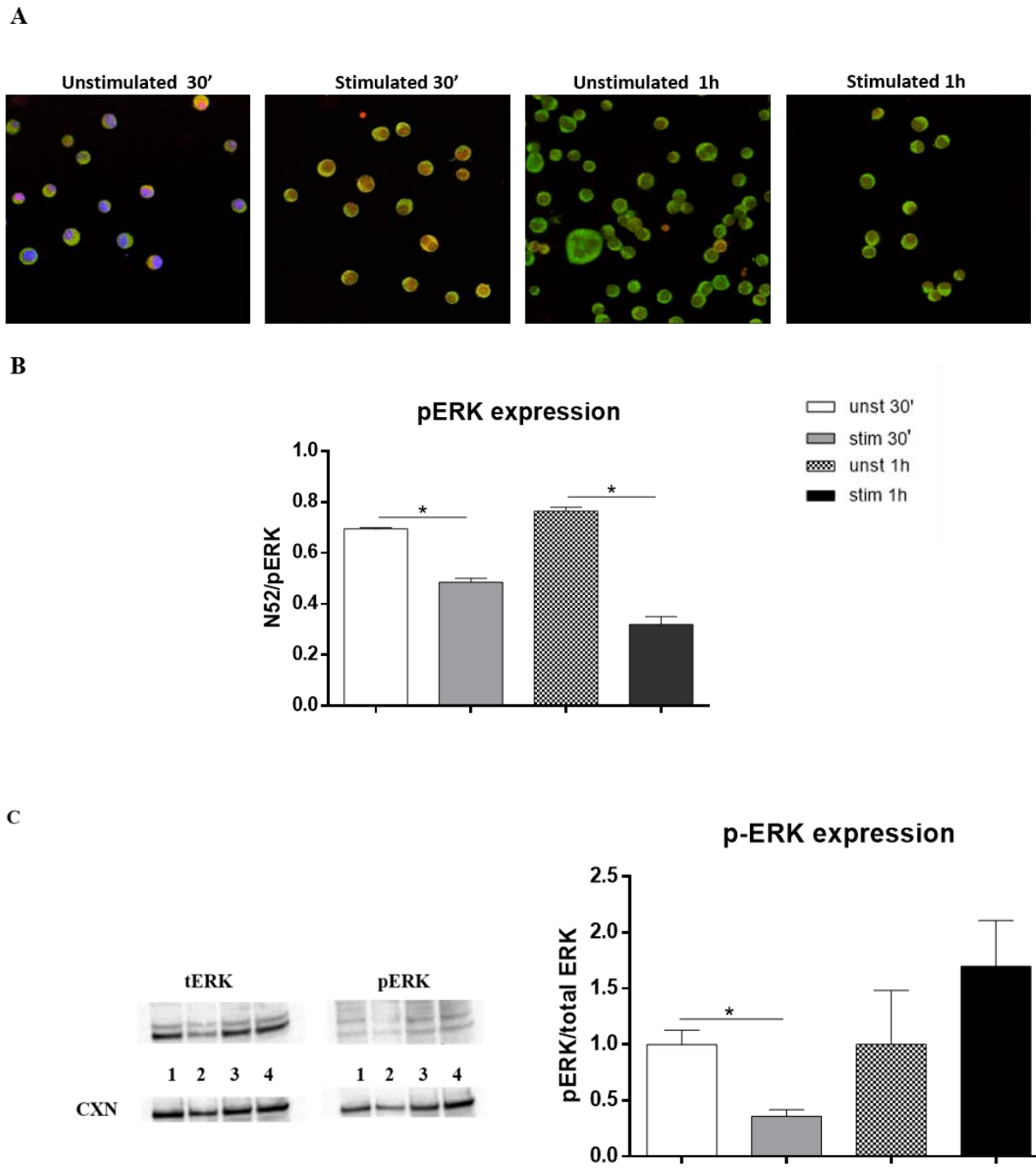


Fig. 26 The reduced phosphorylation of ERK in inflammatory conditions is associated to REST up-regulation in N2a cells. (A) Analysis of phospho-ERK fluorescence intensity in undifferentiated N2a cells exposed for 30 minutes and 1 hour to supernatant from T cells unstimulated (unst) or stimulated with anti-CD3/CD28 antibodies (stim). Nuclei are stained in blue with DAPI; N52, marker of high molecular weight neurofilaments, in green; phospho-ERK in red. **(B)** Quantification of N52/phospho-ERK ratio from **(A)**. **(C)** Representative Western blots (left panel) and quantification from two independent experiments (right panel) of phospho-ERK (pERK) proportionally to total ERK (tERK) (pERK/tERK ratio) from differentiated N2a cells exposed to supernatant from unstimulated (lane 1 for 30 minutes; lane 3 for 1 hour) or stimulated (lane 2 for 30 minutes; lane 4 for 1 hour) T cells. Data are shown as ratio ± SEM. * $P < 0.05$; ** $P < 0.01$; unpaired Student's t-test of N2a cells exposed to supernatant from unstimulated vs stimulated T cells.

7.5.3 *The neural splicing factor, nSR100, is involved in regulation of REST transcripts and in the control of the balance between REST and REST4*

We have analyzed by Western blotting a Neural-Specific Serine/Arginine Repetitive Splicing Factor of 100 KDa (nSR100), responsible for alternative splicing of REST transcripts and the control of the balance between fREST and REST4 (**Fig. 6**). nSR100 expression correlates with high levels of REST4 and low fREST expression. Our experiments in vitro, demonstrated that we observed an increase at mRNA level in both REST isoforms in N2a cells exposed to activated T-cell supernatant, with the fold increase in REST4 significantly greater than that of fREST (**see Fig. 22**). Western blotting analysis confirmed the increased expression of REST4 in our experimental conditions, through an increase of nSR100 protein in differentiated N2a cells exposed to activated T-cell supernatant after 3 hours (**Fig. 27**).

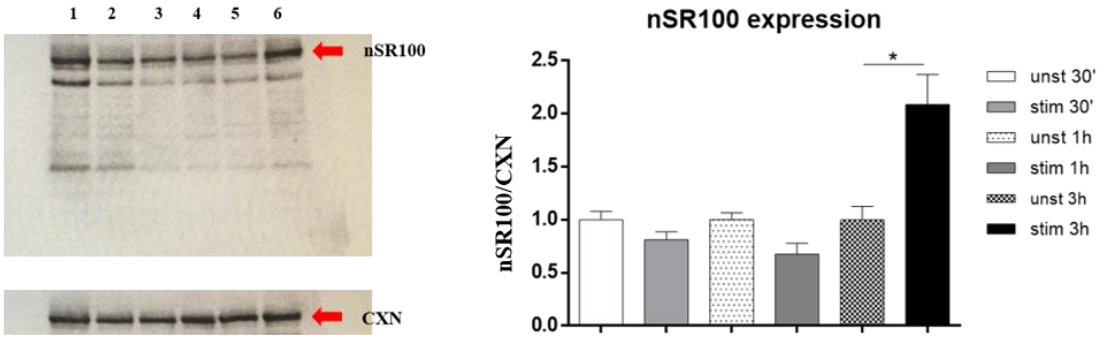


Fig. 27. Neural-Specific Serine/Arginine Repetitive Splicing Factor of 100 KDa (nSR100), an activator of REST alternative splicing is increased in addition to activated T-cell supernatant in neurons. (A) nSR100 regulates REST splicing isoforms at transcriptional level, inducing a switch between REST full-length and REST4 proteins. REST4 is known to induce a de-repression of REST targets genes and, through a negative feedback, inhibits a further activation of nSR100 transcription. Overexpression of REST, on the other hand, causes repression of REST4 and nSR100 (Upper panel). **(B)** Western blot analysis of nSR100 signaling pathways relevant to REST expression in N2a cells under inflammatory conditions. Representative Western blot (Left panel) and quantification (right panel) from three independent experiments. Protein lysates of differentiated N2a cells cultured with supernatant of T cells stimulated with anti-CD3/CD28 antibodies (stim) or not (unst) were analyzed by Western blotting. nSR100 protein was detected using a specific antibody and GAPDH or CXN as a control loading for samples. Data are shown as ratio (right panel) of nSR100 to GAPDH expression in N2a cells exposed (lanes 2, 4, 6) or not (lanes 1, 3, 5) to activated T-cell supernatant at the indicated time points. Data are shown as ratio ± SEM. * $P < 0.05$; unpaired Student's t-test of N2a cells exposed to supernatant from unstimulated vs stimulated T cells.

These data therefore support the involvement of nSR100 signaling pathway in regulation of REST activity and in the balance of the splicing isoforms, responsible for the differential expression of REST target genes in the different areas of CNS and in neurons under inflammatory conditions in vitro. In addition, confocal microscopy analysis has confirmed that two opposite signals regulate REST expression in vitro, under conditions mimicking EAE microenvironment. In fact, we observed a reduction of phospho-ERK, which is in line with the observed REST up-regulation in N2a cells in our experimental conditions. The decrease of p-ERK activity prevents REST phosphorylation-dependent degradation, supporting its role in the context of neuroinflammation. Together with the increase in nuclear-β-catenin levels and the following activation of Wnt target genes transcription, including REST, these two signals, in our experimental conditions, are, at least in part, responsible for regulation of REST in neuronal cells.

8. DISCUSSION

Multiple sclerosis is a demyelinating autoimmune disease of CNS associated with neuronal and axonal damage and loss, where the balance between inflammation and the remaining capacity of neuronal self-protection and repair influences the clinical outcome. Hence, new therapeutic strategies for halting neurodegeneration and promoting tissue repair could be focused on modification of molecular pathways involved in neuronal development, in particular that involving REST which regulates neurogenesis, and whose dysregulation has been observed in several neurological diseases [62, 63, 67]. Here, we show that REST dysregulation also occurs in EAE, suggesting a possible dysfunction also in MS [45]. The complex role of REST in CNS pathology has highlighted a controversial and opposite function of REST, acting in some circumstances in promoting neuronal repair [48, 70], whilst contributing to neuronal death under other pathological conditions [62, 63], suggesting the activation status of REST in neurons might differentiate between neuroprotection and neurodegeneration. It is now largely recognized that neurons can modulate REST expression in response to both environmental and pathological stimuli, suggesting that by its mechanism of target repression, REST is actively involved in controlling gene expression in the adult brain and is essential for the determination of neuronal fate. Our data demonstrated that exposure to inflammatory conditions, both during EAE and in vitro, induces REST up-regulation. Experiments were directed to outline temporal and spatial profile of REST overexpression together with the expression of its target genes. Although REST was originally identified as a neuronal repressor, depending on the cellular context, the binding of REST to a variety of cellular factors can produce an array of mechanisms that can be used to differentially influence transcription of each of its many target genes in a cell context-dependent manner. The specific effect generated by the REST complex can be further affected by the neighboring transcriptional activators or repressors bound to the particular target gene chromatin, and the resultant effect then will determine the gene expression [5]. While in physiological processes the neuronal expression of REST is low, pathological stimuli might trigger an activation of REST

associated with increased transcript and protein levels [48], even if the meaning of such dysregulation is poorly understood. In our experimental conditions, we have confirmed that REST dysregulation occurs at both transcript and protein levels. The results are in line with a recent paper, in which REST up-regulation was firstly demonstrated in EAE model, pointing to its possible involvement also in MS pathology [79]. The hypothesis is that the neuronal down-regulation of the adhesion molecule L1, controlled by REST, is a compensatory response for promoting neuronal self-protection during neuroinflammation [79]. However, the work points to the beneficial role of L1 during EAE, while REST up-regulation is only described as a phenomenon associated with L1 down-regulation. In our study, we focused our attention on changes in the expression of REST transcripts occurring in CNS of EAE-affected mice. Our results have enabled the definition of the most relevant time points of REST dysregulation in EAE, as well as the CNS areas where this dysregulation mainly occurs. In particular, we have observed REST dysregulation in the spinal cord and striatum of EAE-affected mice at early acute phase. In EAE, an altered synaptic transmission has been extensively described occurring in several areas of CNS, including cortex and spinal cord, before or at the onset of clinical symptoms [128, 160-162]. Neuroinflammation might contribute to such synaptic dysfunction, which reflects an unbalance between glutamatergic and GABAergic transmission in the brain and spinal cord, and is involved in the induction of motor deficits observed in EAE mice [128]. These deficits occur as result of the caudo-rostral ascending paralysis, with the lumbar spinal cord as the most affected area at the early phase of the disease. The dysregulation of REST expression and the impact of its target genes in the lower spinal cord of EAE-affected mice at acute phase, reflect the main involvement of the lower part of spinal cord in EAE pathology. Therefore, the dysregulation of transcriptional factors that control neuronal networks, such as REST, in the spinal cord is not unexpected as it could promote synaptic instability driven also by the release of inflammatory mediators, characteristic of the disease. Although we have analyzed the expression of some REST

relevant target genes involved in synaptic transmission, future experiments should determine functional consequences of transcriptional dysregulation of such targets on neural excitability and synaptic transmission. This issue will be investigated by ex-vivo patch-clamp and multielectrode array recordings in acute brain slices of EAE mice, through the determination of intrinsic and network excitability, sodium current density and synaptic strength and plasticity. Several studies [70, 163], have also shown striatal alterations of both GABAergic and glutamatergic transmission in EAE-affected mice. Our results showing REST dysfunction in this area support its involvement in the pathology, and is presumably related to an increased expression of its truncated isoform, REST4. The increase of REST4 expression in striatum associated with the increased transcription of REST-target genes involved in synaptic transmission, corroborate previous studies that demonstrated striatal alteration. In the last years, different mechanisms have been proposed being a part in controlling REST activity. REST expression is known to depend on the context (environmental conditions) and to be regulated, in part, also by mRNA splicing [36]. Based on these observations, we had speculated that the differential effect of REST dysregulation on its target genes, in striatum and in the lower spinal cord of EAE-affected mice at acute phase, could be related to an unbalance of REST transcripts. In particular, the concomitant up-regulation of REST with the unexpected up-regulation of its target genes in striatum led us to consider that a possible unbalance in expression between REST full-length (fREST) and the splicing isoform REST4, which acts by repressing REST function, could be involved. Indeed, our data, which showed up-regulation of REST4 in the striatum together with an up-regulation of REST target genes supported such a possibility. Similarly, in the lower spinal cord of EAE-affected mice up-regulation of fREST is accompanied by down-regulation of its target genes, as expected, with a parallel decrease of REST4, a data that supports the down-regulation of REST target genes. So we have hypothesized that the lack of repressor effect of REST overexpression on its targets could be affected by REST4-dependent effect. Taken together, the data show the complexity of REST

regulation and function, highlighting that the balance between fREST and REST4 is important not only during neural differentiation/development, but also in mature CNS cells for modulation of gene expression. Therefore, the unbalance between fREST- and REST4 and its impact on gene transcription could represent a common pathogenic mechanism that links REST dysregulation to aberrant gene expression observed in some neurological diseases [36]. Based on the observation of the mechanism of regulation that correlates nSR100 expression with REST4 and fREST, we have ascertained this feedback loop by Western blotting analysis. Our experimental conditions have shown an increase of nSR100 in neuronal-like cells, supporting the involvement of nSR100 in the differential expression of REST target genes that we observed in the different areas of CNS during EAE, and in neurons, in vitro, in the context of neuroinflammation [40]. Although the molecular weight calculated on REST amino acid sequence is about 116 kDa, Western blotting analysis has demonstrated that this transcription factor could appear at distinct apparent molecular weights, a difference probably related to post-translational modifications of the proteins. In addition, several splice variants of REST were discovered encoding proteins with five or four zinc finger motifs [41]. In line with previous works, we observed a double immunoreactive band for REST around 180 kDa, according to what occurs in mouse cortical neurons after treatments that are known to induce REST up-regulation [47, 74]. The observed molecular weight could be explained by the post-translational modifications of REST protein, such as phosphorylation and glycosylation, which are known to reduce the migration of proteins upon electrophoresis. The increase of REST also at protein level is in line with REST transcript up-regulation in lower spinal cord samples of EAE-affected mice at disease peak, fully confirming the results previously obtained at the transcriptional level. To investigate the molecular signature of pathways involved in REST regulation in neuronal cells under inflammatory conditions, in-vitro studies were performed using a neuron-like cell line. The expression of REST was evaluated in vitro in differentiated N2a cells, showing that the exposure to inflammatory conditions, in order to mimick

the EAE microenvironment, induces REST up-regulation in these cells. In N2a cells, which are thought to have extremely low levels of REST, our results have demonstrated that during neuroinflammation, neurons are able to modulate REST expression. While the complexity of the downstream targets of REST is well understood, the upstream regulators are somewhat less well known. Neuronal cell differentiation is regulated by several signaling pathways, including Wnt and β-catenin, and REST is one factor contributing in the regulation of neuronal gene expression, through epigenetic transcriptional changes across the genome [9]. The low levels of REST protein during neuronal differentiation are maintained via the ubiquitin ligase SCFβ-TrCP, that directs the protein for proteasome-mediated degradation by phosphorylation [56], while REST activity is maintained by dephosphorylation on the same regulatory site. Thus, two opposite signals are responsible for regulation of REST activity in neuronal cells. Therefore, we have investigated the transduction pathways triggering REST overexpression during neuroinflammation in neurons, to understand how inflammatory conditions affect REST expression and its activity. According to previous studies, we have confirmed through confocal microscopy and Western blotting analysis, the involvement of Wnt/β-catenin and ERK signaling in regulation of REST activity. In particular, β-catenin translocation into nucleus is associated with the activation of transcription of Wnt target genes, including REST, and we have found an increase in β-catenin in the nucleus, line with REST mRNA up-regulation in stimulated N2a cells. Such an upregulation was also demonstrated in AD, where nuclear β-catenin was significantly elevated in aging PFC neurons and co-localized with REST [70]. In parallel, we have observed in these cells a reduction in the ERK signaling pathway responsible for REST degradation. Recent studies have demonstrated a correlation between reduced activity of ERK1/2 and up-regulation of REST mRNA [164] and REST protein stabilization [159]. Our results corroborate the observed reduction in the phosphorylation of ERK1/2 in neurons in a T cell/neuron co-culture model [79] and the associated REST up-regulation. Our results support a well-defined mechanism of action of REST in the control of the

expression of its target genes in response to neuroinflammation. Although the data confirmed that REST is an important factor in regulating the expression of its targets, other possible mechanisms are also involved and could contribute to aberrant gene expression of its targets during EAE and in neuroinflammation. Nevertheless, altogether, these data strongly suggest the involvement of REST in the pathological process of EAE, indicating that such a pathway should also be considered in MS. Accordingly, our data provide the basis for future studies aimed at finding modulators of REST in EAE that could eventually be envisaged in the translation to the human disease and therefore towards possible therapeutic approaches for MS. The possibility to modulate REST expression and/or activity expression represents a tool to dissect the molecular mechanisms underlying REST activity in the onset and progression of EAE. Time profiling of REST levels in the context of disease expression and stages allowed us to ascertain if REST could be used as biomarker potentially useful in MS to monitor treatment efficacy. In fact, a number of pharmacological treatments verified in EAE have been approved for relapsing-remitting MS. To understand if REST expression can be monitored as biomarker for therapeutic efficacy, we are performing experiments to identify and correlate changes in expression of REST and REST target genes with disease amelioration upon treatment. In particular, we are addressing this issue with specific drugs that cross the blood-brain barrier, such as fingolimod and monomethyl fumarate. These studies will establish whether REST modulation could represent a potential therapeutic approach to be translated in MS, outlining the perspective to design drugs able to modulate its activity and interfere with neuronal fate in neurodegeneration processes.

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