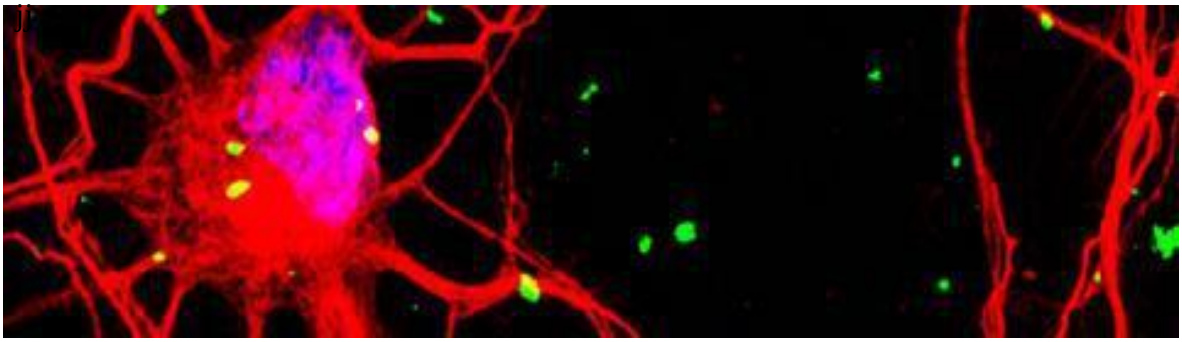

Corso di Dottorato in Neuroscienze
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Ciclo XXXI

In vitro and *in vivo* characterization of the RE-1 Silencing Transcription Factor (REST) activity under neuroinflammatory conditions

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ABSTRACT

The ability to specifically target epigenetic and molecular mechanisms involved in neuronal development could be an alternative therapeutic strategy for neuroinflammatory/neurodegenerative disorders such as Multiple Sclerosis (MS). The transcriptional repressor RE1-Silencing Transcription Factor (REST) regulates neurogenesis and neuronal identity through cell-specific gene repression, allowing expression of its targets in mature neurons where REST is quiescent. REST dysregulation has been implicated in several neurodegenerative disorders, including Alzheimer and Huntington diseases, tumors of the nervous system, and epilepsy.

We found that REST is overexpressed in the spinal cord of mice with experimental autoimmune encephalomyelitis (EAE), suggesting that its dysregulation might be an important factor in the pathogenesis of the disease. Starting from these observations, we have firstly analyzed the expression of REST target genes in EAE and characterized the cell-specificity of REST overexpression, investigating the differential contribution of neuronal and glial cell populations to REST upregulation. Moreover, in order to mimic the inflammatory EAE scenario, we have analyzed REST activity in primary neuron cultures treated with various pro-inflammatory cytokines. Altogether, this study provides the basis for understanding the molecular mechanisms of REST expression during brain inflammation and its implication in the subsequent neurodegenerative processes.

1. INTRODUCTION

1.1. The RE-1 Silencing Transcription Factor (REST)

The specification of cell identity during central nervous system (CNS) development is regulated by the transcriptional machinery, made by positive and negative transcriptional regulators that act simultaneously to shape the cell-specific transcriptome. Transcription factors (TFs) play a fundamental role in development as they ensure that the right genes are expressed in the right cells of the body, at the correct time. In 1995 the laboratories of Drs. Mandel and Anderson independently discovered the RE1-silencing transcription factor (REST), also known as neuron-restrictive silencer factor (NRSF)[1], a transcriptional repressor that binds a specific consensus sequence named repressor element 1 (RE-1)[2].

REST is a member of the Kruppel-type zinc finger transcription factor family, as it is characterized by the presence of nine zinc finger repetitions, eight localized near its N-terminal domain, and one near the C-terminus. Its repressive functions are mediated by two repressor domains: the N-terminal domain interacts with Sin3 [3], while the C-terminus recruits CoREST [4], [5]. In turn, each co-repressor recruits other associated proteins and chromatin remodeling factors, including histone deacetylases (e.g. HDAC1/2), demethylases (e.g. LSD1), and methyltransferases (e.g. G9a) that mediate the transcriptional

repression of target genes, densely packing the genomic material and ultimately inhibiting the activity of RNA polymerases [6] (**Fig. 1**). Translocation of REST into the nucleus is essential for its function. Several studies have highlighted that the nucleocytoplasmic shuttling of REST is influenced by its reversible association with other proteins such as RILP [7], Dynactin p150-Glued [8] and huntingtin (htt) [9]. In particular, wild type htt sequesters REST in the cytoplasm, thus preventing it from repressing its target genes, while mutated htt does not retain the transcription factor anymore, allowing REST translocation to the nucleus and the consequent pathological repression of target genes, as shown for BDNF in Huntington Disease [9], [10].

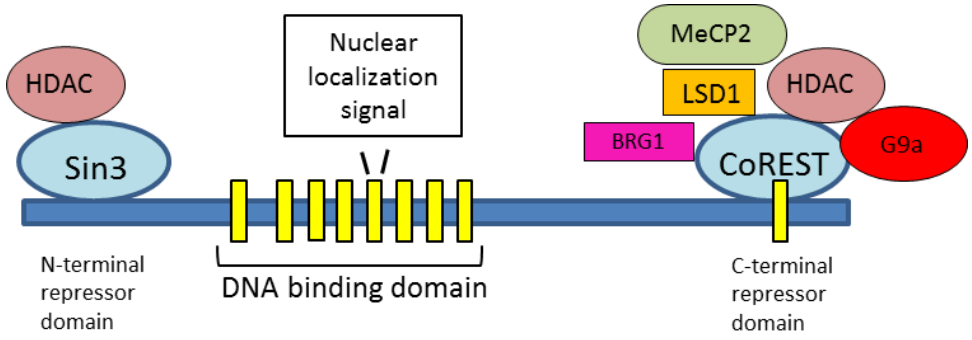


Figure 1. REST protein structure. Diagram showing the various functional domains of REST. The DNA binding region encompasses eight of the nine zinc finger domains of the protein. The co-repressors Sin3 and CoREST bind to the N- and C-terminus of the protein, respectively. Both co-repressors recruit histone deacetylases (HDAC), while CoREST recruits also other enzymes like the histone methyltransferase G9a, the histone demethylase LSD1, the ATP-dependent chromatin remodeling enzyme BRG1 and the methyl-CpG2 binding protein MeCP2.

REST binds its target genes at the level of RE-1, a 21 bp motif that was identified for the first time in the 5' flanking region of the voltage-gated sodium type II channel ($Na_V1.2$) and superior cervical ganglion 10 (SCG10) genes [11], [12]. Multiple genome-wide studies, including several in silico and biochemical analyses, have predicted the complete list of RE-1 sites and their associated target genes [13]–[15]. RE-1, whose computational derived sequence is represented in Fig. 2, is evolutionarly conserved and has been initially identified in 1,892, 1,894 and 554 sites within the human, mouse and pufferfish genomes, respectively [13]. Subsequent studies have increased this number to nearly 2000 sites within the human genome, of which amost 900 are conserved between humans and mice [14], [16]. RE-1 containing genes code for proteins, like growth factors, ion channels, and molecules involved in intracellular signaling, synaptic plasticity, metabolism, and neurotransmission [13]–[15]. Moreover, the RE-1 sequence is present also in the promoter of non-coding RNAs (ncRNAs). Indeed, REST is known to control, and be controlled by, various classes of ncRNAs, like microRNAs (miR-124, miR-9, miR-132) [17]–[19] and long ncRNAs [20],[21], and thus it is involved in the transcriptional and post-transcriptional regulatory activity of the ncRNA network [22].

In 2008, Jothi and colleagues performed a genome-wide sequencing analysis from ChIP-seq experiments, identifying 5813 putative RE-1 sites, most of them lying in intergenic regions of the genome (40%), about 24% in intronic regions, and only 15% in promoter sequences [23]. Among the RE-1 sequences located in and near promoter regions, most of them are found in neuron-specific genes.

This is why REST was initially thought to act as an inhibitor of neuronal genes in non-neuronal cells. However, subsequent studies have shown that REST is a master regulator of neurogenesis, and has other essential functions in both neuronal and non-neuronal cells. REST levels are progressively downregulated during neuronal differentiation, from embryonic stem cells to mature neurons. This process is regulated both by transcriptional and post-translational mechanisms [6]. In order to maintain low REST levels, a degradation signal sequence is located near the C-terminus domain of the protein, recognized by the ubiquitin ligase SCFβ-TrCP, which directs the protein for proteasome-mediated degradation [24]. More recently, REST has been identified not only as a repressor, but also as an activator of neuronal gene transcription, specifically recruiting TET3, the major methylcytosine dioxygenase expressed in neurons, which catalyzes the conversion of 5 methylcytosine to 5 hydroxymethylcytosine [25]. The interaction between REST and TET3 has been shown to activate the transcription of REST target genes, through the enhancement of TET3 hydroxylase activity and the consequent remodeling of the chromatin state.

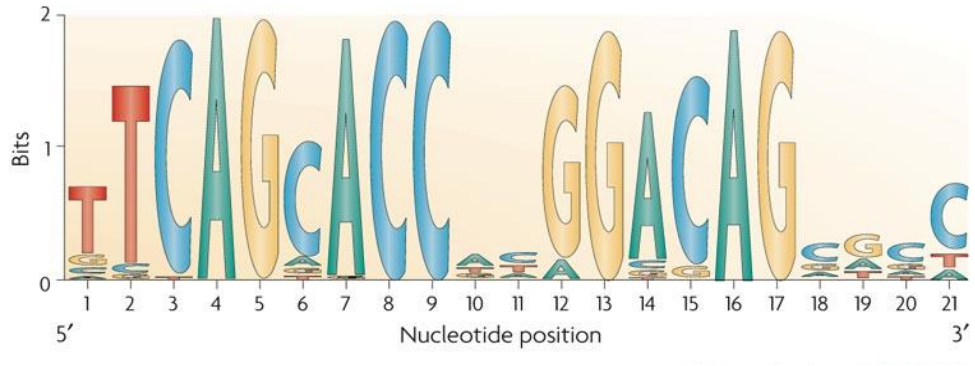


Figure 2. **RE-1 *cis* site sequence**. The 21 bp canonical REST binding site. The dimension of each nucleotide relates to the probability of that nucleotide at the indicated position within the transcription factor-binding site. From: *Ooi, L., and Wood, I. C. Chromatin crosstalk in development and disease: lessons from REST. (2007) Nat Rev Genet **8**, 544-554* [26].

The repressive function of REST is relevant for CNS physiology. As previously mentioned, it regulates the expression of many presynaptic and postsynaptic proteins, modulating membrane excitability and synaptic transmission. REST is well-known to repress the expression of various channels, such as Na_v [2], [27], calcium [28], [29] and potassium channels [30]. The role of REST is not limited to the developmental period, but it is noteworthy also during postnatal development; for example, REST mediates the transcriptional downregulation of the KCC2 chloride transporter, which is involved in the GABAergic switch from excitatory to inhibitory transmission during neuronal maturation [31]. Likewise, REST has been shown to downregulate the expression of Grin2b and GluR2, which code for the NMDA and AMPA receptor subunits, respectively [32], [33],

[34], providing further support to its fundamental role in the modulation of genes involved in synaptic activity and plasticity. Another REST is also involved in the control of transmitter release, whereby it represses several genes involved in neurosecretion, like SNAREs [35], and in vesicle trafficking, like synapsin 1 [36]. All the above-reported functions have profound implications for the role of REST in a wide spectrum of disorders, as described in the following paragraphs.

1.1.1. REST gene structure and isoforms

The human REST gene spans 24 kb of genomic DNA; it is composed of three alternative 5' non-coding exons associated with different promoters, three coding exons and an internal alternative exon that can be spliced into six alternative neuron- and disease-associated transcripts [37], [38]. At least six different splice variants of REST mRNA have been recognized, associated with neural gene expression and various pathological conditions [37], [39]. REST 1 and REST-5FΔ code for isoform 2 and isoform 4 respectively, while the alternative exon present in REST-N62, REST-N4 and sNRSF introduces a premature stop codon, so that all the three transcripts encode isoform 3, also called sNRSF or REST4 (**Fig. 3**).

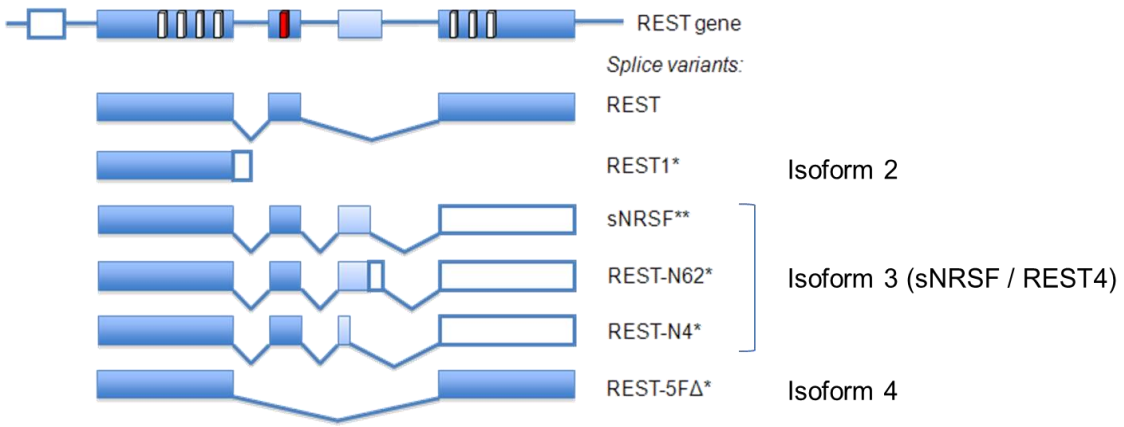
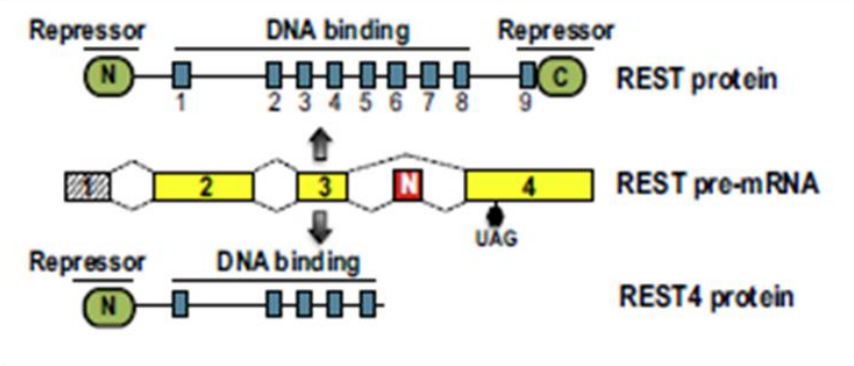


Figure 3. The human REST gene is characterized by three alternative 5' non-coding exons (all together represented by the first white box), three coding exons (dark blue) and one internal alternative exon (light blue). Small boxes indicate the position of zinc finger domains that encompass the DNA binding domain (white), and the nuclear localization signal (red). The gene can be transcribed into 6 distinct splice isoforms, which codify for 4 different REST protein isoforms as indicated. From: *Faronato and Coulson, REST (RE1-silencing transcription factor), Atlas Genet. Cytogenet. Oncol. Haematol. vol. 3, no. 2, pp. 208–213, 2011.* [40]

Further studies in humans extended the amount of context-specific alternatively spliced isoforms, linking them to different types of cancer and providing more cues on the complexity of REST gene regulation [41], [42]. Among all these splice variants, the most studied is REST4, which encompasses the N terminal repressor domain and 5 of the 9 zinc fingers of the full-length REST sequence, thus lacking the critical domains required for REST-mediated transcriptional silencing of target genes. This neuron-specific isoform is conserved in human, mouse and rat [38], but its biological function is not completely understood. REST4 was first identified in 1998 by Palm and colleagues, who showed that it

has repressor activity even without a direct DNA binding [37]. In the following years, various papers were published supporting the idea of REST4 as a de-repressor. REST4 is not able to bind the RE-1 site, as it lacks the 7th zinc finger domain, thought to be the responsible of DNA binding. Thus, a model was proposed whereby REST and REST4 interact to form an inactive heterodimer complex. REST4 is thus able to prevent the binding of REST to the RE-1 sequence, causing de-repression [32]–[34]. Later, this function was also observed in the context of the regulation of glutamine synthase expression in the nervous system, where REST4 is thought to act by enhancing the hormonal response, while REST inhibits it in the non-neuronal tissues [47]. REST4 levels increase in response to a variety of stimuli in neuronal cells, such as chronic stressful events [48]. The REST4 sequence is formed by an insertion of 16 nucleotides of the neural-specific N-exon, in the gene region between zinc fingers 5 and 6, followed by an in-frame stop codon (**Fig. 4A**). Its expression is regulated by the alternative splicing of a neural-specific Ser/Arg repeat-related protein of 100 kDa (nSR100/SRRM4), which mediates the inclusion of the above-mentioned neural-specific exon in the transcript. Moreover, REST directly silences the expression of nSR100, thereby preventing the expression of REST4 and other neural-specific AS variants in non-neural cells [45], [49] (**Fig. 4B**). The presence of distinct REST isoforms is frequently overlooked in literature, however it has to be considered in order to avoid data misinterpretation [42].

A



B

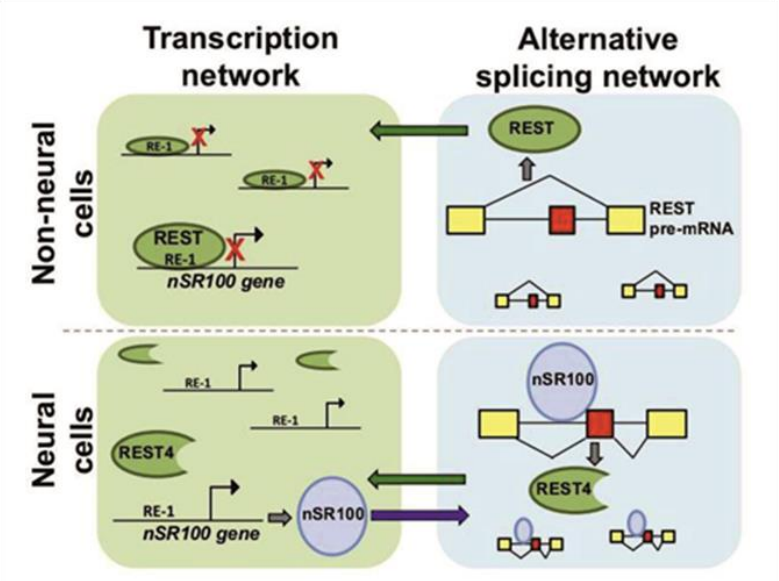


Figure 4. REST4 structure and activity are mediated by nSR100. (**A**) REST pre-mRNA can be spliced into either the full-length form of REST protein or into its splicing variant REST4, lacking the C-terminal repressor-binding domain. Red box corresponds to the neural-specific exon (N). (**B**) Scheme of the reciprocal inhibition between REST and REST4 mediated by nSR100 in non-neuronal and neuronal cells. From: *Raj B, et al. Cross-regulation between an alternative splicing activator and a transcription repressor controls neurogenesis. Mol Cell 2011;43: 843–50.* [49]

1.1.2. Animal models to study REST function

Several animal models have been developed over the years, which have been instrumental to better understand and define the function of REST in physiology and pathology. The first evidence of the requirement of REST for neuronal gene repression *in vivo* was demonstrated in 1998 by Chen and colleagues, through two different approaches [50]. Targeted gene deletion in mouse embryonic stem cells demonstrated the essential role of REST in development, as embryos bearing the complete deletion of REST did not survive beyond embryonic day 11.5. At the same time, the mosaic expression of a dominant negative form of REST in chick embryos resulted in the alteration of the physiological pattern of expression of several genes, confirming the function of REST in controlling the proper spatial and temporal expression of neuronal genes [50], [51]. As the REST gene is conserved across vertebrates, its role in the acquisition of the neuronal phenotype has been evaluated in animal models from different species. REST is involved in the early ectodermal patterning in *Xenopus laevis* [52], [53], and in the proneural development of sensory neurons, through its homologue gene *Charlatan,* in *Drosophila* [54], [55]. The zebrafish model allowed studying behavior upon full deletion of REST. Mutant REST zebrafish presented locomotion defects and erratic swimming [56], a phenotype that was ascribed to maternal REST, which is involved in neurogenesis and responsible for the regulation of several target genes during development, including *snap25a/b* [57], [58].

In more recent years, a number of REST conditional KO mouse lines have been generated, lacking REST in the excitatory neurons of the forebrain (CaMKII-Cre-driven) [59] or during neurogenesis (Nestin-Cre-driven) [60]. In 2011, Hu and colleagues studied the progression of kindling-induced seizures in the CaMKII-Cre driven REST conditional mouse model. In the absence of REST, they observed an acceleration in the development of seizures, with a concomitant worsening in mossy fiber sprouting, proposing REST as a modulator of the epileptic phenomenon [59]. In contrast, the following year, the same group reported an opposite effect in a Nestin-Cre driven REST conditional mouse model of pentylenetetrazol (PTZ)-induced epileptogenesis. In this case, ablation of REST attenuated the susceptibility to seizures [61]. These conflicting data could be explained by the different neurochemical pathways activated by the different seizures model, and / or by the different cell populations where REST is selectively deleted (excitatory neurons of the basal forebrain in CaMKII-Cre mice, *vs* all neuronal cells in the Nestin-Cre model). This suggests that REST may have different functions in the signaling pathways activated by the various seizure-inducing treatments, and/or in the various targeted cell types.

REST was demonstrated to have a central role in the determination of neuronal fate [62]. By using a mouse model lacking REST specifically in the neural stem cell population (NSC) (Nestin-Cre-driven), Gao and colleagues identified REST as a master negative regulator of adult neurogenesis, able to accelerate neuronal differentiation and the exit from the quiescent stem cells cycle [60].

The following year, Aoki and colleagues confirmed the repressive role of REST on the expression of neuronal genes in neuronal progenitor cells *in vitro*, as well as in non-neuronal cells outside the CNS; however, they failed to identify significant abnormalities in the neurogenesis of the developing or adult brain in the absence of REST *in vivo,* showing that mice lacking REST in the brain grow into normal adults [63]. The evident discrepancy between the two studies is likely due to the different experimental models employed. Gao et al. deleted REST acutely in the adult dentate gyrus (DG), while, in the work of Aoki et al, REST deletion was performed at early embryonic stages, which possibly triggered the activation of complementary mechanisms in the brain, which would mask REST function in adulthood. Overall, these data support the idea that REST repressive function has to be studied in a cell-type, time-specific and tissue-specific manner.

Furthermore, in the last years, attention has been focused on the consequences of REST overexpression. In wild type mice, an increased REST expression was shown to have a beneficial role in ageing, further improved by physical activity [64]. On the other hand, in a REST conditional overexpression model expressing the human REST gene in neural stem cells (Nestin-Cre-driven), REST has been shown to repress the Drd2 gene, which encodes a nigrostriatal receptor involved in the regulation of motor behavior, leading to locomotion deficits. Moreover, the homozygous overexpression of REST is embryonically lethal [65]. Once again, the overexpression of REST in different cells and tissues leads to very different, and sometimes opposite outcomes. Both the

complete deletion and overexpression of REST are lethal at the embryonic level, suggesting a physiological amount of REST is critical during embryonic neurogenesis.

In 2016, Nechiporuk and colleagues developed a novel REST transgenic mouse line where REST is eliminated at the transcriptional level from all the coding exons, in order to remove it prematurely from neural progenitors. This is different from the previously published models, characterized by single coding exons deletion, without a complete loss of REST sequence. In fact, as demonstrated in the same work, the use of a conditional REST KO mouse that target the deletion of exon 2 (as in [59] and [60]), maintains the expression of the C-terminal part of the protein, that can mediate gene repression itself, through the recruitment of HDAC molecules to the chromatin remodeling complex. Similarly, previous transgenic models where exon 4 is specifically deleted (as in [63]), leave intact the sequence which codifies for the N-terminal domain of the protein, suggesting that each terminal domain can maintain repressive function even singularly [66].

The Nechiporuk model carries a GT cassette between non-coding exon 1a-c and the first coding exon, exon 2 ($REST^{GT}$). The GT cassette includes a splicing acceptor site (SA), a reporter gene encoding a β-galactosidase neomycin fusion gene (β-geo) and a polyadenylation sequence (pA) (**Fig. 5A**). Researchers used a two-step breeding scheme: firstly they crossed $REST^{GT}$ mice to mice expressing Flp recombinase, obtaining the inversion of the GT cassette and the normal splicing of REST exons ($REST^{GTi}$) (**Fig. 5B**). Then, mice heterozygous

for the inverted allele were bred with mice bearing the pan-neuronal nestin-Cre transgene, resulting in re-inversion of the cassette, with the introduction of a stop codon upstream the SA and the remaining sequence of REST, obtaining the conditional knock out model (**Fig. 5C**) [67]. Animals bearing the pan-neuronal ablation of REST survived into adulthood, even if they show smaller brains, with widespread apoptosis and high levels of DNA damage during the S-phase of the cell cycle. These features are more severe when compared to the other previously developed models, since in those transgenic lines the loss of REST was not complete, as described before. By using this more precise model, authors suggest a functional role for REST repressor complex during neurogenesis, which results in the exit from the cell cycle, and the consequent neuronal differentiation.

The mice we are currently breeding are those reported in **Fig. 5B**, with the inverted GT cassette.

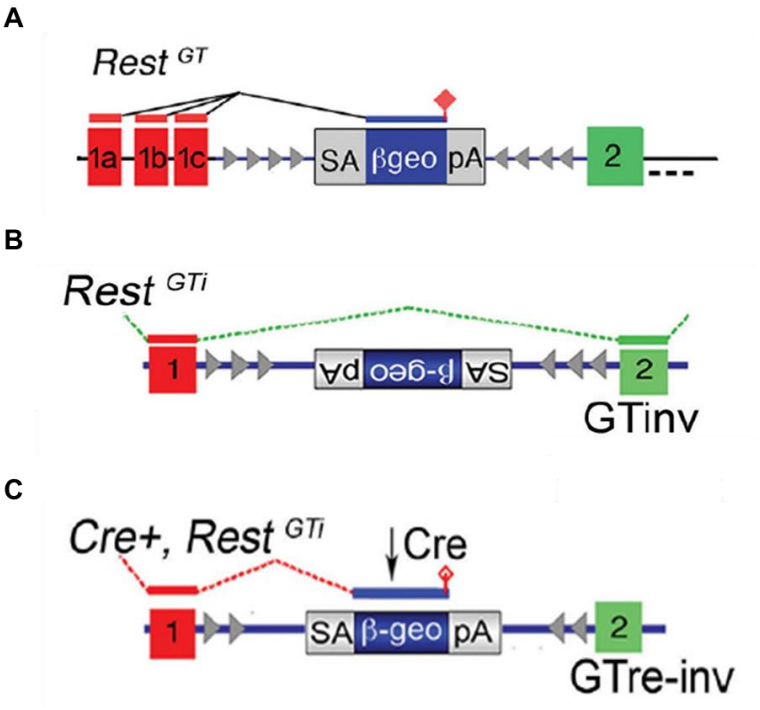


Figure 5. REST cKO model with a GT approach. (**A**) A floxed REST mouse model was generated by introducing a gene trap in the REST gene, between non-coding exon 1a-c and the coding exon 2. The GT cassette contains a SA site, a reporter gene encoding a β-galactosidase neomycin fusion gene (β-geo), and a pA sequence. Inverted triangles indicate target sites for Flp and Cre recombinases. (**B**) $REST^{GT}$ mice crossed with mice containing the Flp transgene generate $REST^{GTi}$ mice, with a correct splicing of REST transcript. (**C**) Conditional mutants result from mating $REST^{GTi}$ mice to mice bearing the Cre transgene. From: *Nechiporuk T, et al. The REST remodeling complex protects genomic integrity during embryonic neurogenesis. eLife. 2016; 5: e09584.* [67].

1.1.3. REST associated diseases

Given the well-defined role of REST in development, it is understandable how alterations of its expression and/or activity have been linked to several disorders, making REST a potential molecular target for therapeutic approaches [68], [69]. The expression levels of REST are altered in a number of neurological diseases and, depending on the tissue and the pathology, REST acts under some circumstances as a protective factor, and under other conditions as a promoter of insult-induced neuronal death or dysfunction, as discussed in the following paragraphs.

In brain, increased REST levels have been observed after epileptic or ischemic insults. In epilepsy, the role of REST is still debated. On the one hand, it seems to have a protecting role as it maintains cell homeostasis by downregulating genes like BDNF [70]; on the other hand, it appears to participate in the induction of the disease, mediating epileptogenesis by inhibiting genes such as HCN1, a hyperpolarization-activated, cyclic nucleotide-gated channel, involved in synaptic transmission and neuronal excitability. *In vitro* and *in vivo* studies with kainate, an agonist of glutamatergic receptor, have shown the upregulation of REST levels in hippocampal and cortical neurons [37], [59], [71], but whether such increase is protective or deleterious, is still not understood. In a rat model of global ischemia, REST is strongly upregulated in post-ischemic CA1 neurons, and linked to neuronal death through the suppression of the AMPA receptor subunit GluR2 [34], modulation of calcium permeability and the silencing of the

μ-opioid receptor 1 (MOR-1), which belongs to the family of G protein-coupled receptors, abundantly expressed in basket cells and GABAergic inhibitory interneurons of the CA1 region [72].

Only in recent years, research has focused on the involvement of REST in Alzheimer's disease. Two initial studies have shown the relationship between REST overexpression and choline acetyltransferase (ChAT, the enzyme responsible for the synthesis of acetylcholine) downregulation in specific brain areas affected by the disease, both in patients' brains and in a transgenic mouse model of AD [73], [74]. First evidences of the protective role of REST in neuropathologies appeared in the same year, with the work of Lu and colleagues, which investigated the role of REST in aging and AD. According to their data, REST increases during normal ageing in the brain, regulating the inhibition of a class of genes involved in cell death. On the contrary, in AD, frontotemporal dementia and dementia with Lewy bodies, REST is lost from neuronal nuclei and accumulates within autophagosomes in the cytoplasm, together with other pathological misfolded proteins, specifically in the regions more affected by the diseases (prefrontal cortex and hippocampus). As a consequence, several REST target genes are dysregulated, contributing to the pathogenesis of the disorder. On the basis of these observations, REST was presented for the first time as a neuroprotective modulator, able to protect neurons from oxidative stress and amyloid β-induced toxicity [75]. Similar conclusions were also reached in the context of prion diseases. In this case, REST was shown to protect neurons both *in vitro* and *in vivo*, in prion-infected

animals. In diseased cells, REST translocates from the nucleus to the cytoplasm, where it is targeted by the autophagy pathway, while in healthy neurons REST mediates neuronal survival by regulating the Akt-mTOR and Wnt-β-catenin signaling pathways [76], [77].

The translocation of REST between nucleus and cytoplasm is therefore fundamental for its function, as initially shown by its involvement in Huntington's disease (HD). Nuclear accumulation of REST in neurons of the striatum and cortex correlated with the expression of the mutant form of huntingtin (htt), the main protein responsible for the pathology. In this case, the nuclear accumulation of REST was shown to induce the repression of important target genes, amongst which BDNF [9], [10]. Subsequent studies have shown that the retention of REST into the cytoplasm is mediated by a complex between wild type htt, REST, RILP and Dynactin p150-Glued; in the diseased brain, instead, mutant htt does not support the formation of such complex, which eventually results in the pathogenic accumulation of REST inside the nucleus [7], [8].

In recent years, many studies have described alterations of REST in cancer, where it can act as either tumor suppressor or oncogene, depending on the cellular context [78]. In some cell types, such as neural tumors (medulloblastomas, neuroblastomas and glioblastomas), REST stimulates proliferation, preventing cell differentiation and acting as an oncogene [79]–[81]. In epithelial cells, instead, high REST levels prevent proliferation through the inhibition of various signaling cascades, such as the phosphoinositide 3-kinase (PI3K)–Akt and the inositol 1,4,5-triphosphate (IP_3)-metabolizing F actin-

bundling IP_3 kinase (ITPKA), thus acting as a tumor suppressor in carcinomas of the lung (small-cell lung cancer [SCLC]) and breast [82]–[84].

Altogether, data in the literature indicate that REST plays a multifaceted role in the diseases of the nervous system. It is therefore mandatory to understand the disease-specific molecular mechanisms underlying REST malfunctioning, to devise novel therapeutic approaches.

1.2. Neuroinflammation and Multiple sclerosis

Neuroinflammation is a key process in the complex biological response of the brain to insults. It is a symptom of many diseases and can influence the outcome and the severity of the pathology itself. The overall effect of neuroinflammation is the result of a fine balance between a wide array of cytokines, chemokines and growth factors, all of which may exert either neuroprotective or neurotoxic effects.

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS that affects approximately 2.5 million people worldwide, especially young adults [85]. The pathogenesis of the disease begins with the activation of inflammatory pathways, due to an inappropriate activation of T cells that trigger an immune response against myelin. T cells can penetrate into the CNS through the blood-brain barrier, producing cytokines and causing damage to myelin and surrounding tissues. The resulting demyelinated plaques in the white matter lead to neurodegeneration, with brain atrophy and ventricular enlargement in the progressive stage of the disease [86], [87]. In approximately 85% of patients, the disease starts with a phase of relapses and remissions (relapsing-remitting MS, RRMS) that, in 80% of the cases, evolves and becomes chronic into a progressive phase after 10-15 years (secondary progressive MS, SPMS) [85]. Ten to fifteen percent of patients do not go through the relapsing phase and are affected by an acute form of the disease from the onset, the so-called primary progressive MS (PPMS) [88], [89].

Progressive forms of MS are characterized by chronic inflammation, demyelination in white and gray matter, and diffuse neurodegeneration within the CNS associated with impaired synaptic function, loss of network connectivity and, ultimately, axonal loss. These changes are possibly amplified by pathogenic mechanisms related to brain ageing and accumulated disease burden. Mechanisms leading to neurodegeneration include microglia activation, chronic oxidative injury, altered ion channel activity, accumulation of mitochondrial damage in axons resulting in chronic cell stress and imbalance of ionic homeostasis, ultimately leading to neuronal death. Chronic inflammatory processes that continuously disturb neuronal homeostasis drive neurodegeneration, so the clinical outcome depends on the balance between inflammation and any remaining capacity for neuronal self-protection and repair. In recent years, tremendous progress has been made in identifying novel mechanisms and new medications that regulate immune cell function in MS. However, a significant unmet need is the identification of the mechanisms underlying neurodegeneration that associates with the progressive form of MS, as patients continue to manifest brain atrophy and disability despite current therapies. Due to the complexity and the large spectrum of symptoms of MS, various models have been established in order to experimentally recreate the clinical course, immunology and pathology of the disease.

1.2.1. MS *in vivo* models: the EAE mouse models

Different *in vivo* models are currently being used to study MS to confirm the efficacy and safety of pharmacological treatments tested *in vitro*. Since many human and animal inflammatory diseases are caused by viral infection, several models have been developed using viral agents like the Semliki Forest virus, the Theiler's murine encephalomyelitis virus or the mouse hepatitis virus [90], [91]. Viruses either have a direct effect on neurons, with the myelin damage as a secondary event, or attack directly myelin causing neuronal loss as a consequence. Models based on the delivery of toxins induce demyelination by focal application of specific substances (like ethidium bromide or lysopvhosphatidylcholine, [92], [93]) or by systemic administration of the toxin (such as the cuprizone model [94]). They present some limitations, but remain one of the most useful tools to study human demyelinating diseases. In order to understand the pathogenic mechanisms of MS, several transgenic mice have also been generated with deletion or overexpression of pathogenically relevant genes, such as those encoding T cell receptors, major histocompatibility complex molecules, cytokines and neurotrophic factors and their receptors [95], [96]. Although these mice spontaneously develop the disease, their use has remained limited. Last but not least, immunization of susceptible animals with CNS antigens gives rise to a spectrum of inflammatory disorders collectively named EAE (Experimental autoimmune encephalomyelitis).

EAE is the most common animal model for MS, sharing many clinical and physiopathological features with the human disease. Most of the current knowledge about MS originates from this model. Various types of EAE models have been developed to analyze the pathological features of the human disease. In particular, two approaches can be distinguished: actively-induced EAE (aEAE; active immunization) or passively transferred EAE (pEAE; transfer of encephalitogenic cells from an immunized animal). The easiest inducible model is aEAE in mice, considered as the "gold standard" of neuroimmunological animal models by many researchers in the field [97]. In the aEAE, the animal is immunized with an intraperitoneal injection of the selected antigen, dissolved in complete Freund's adjuvant and injected with pertussis toxin, on the day of immunization and two days later. The immunogenic complex induces the activation of myelin-specific T lymphocytes, which can cross the blood brain barrier and migrate into the CNS, activating the inflammatory processes. Symptoms usually appear 10 - 14 days after immunization, with an ascending flaccid paralysis (**Fig. 6**). The phenotype of EAE varies depending on the genetic background of the animals, the source of the antigenic material and the mode of application of the antigen. Currently, the most used and validated models are based on the injection of proteolipid protein (PLP)139-151, which induces a relapsing-remitted form of the disease in SJL mice, or myelin oligodendrocyte glycoprotein (MOG)35-55 that triggers chronic-progressive EAE in C57BL mice [96], [98].

A

Score	Clinical signs
0	Normal behaviour
1	Tail weakness
2	Limp tail and hind limb weakness
3	Partial hind limb paralysis
4	Complete hind limb paralysis
5	Moribund state; death

B

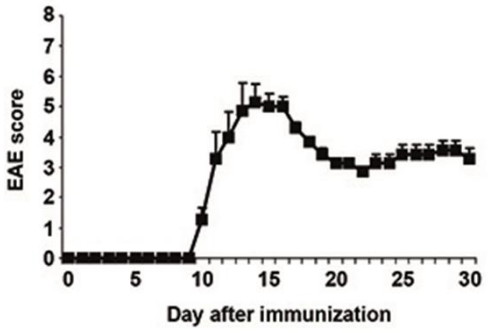


Figure 6. Clinical course of a MOG35-55 EAE mouse model. Clinical scoring system (**A**) and representative disease course (**B**) of EAE induced by MOG35-55 immunization protocol [99].

1.2.2. MS *in vitro* models

A more specific approach to study the cellular and molecular pathways involved in neuroinflammation, concerns the use of single cells or mixed cell cultures. Distinct primary cell cultures are used according to the feature that has to be investigated: microglia cells are key factors to study the inflammatory response against several types of insults, such as trauma, ischemia, and neurodegeneration [100], while oligodendrocyte cultures are used to study the re-myelination process [101]. The contribution of astrocytes to MS remains not fully elucidated, although recent literature indicates they are active players during neurodegeneration, inflammation and re-myelination [102], [103]. Indeed, astrocytes play a role in the evolution of the pathology, contributing to tissue damage on the one side, and confining inflammation on the other side, with the formation of glial scars typical of chronic MS lesions [104].

Neuronal cultures are used to analyze axonal damage and neurodegeneration in MS. When treated with cytokines, which mimic the typical inflammatory milieu of the neuro-inflammation process, they provide a useful acute model of inflammation [105]–[108]. In order to go closer to the physiological scenario, various co-cultures systems have been established, such as dorsal root ganglia cultured together with Schwann cells or neuronal-glia mixed cultures, in direct contact or separated by special inserts, in order to determine whether direct cell-cell contacts are a prerequisite for the observed effects [89], [95], [96].

2. AIM OF THE STUDY

Modifications of molecular pathways involved in neuronal development is an appealing strategy towards therapy for neurodegenerative diseases like chronic MS. REST controls a large cluster of genes regulating neurogenesis and is involved in several neurodegenerative disorders. In a pilot study, we have observed that REST was overexpressed in the spinal cord of mice affected by EAE, suggesting that its dysregulation might be an important factor in the disease. Whether elevated REST levels are pathogenic or the expression of an endogenous protective response to disease is unclear. In both cases, REST appears to be a very promising target to interfere with neuronal fate during neurodegeneration.

Starting from these observations, our overall goal has been to characterize the role of REST overexpression in the CNS of mice with EAE, by assessing (i) the expression levels of different REST isoforms and REST target genes in the spinal cord of EAE-affected mice, (ii) the cell-specificity of REST overexpression in EAE, investigating whether it is attributable to either neuronal overexpression or proliferation of REST-expressing glial cells, (iii) REST activity under inflammatory conditions *in vitro*, testing the hypothesis that neurotoxic pro-inflammatory molecules might trigger an activation of REST in injured neurons.

3. MATERIALS AND METHODS

3.1. Primary cultures of neurons

Primary cortical neurons were prepared from C57BL/6J mice (Charles River Laboratories, Calco, Italy) at embryonic day 17, dissected in ice-cold Hank's Balanced Salt Solution (HBSS), incubated with trypsin 0.25% (#25050-014, Gibco) for 30 min at 37°C, and mechanically dissociated. Postnatal cortical neurons were prepared from REST lox/lox mice [67] at postnatal day 0, dissected in ice-cold HBSS, incubated with trypsin EDTA 0.25% (#25200-056, Gibco) for 6 min at 37°C and mechanically dissociated. Neurons stained with a vital dye (Trypan blue; Sigma-Aldrich) were counted by using a Burker chamber. Neurons were plated on poly-L-lysine (0.1 mg/ml; Sigma-Aldrich)-treated 18 mm glass coverslips at a density of 50,000 cells per well, or on poly-D-lysine 6-well Petri dishes (0.1 mg/ml in H_2O, #P6407-5mg, Sigma-Aldrich) at a density of 500,000 cells per well. Cells were grown in Neurobasal Medium for embryonic neurons (#21103049, Gibco) or Neurobasal A Medium for postnatal neurons (#10888022, Gibco) plus B-27 2% (#17504044, Gibco), Glutamax 1% (#35050038, Gibco) and penicillin-streptomycin 1% (#15140122, Gibco).

3.2. Pharmacological treatments

Neuronal cultures were treated with pro-inflammatory cytokines, singularly or in combination: IL-6 (20 ng/ml), TNF-α (20 ng/ml), IL-1β, (20 ng/ml), IFN-γ (20 ng/ml) (PeproTech Inc., Rocky Hill, NJ, USA) or with the same volume of BSA 0.1% in H_2O as control. Drugs were added at different time points and cells collected at 7 DIV or 14 DIV.

3.3. RNA preparation and qRT-PCR

Total cellular RNA was extracted using Trizol (Qiagen) and RNeasy MinElute Cleanup Kit (#74204, Qiagen), and cDNA was synthesized starting from 0.5 µg of RNA, using the SuperScript IV Reverse Transcriptase kit (Invitrogen) and following manufacturer's instructions. The cDNA was amplified and quantified by quantitative real-time PCR with the SYBR Green Master Mix (Qiagen) and Bio-Rad CFX96 Real-Time PCR Detection System. Transcript levels from each sample were normalized to the following housekeeping genes: 18S, β-actin, GAPDH, HPRT1 and Tubulin2. The sequences of the primers used are the following:

18S-Fw 5'-CGGACAGGATTGACAGATTGAT-3'

18S-Rv 5'-CCAGAGTCTCGTTCGTTATCG-3'

β-Actin-Fw 5’-AAGTGGTTACAGGAAGTCC-3’

β-Actin -Rv 5’-ATAATTTACACAGAAGCAATGC-3’

GAPDH-Fw 5’-GAACATCATCCCTGCATCCA-3’

GAPDH-Rv 5’-CCAGTGAGCTTCCCGTTCA-3’

HPRT1-Fw 5’-AAGCTTGCTGGTGAAAAGGA-3’

HPRT1-Rv 5’-TTGCGCTCATCTTAGGCTTT -3’

Tubulin2-Fw 5’-CAAGGCTTTCCTGCACTGGT-3’

Tubulin2-Rv 5’-AACTCCATCTCGTCCATGCC-3’

REST-Fw 5’-ACCACTGGAGGAAACACCTG-3’

REST-Rv 5’-ATGGCTTCTCACCTGAATGAGTC-3’

REST4-Fw 5’-ACCACTGGAGGAAACACCTG-3’

REST4-Rv 5’-CTCACCCAGCTAGATCACACTC-3’

For the Nanostring analysis of REST target and non-target genes, fluorescently labeled probes were designed and synthesized by Nanostring Technologies Inc. (Seattle, WA). One hundred ng of total RNA per sample, prepared as described above, was processed in the Center for Genomic Science, Istituto Italiano di Tecnologia, Milano (Italy), following standard procedures. For the sequence of the probes refer to Paonessa et al. 2016. Data were analyzed by using the nSolver^{TM} Analysis Software Version 2.5.

3.4. Immunoblotting

Dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed according to standard procedures. Both tissues and neuronal cells were 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, #4693116001, Roche Diagnostic; serine/threonine phosphatase inhibitor and tyrosine phosphatase inhibitor, #P5726, #P0044, Sigma) and equal amounts of proteins were loaded, as determined by BCA assay (#23225, Thermo Scientific). Samples were separated on 6-10% SDS polyacrylamide gels and proteins transferred to a nitrocellulose membrane with 0.2 µm pore size (#10600001, GE Healthcare). Membranes were washed in TBS containing 0.1% Tween (TBST) and blocked with 5% BSA in TBST buffer for 1 h at room temperature (RT). Primary antibodies were diluted in blocking solution and incubated overnight at 4°C in a humidified chamber. Primary antibodies used: anti-REST 1:1000 (#07-579, Millipore), anti-Calnexin 1:70000 (#ADI-SPA-860, Enzo Life Sciences), anti-REST4 1:1000 (homemade, kindly gifted by Dr. Uchida, Yamaguchi University Graduate School of Medicine), anti-pCREB 1:500 (#87G3, Cell Signaling), anti-CREB 1:1000 (#86B10, Cell Signaling). Membranes were washed 3 times in TBST to eliminate primary antibody in excess. Appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies were diluted in blocking

solution and incubated for 1 h at RT. Membranes were washed 3 times in TBST to remove secondary antibodies in excess and detected using the ECL™ Western Blotting Detection Reagents (#GEHRPN2106, GE Healthcare BioSciences, Buckinghamshire, UK). Images were acquired via the ChemiDoc MP System (BioRad).

3.5. EAE induction and scoring

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 (200 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 [110]. Body weight and clinical score (0, healthy; 1, limp tail; 2, ataxia and/or paresis of hind limbs; 3, paralysis of hind limbs and/or paresis of forelimbs; 4, tetra paralysis; 5, moribund or death) were recorded daily.

3.6. Immunohistochemistry and microscopy

Different immunostaining protocols have been followed to label cells cultured *in vitro* and sections of tissue samples.

Brains and spinal cords were obtained from transcardially perfused naïve and EAE adult animals and used for immunohistochemistry. Tissues were fixed in 4% PFA in PBS at 4°C overnight, equilibrated in 30% sucrose and embedded in OCT tissue frozen medium. Coronal sections (14 µm) were cut with a cryostat and stored at −20°C before immunostaining. Sections were rehydrated in PBS for 5 min and incubated in the antigen retrieval solution (10 mM sodium citrate, pH 6.0, 0.1% Tween-20) at 95°C for 10 min. Slices were subsequently blocked with 5% BSA in PBS and incubated overnight at 4°C in the following primary antibodies: anti-REST 1:300 (homemade, kindly gifted by Prof. Gail Mandel, Howard Hughes Medical Institute), anti-NeuN 1:1000 (#DAB377, Millipore), anti-s100β 1:200 (#287003, Synaptic Systems), anti-Iba-1 1:1000 (#019-19741, Wako). Sections were then stained with secondary species-specific antibodies conjugated to Alexa-488 or Alexa-647 (Invitrogen, Waltham, MA), and counterstained with DAPI to reveal nuclei. After washes, sections were mounted on glass coverslips with Mowiol. Images were acquired at an SP8 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with 63x and 40x objectives. Images were visualized and processed by using the Leica LAS X, ImageJ and Photoshop software programs.

3.7. Patch-clamp electrophysiology

All experiments were performed using an EPC-10 amplifier controlled by the PatchMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and an inverted DMI6000 microscope (Leica Microsystems GmbH). Patch electrodes fabricated from thick borosilicate glasses were pulled to a final resistance of 4−5 MΩ. Recordings with leak current > 100 pA were discarded. All recordings were acquired at 10-20 kHz. Primary mouse cortical neurons exposed at 7 DIV for 20 min to IL-1β or to the respective vehicle were used for patch-clamp recordings at 14 DIV. The standard Tyrode's extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, 5 glucose, pH 7.4, with NaOH and osmolarity adjusted to ~315 mOsm/l with mannitol. The intracellular (pipette) solution was composed of (in mM): 126 K gluconate, 4 NaCl, 1 MgSO4, 0.02 CaCl2, 0.1 BAPTA, 15 glucose, 5 Hepes, 3 ATP, and 0.1 GTP, pH 7.3. Experiments were carried out at RT (20–24°C). All parameters were analyzed using the Minianalysis program (Synaptosoft, Leonia, NJ, USA) and Prism6 (GraphPad Software, Inc.) software. Miniature postsynaptic currents (mPSCs) were recorded in voltage-clamp configuration at -70mV of membrane potential in the presence of tetrodotoxin (TTX, 300 nM) in the extracellular solution to block the generation and propagation of spontaneous action potentials. To isolate mEPSCs currents, bicuculline methiodide (30 μM), and (2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid hydrochloride (CGP 55845; 5 μM) were added to block GABA A and GABA B

receptors, respectively. All reagents were purchased from Sigma Aldrich or Tocris (Tocris, Avonmouth, Bristol, UK).

3.8. Lentivirus production and infection procedures

Third-generation lentiviruses were produced by transient four-plasmid co-transfection into HEK293T cells using the calcium phosphate transfection method. Supernatants were collected, passed through a 0.45 μm filter, and purified by ultracentrifugation as previously described. Viral vectors were titrated at concentrations ranging from 1×10^8 to 5×10^9 transducing units (TU)/mL and used at a multiplicity of infection (MOI) of 10. The efficiency of infection calculated from the ratio between neurons expressing GFP and total cells stained with DAPI, ranged between 70% and 90%. Primary cortical neurons were infected at 1 DIV and treated with IL-1β at 7 DIV for 20 min. Experiments were performed at 14 DIV.

3.9. Statistical analysis

The statistical analysis is described in the figure legends. Data are given as means ± SEM for n = sample size. To check for normal distribution of data, the D'Agostino-Pearson's normality test was used. To compare data between two groups that were not normally distributed, we used the non-parametric Mann–

Whitney's *U-test*. Two-way ANOVA was used for comparison among multiple experimental conditions. Statistical significance was set at P-value < 0.05, using the GraphPad Prism statistical software 7.03.

4. RESULTS

4.1. Optimization of an experimental toolkit to unambiguously identify full-length REST and REST4.

Most published studies failed to report the size of the immunoreactive band of REST when examined by western blotting, eventually leading to data misinterpretation and controversial findings. Because of this and because of the very low expression of REST in the adult brain, the detection of REST-specific bands by biochemical experiments is a challenge. Moreover, as discussed in the introduction, in mice the REST gene is mostly expressed as a full-length transcript, but the REST4 splicing variant can also occur. Thus, we first optimized a western blotting protocol to unambiguously identify the REST messenger and protein. To distinguish between REST- and REST4-dependent effects, specific qRT-PCR primers were designed, and specific antibodies were tested, to selectively recognize full-length REST and REST4 at the mRNA and protein level.

Kainic acid (KA) treatment has been reported to increase REST protein levels in the cortex and hippocampus in rodents [37], [59]. Thus, this treatment was chosen to identify REST-specific immunoreactive bands. Primary mouse cortical neurons *in vitro* were exposed to KA (100 μM) for 20h and the nitrocellulose membrane was probed with the Millipore #07-579 anti-REST antibody. An

appreciable increase in the intensity of a double band around 180 kDa was observed, compared to non-treated, control samples. This band was identified as phosphorylated REST (**Fig. 7A** left, experiment performed by Dr. Rocchi). Similarly, cell extracts of REST lox/lox primary cortical neurons infected with CRE or deltaCRE expressing lentiviral vectors, were subjected to KA treatment. We observed a significant decrease in the intensity of REST immunoreactive band in samples infected with the CRE-carrying lentivirus, ultimately confirming the specificity of the REST antibody used (**Fig. 7A**, right). Subsequently, mice were treated with saline or KA (30 mg/Kg) and followed for 24 h and 48 h according to the published protocols. Various CNS areas were isolated from vehicle- and KA-treated animals and analyzed by western blotting: cerebellum, cortex, hippocampus, striatum, lower and upper spinal cord. The nitrocellulose membrane was probed with the same Millipore #07-579 anti-REST antibody, which recognizes only the full-length form of the protein (**Fig. 7B**). According to the quantification of the double band just above the 150 kDa marker, REST immunoreactivity increased in cortex of the animals treated with KA, as reported in literature. The western blotting protocol was thus optimized to clearly determine REST immunoreactivity and employed for all the subsequent biochemistry experiments.

In order to distinguish between REST and REST4 mRNAs, we designed specific primers able to selectively recognize the full-length form of REST (REST-FL), but not REST4, and vice versa. **Figure 7C** shows the amplification

curves obtained using REST-FL and REST4-specific primers on plasmids coding full-length REST (myc-REST, left) and REST4 sequence (right).

Moreover, REST4-specific antibodies were tested transfecting Neuro2a neuroblastoma cells (N2a) with three distinct plasmids coding for REST4, human REST (hREST) and full-length myc-tag REST (myc-REST), respectively. This antibody is able to recognize the C-terminal epitope SECDLVG of REST4 sequence, which belongs to the first part of N-exon [48]. In figure 7D, the first membrane was probed with an anti-GFP antibody able to recognize the GFP sequence cloned in the REST4 and hREST vectors (**Fig. 7D** left). The second and third membranes were probed with the anti-REST4 and anti-REST full-length antibodies respectively, confirming the specificity of the two antibodies for their respective REST isoforms (**Fig. 7D** middle and right).

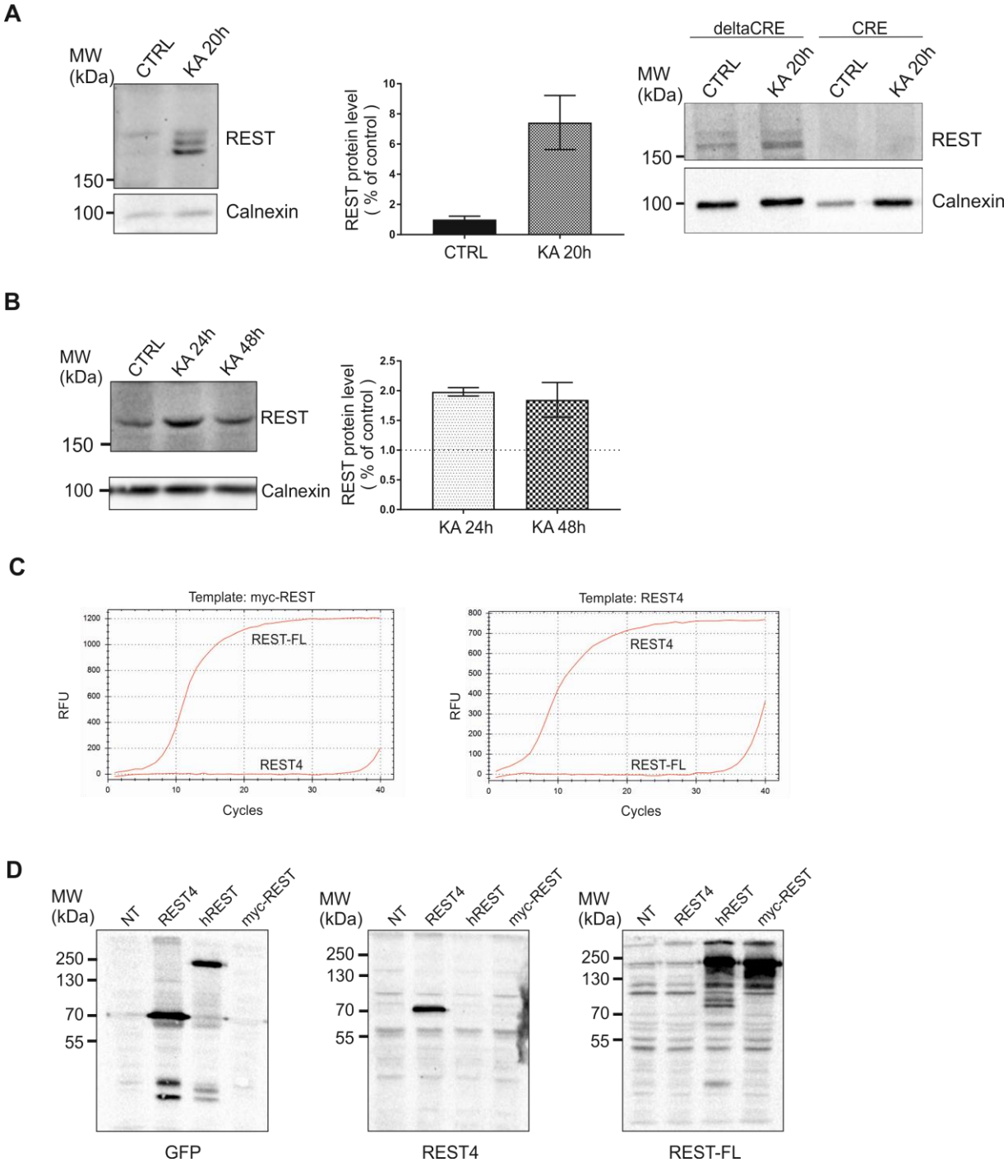

A
MW (kDa)
CTRL
KA 20h
REST
150
100
Calnexin
REST protein level (% of control)
CTRL
KA 20h
deltaCRE
CRE
B
KA 24h
KA 48h
C
Template: myc-REST
REST-FL
REST4
RFU
Cycles
Template: REST4
D
NT
REST4
hREST
myc-REST
250
130
70
55
GFP
REST4
REST-FL

Figure 7: Different REST isoforms are discriminated at the mRNA and protein level. (**A**) Representative western blot analysis (left) and quantification (middle) of REST protein levels in mouse cortical neurons treated with vehicle (CTRL) or KA (100 μM) for 20 h. Representative western blot of REST lox/lox mouse cortical neurons infected with CRE or deltaCRE expressing lentiviral vectors and treated with vehicle (CTRL) or KA (100 μM) for 20 h (right). (**B**) Representative immunoblot (left) and quantification (right) of brain samples from mice treated with KA (30 mg/Kg), for the indicated times. Values of KA animals are expressed as % of control mice. Data are expressed as means ± s.e.m. Anti-REST antibodies: Millipore #07-579; anti-calnexin antibodies were used to verify equal loading. (**C**) qRT-PCR amplification curves obtained using REST-FL and REST4-specific primers, using a vector containing the full- length REST sequence (myc-REST, left) or the REST4 sequence (right) as template. (**D**) Representative western blot experiment showing N2a cells not transfected (NT) or transfected with plasmids coding REST4, hREST and myc-REST. Membranes were probed with different antibodies: anti-GFP (left), anti-REST4 (middle) and anti-REST full-length (#07-579, right).

4.2 REST is overexpressed in the spinal cord of mice affected by EAE

From a pilot study conducted on spinal cord samples of EAE-affected mice, full-length REST appeared to be overexpressed immediately after the onset of the disease symptoms. Such overexpression was accompanied by downregulation of one of its target genes, the voltage-gated sodium channel Nav1.2, confirming REST transcriptional repression and suggesting neuronal dysregulation at this early stage (data not shown). Starting from this observation, full-length REST mRNA levels were firstly measured at different relevant stages of EAE, from the asymptomatic (7 days post immunization, dpi) to the chronic phase (14 days post-onset, dpo), and in various CNS regions. The most significant increase of REST expression was observed at 4 dpo, during the peak of the disease symptoms, in both the upper and lower regions of the spinal cord (**Fig. 8A**, experiments performed by Ms. V. Petrosino). Spinal cords were extracted from naïve and EAE-affected mice at the same time point and subjected to SDS-PAGE and immunoblotting analysis. The results showed a significant upregulation of REST protein levels in the lower spinal cord (**Fig. 8B** left), while in the upper spinal cord REST levels were comparable between EAE-affected and naïve mice (**Fig. 8B** right).

Analysis of REST4 at the same disease point revealed a significant upregulation of mRNA levels restricted to the upper spinal cord of EAE-affected mice (**Fig. 8C** left). This, however, was not paralleled by an increase of protein levels,

which instead showed a trend to be downregulated in both upper and lower spinal cord samples (**Fig. 8D** left).

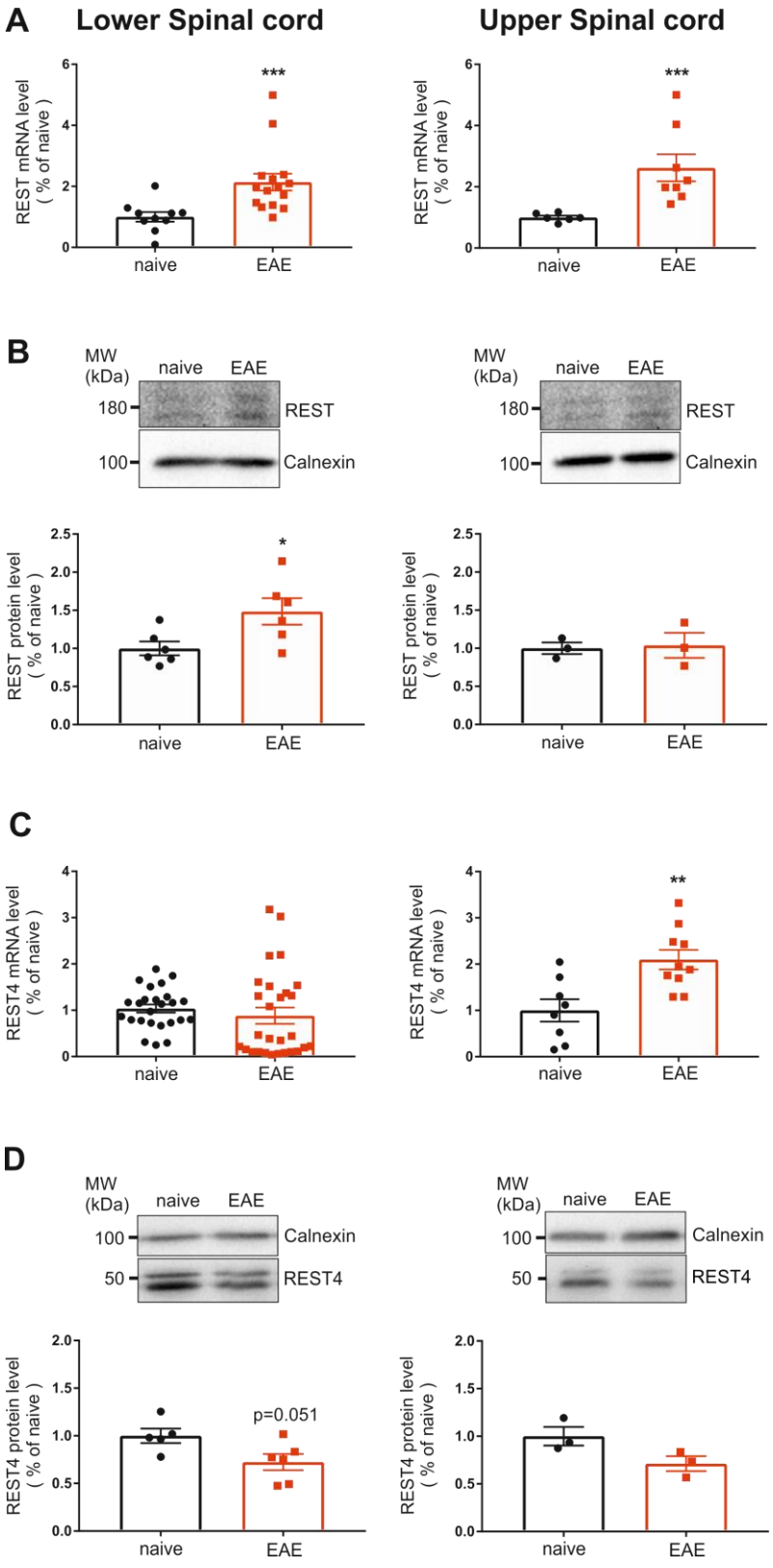

A
Lower Spinal cord
Upper Spinal cord
REST mRNA level (% of naive)
***
naive
EAE
B
MW (kDa)
naive
EAE
180
REST
100
Calnexin
REST protein level (% of naive)
*
C
REST4 mRNA level (% of naive)
**
D
REST4
50
REST4 protein level (% of naive)
p=0.051

Figure 8. Expression levels of full-length REST and REST4 mRNA and protein in the spinal cord of EAE mice at 4 dpo. (**A**) qRT-PCR analysis of full-length REST mRNA levels in the lower (left) and upper (right) spinal cord samples from naïve and EAE mice. Lower spinal cord: n = 10 naïve and 15 EAE animals; upper spinal cord: n = 6 naïve and 8 EAE animals. (**B**) Representative immunoblots (top) and quantification of REST protein level (bottom). Lower spinal cord: n = 6 naïve and 6 EAE animals; upper spinal cord: n = 3 naïve and 3 EAE animals. (**C**) qRT-PCR analysis of REST4 mRNA levels. Lower spinal cord: n = 25 naïve and 28 EAE animals; upper spinal cord: n = 8 naïve and 10 EAE animals. (**D)** Representative immunoblot (top) and quantification of REST4 protein level (bottom). Lower spinal cord: n = 5 naïve and 6 EAE animals; upper spinal cord: n = 3 naïve and 3 EAE animals. Anti-calnexin antibodies were used in all blots to verify equal loading. Values of EAE animals are expressed as % of naïve mice. Data are expressed as means ± s.e.m. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U-test.

4.3. The expression of REST target genes is reduced in the spinal cord of EAE-affected mice

A comprehensive analysis of REST-target and non-target genes expression in the spinal cord was performed using the NanoString nCounter™ gene expression system, a technology able to capture and count individual mRNA transcripts without amplification [111]. **Fig. 9** summarizes the results of two independent experiments, for a total of 4 mice per experimental group. The fold change EAE-affected / naïve is reported for the selected 80 genes, including REST-target (RE1-containing) and non-REST-target (non-RE1) genes, and keeping the results of the first and second experiment separated to appreciate the inter-experimental variability. Values are reported in a color-coded fashion, where blue / red colors correspond to genes that are respectively less / more expressed in EAE samples than in naïve samples. The data from the two experiments are consistent, showing a trend for RE1 genes to be downregulated in the spinal cord region. On the other side, the expression of most 'non-RE1' genes is very similar between EAE-affected and naïve mice, with some genes (particularly those belonging to cytokine signaling pathways) being clearly upregulated in samples from EAE-affected mice.

		LSC1	LSC2
RE1	Aplp1		
RE1	Bdnf		
RE1	Cacna1h		
RE1	Cacna1i		
RE1	Cacng2		
RE1	Calb1		
RE1	Cdk5R1		
RE1	Cdk5R2		
RE1	Celsr3		
RE1	Chat		
RE1	Chrm4		
RE1	Chrnb2 (Acrb2)		
RE1	Drd3		
RE1	DSCAM		
RE1	Gabrb3		
RE1	Gabrg2		
RE1	Gad1		
RE1	Glra3		
RE1	Gria2		
RE1	Grik4		
RE1	Grin1		
RE1	Grin2a		
RE1	Grin2b		
RE1	Kcnq2		
RE1	L1cam		
RE1	Ncam1		
RE1	Nefh		
RE1	Nefm		
RE1	Nes		
RE1	Neurod1		
RE1	Neurod2		
RE1	Nfasc		
RE1	Npas4		
RE1	Nrxn1		
RE1	Nrxn3		
RE1	Ntrk3		
RE1	Oprm1 (MOR-1)		
RE1	P2ry4		
RE1	Penk		
RE1	Pou4f3		
RE1	Scn2a1		
RE1	Scn3b		
RE1	Snap25		
RE1	Sst		
RE1	Stat1		
RE1	Syn1		
RE1	Syn2		
RE1	Syp		
RE1	Syt2		
RE1	Syt4		
RE1	Syt6		
RE1	Tubb3		

		LSC1	LSC2
non-RE1	App		
non-RE1	Camkk2		
non-RE1	GAP43		
non-RE1	Gpi1		
non-RE1	Gtf3c1		
non-RE1	Map2k2		
non-RE1	Map3k5		
non-RE1	Nos2		
non-RE1	Nox1		
non-RE1	Rcan1		
non-RE1	Stat3		
non-RE1	Stat4		
non-RE1	Syt17		
non-RE1	Tab1		
non-RE1	Tap2		
non-RE1	Tapbp		
non-RE1	Tbx21		
non-RE1	Tdo2		
non-RE1	Tfe3		
non-RE1	Tfeb		
non-RE1	Tnf		
non-RE1	Tuba1a		
non-RE1	Ubc		

RATIO EAE/naive	
	0 - 0.4
	0.4 - 0.6
	0.6 - 0.8
	0.8 - 1.0
	1.0 - 1.5
	1.5 - 2.0
	2.0 - 5.0
	5.0 - 10.0
	10.0 - 20.0

Figure 9: Transcriptional analysis of lower spinal cord samples from naïve and EAE-affected mice at 4dpo. mRNA from lower spinal cord (LSC) samples of EAE (4 dpo) and naïve animals was analyzed through the nCounter system. Two animals per group for the first experiment (values averaged in LSC1) were analyzed, and two for the second experiment (values averaged in LSC2). Values are normalized against five housekeeping genes (PPIA; Pgk1; Hdac3; GAPDH; HPRT) and expressed as fold change EAE/naïve. The blue color corresponds to genes that are less expressed in EAE compared to naïve, while red color indicates a higher expression in EAE samples.

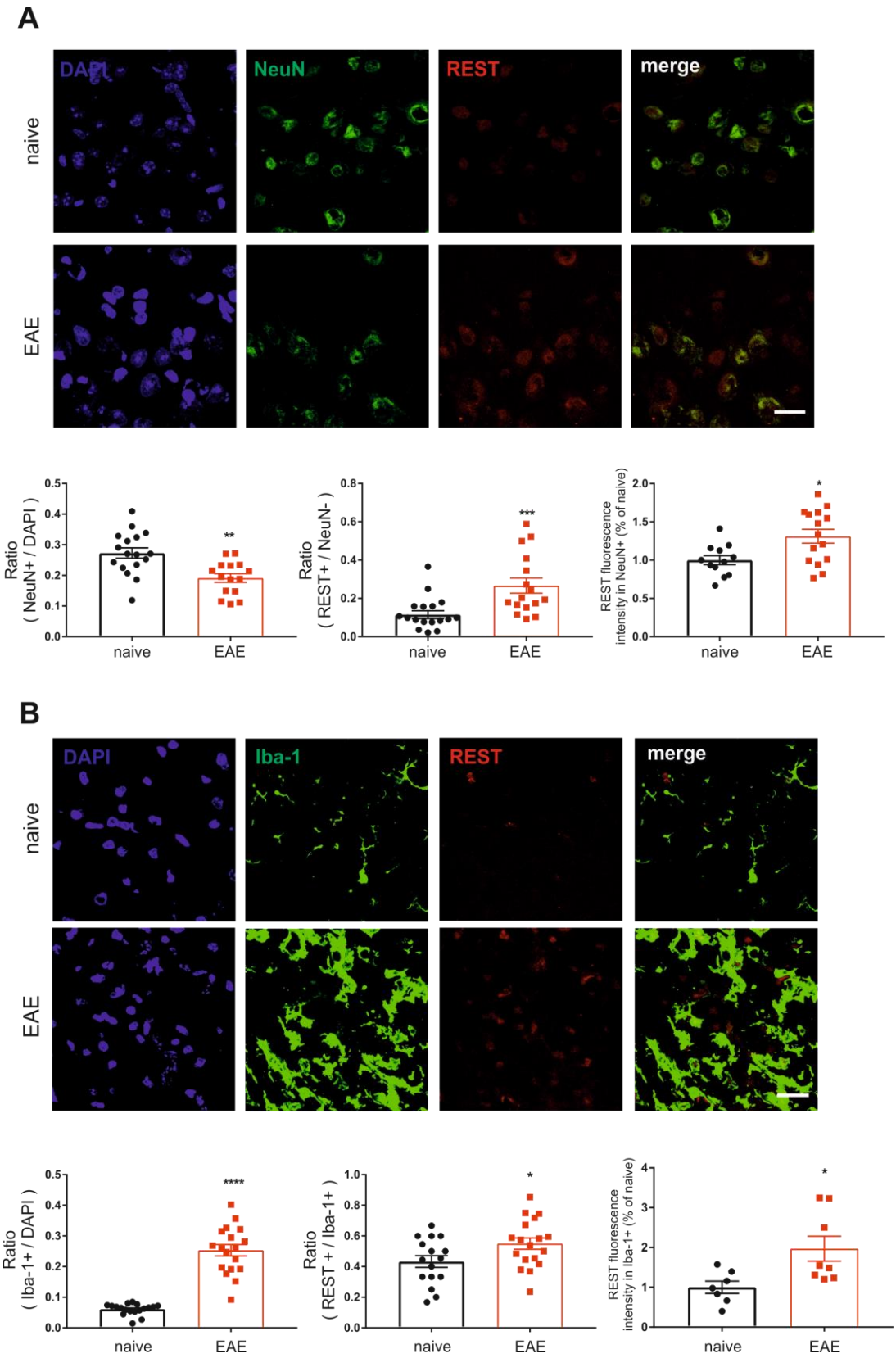
4.4. Characterization of the cell-specificity of REST overexpression in EAE-affected mice

Western blotting analysis does not allow to identify which cell population(s) are involved in REST overexpression. Thus, we sought to visualize REST localization by immunohistochemistry and confocal microscopy in spinal cord tissues derived from naïve and EAE mice. Immunostaining was performed on spinal cord slices by using anti-REST antibodies and anti-NeuN antibodies as marker of neuronal nuclei (**Fig. 10A**). REST immunoreactivity was localized mainly to nuclei, and all neuronal nuclei were REST-positive. The total number of REST-positive cells remained constant overall but, in samples derived from EAE-affected mice, neurons decreased significantly, most likely as a consequence of the pathology that can induce appreciable neuronal death in the lower part of the spinal cord at this stage of the disease (**Fig. 10A**, left). Consistent with these observations, there was a striking increase in the number of REST-positive non-neuronal cells in samples from EAE-affected mice (**Fig. 10A**, middle). In order to address the contribution of neuronal REST to the global REST overexpression, the intensity of REST fluorescence was measured in NeuN-positive nuclei, unveiling a significant increase in neuronal REST expression in EAE samples, compared to naïve (**Fig. 10A**, right).

To assess the identity of the glial cell population(s) that contributed to REST overexpression, co-immunostaining with anti-REST antibodies and anti s100β or anti-Iba-1 antibodies to label astrocytes or microglial cells, respectively, was

performed, and slices were analyzed by confocal microscopy. Quantification of Iba-1-positive cells showed a noticeable upregulation of microglial cells, as consistent with the fact that microgliosis is a key neuropathological feature of EAE (**Fig. 10B**, left). An increased number of REST-positive microglial cells in EAE samples (**Fig. 10B**, middle) and a concomitant increase in REST fluorescence intensity in these cells (**Fig. 10B**, right), were observed. Thus, more microglial cells express REST in EAE, and at higher levels.

Similarly, the analysis of s100β immunoreactivity showed an upregulation also in the number of astrocytes (**Fig. 10C**, left), while, the number of REST-positive astrocytes remains constant between the two experimental groups (**Fig. 10C**, middle). Nevertheless, they show an upregulation in the expression of REST fluorescence intensity (**Fig. 10C**, right).


A
DAPI
NeuN
REST
merge
naive
EAE
Ratio (NeuN+ / DAPI)
Ratio (REST+ / NeuN-)
REST fluorescence intensity in NeuN+ (% of naive)
naive
EAE
B
DAPI
Iba-1
REST
merge
naive
EAE
Ratio (Iba-1+ / DAPI)
Ratio (REST + / Iba-1+)
REST fluorescence intensity in Iba-1+ (% of naive)
naive
EAE

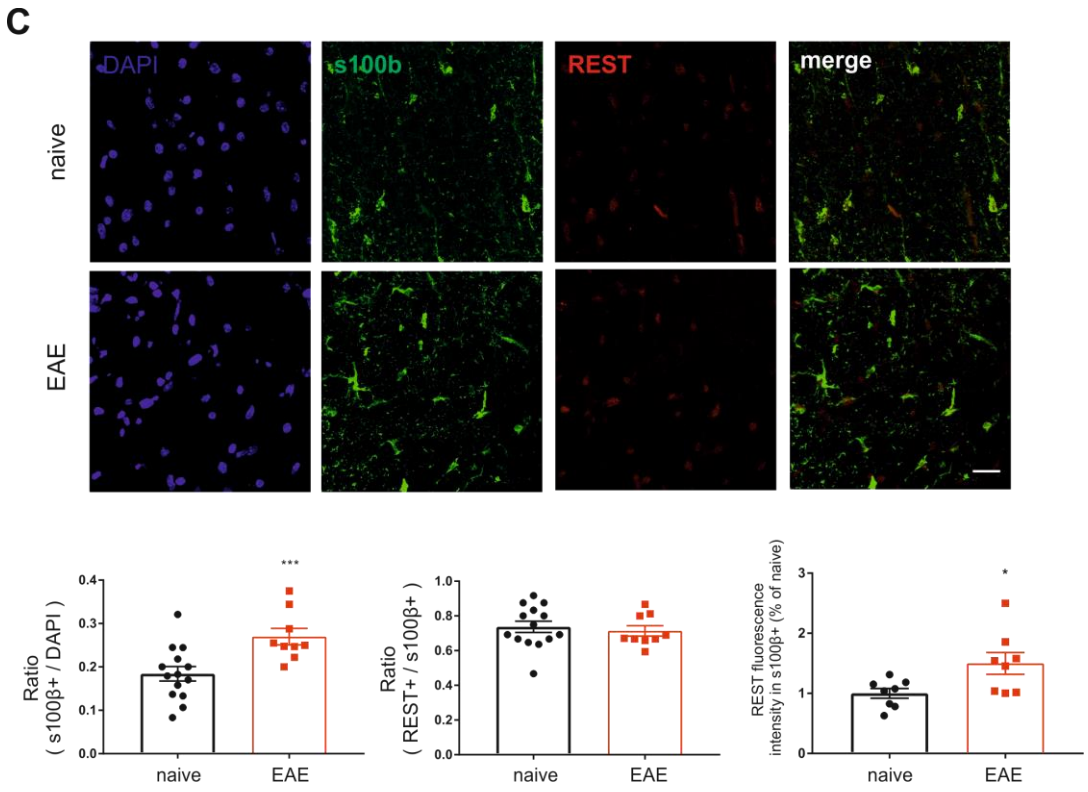


Figure 10. Cell-specificity of REST overexpression in lower spinal cord samples from naïve and EAE mice at 4 dpo. Lower spinal cord tissue samples from naïve and EAE mice at 4 dpo were processed for immunofluorescence with the following antibodies: anti-REST antibodies (red in all panels); anti-NeuN (neurons, panel A), anti-Iba1 (microglia, panel B) and anti-s100β (astrocytes, panel C); nuclei were stained with DAPI (blue in all panels). Scale bars: 20 μm. In each panel, representative confocal images are shown at the top, while the corresponding quantification of fluorescence data is at the bottom. (**A**) Left: Quantification of NeuN+ cells. All NeuN+ cells are also REST+. Middle: Quantification of REST+ /NeuN- cells. Right: Quantification of REST fluorescence intensity in NeuN+ nuclei. n = 12-18 slices, from 4 animals per experimental group. (**B**) Left: Quantification of Iba1+ cells. Middle: Quantification of REST+/Iba1+ cells. Right: quantification of REST fluorescence intensity in Iba1+ cells. n =7-18 slices, from 4 animals per experimental group. (**C**) Left: Quantification of s100β+ cells. Middle: Quantification of REST+/s100β+

cells. Right: Quantification of REST fluorescence intensity in s100β+ nuclei. n =8-14 slices, from 4 animals per experimental group. Data are expressed as means ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U-test.

4.5. Characterization of REST expression under inflammatory conditions *in vitro*

4.5.1. IL-1β treatment at 7 DIV induces a transient increase in REST mRNA/protein expression and CREB phosphorylation

To test the postulate that neurotoxic pro-inflammatory molecules might trigger an activation of REST in injured neurons, young (7 DIV) and mature (14 DIV) primary mouse cortical neurons were exposed for 24 h to specific inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6 and a mix of all of them) (20 ng/ml) involved in EAE pathogenesis, and REST expression was assessed at the mRNA and protein level. REST mRNA and protein were significantly upregulated selectively upon IL-1β treatment in 7 DIV neurons (**Fig. 11A-B**). Longer treatments (48 and 72 h) were also tested, but no significant effects on REST levels were observed (data not shown).

IL-1β is one of the most important mediators of the inflammatory response and modulates some of the inflammation-induced alterations of synaptic plasticity and structure [112]. In consideration of the fast kinetics through which IL-1β exerts its neuroprotective and neurotoxic actions [113], a time response curve of REST mRNA and protein was performed by treating 7 DIV neurons with IL-1β (20 ng/ml) at various time points, ranging from 20 min to 72 h. REST transcription markedly increased at shorter times (20 min, **Fig. 11C**), while 24 h were needed to observe an effect at the translational level (**Fig. 11D**). Furthermore, we

speculated about some possible kinase pathways involved downstream to IL-1β and REST. Since CREB signaling is activated by IL-1β and evidence exists of a correlation between REST and CREB, we evaluated the expression of the Ser-133-phosphorylated isoform of CREB (pCREB) in parallel with REST expression after 20 min and/or 24 h treatment with IL-1β. Indeed, we showed that IL-1β-induced REST overexpression triggered the activation of CREB signaling, as showed by the significant increase of Ser-133-phosphorylated CREB at shorter times (**Fig. 11E**).

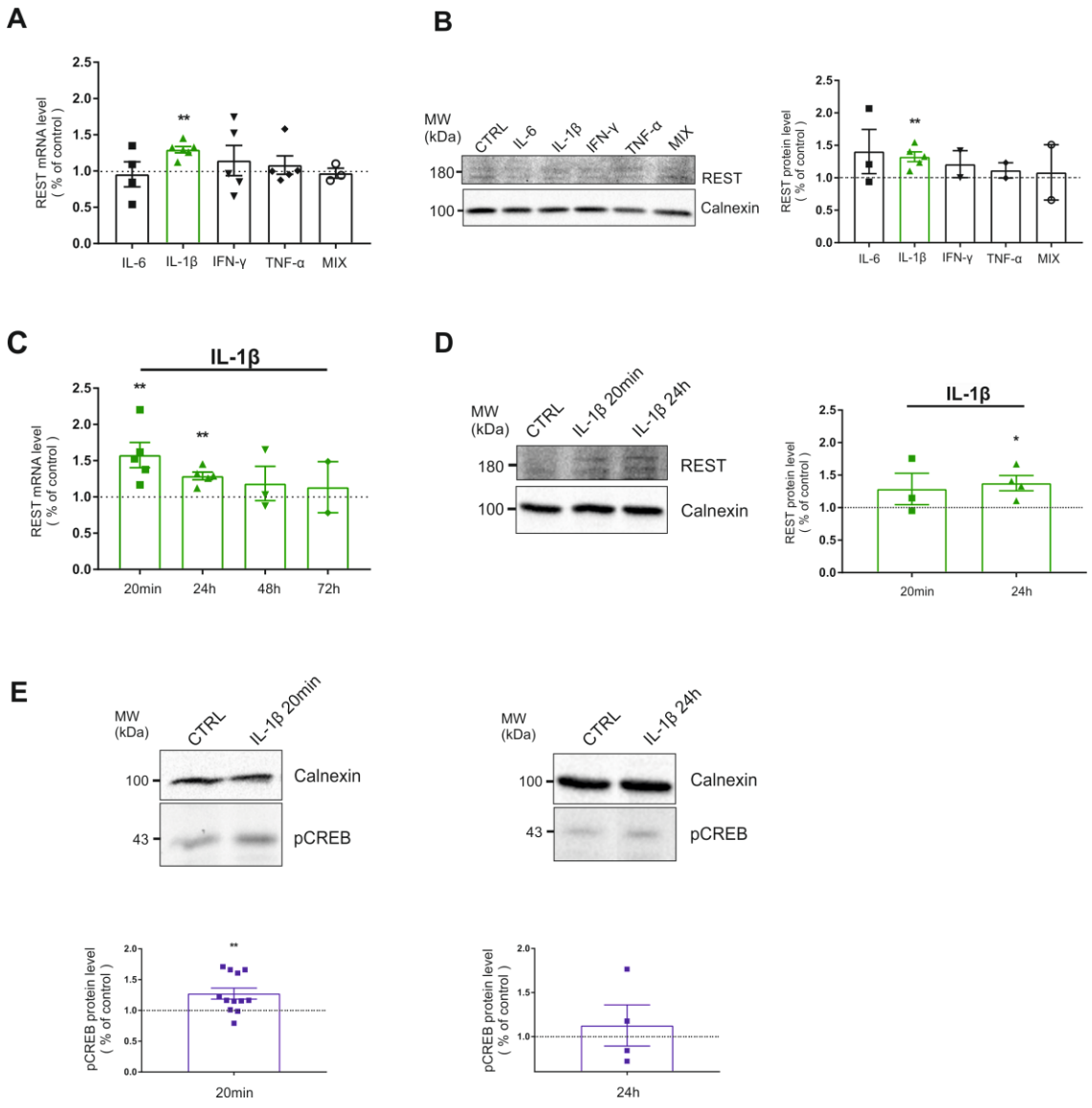


Figure 11: IL1-β treatment induced an increase in REST mRNA and protein levels in 7 DIV cortical neurons. (**A**) qRT-PCR analysis of REST mRNA levels upon treatment with various pro-inflammatory cytokines for 24 h. n=3-6 independent preparations. (**B**) Representative immunoblot (left) and corresponding quantification (right) of REST protein levels under the same experimental conditions. n=2-5 independent preparations. (**C**) qRT-PCR analysis of REST mRNA levels upon IL-1β treatment for different times, as indicated. n=2-5 independent preparations. (**D**) Representative immunoblot (left) and corresponding quantification (right) of REST protein levels upon treatment with IL-1β for 20 min and 24 h. n=3-4 independent

preparations. (**E**) Representative immunoblots (top) and corresponding quantification (bottom) showing the levels of pCREB in samples treated with IL-1β for 20 min (left, n=12 independent preparations) or 24 h (right, n=4 independent preparations). pCREB values were normalized to the housekeeping gene calnexin. In all panels, values of treated samples are expressed as % of vehicle-treated samples (CTRL). Data are expressed as means ± s.e.m. *p < 0.05, **p < 0.01, Mann-Whitney's U-test.

4.5.2. A short IL-1β treatment at 7 DIV impacts on neuronal physiology at later stages *in vitro*

To gain some insights into the role of REST in synaptic integrity and neuroprotection, the effect of the 20 min treatment of IL-1β was evaluated in mature neuronal networks at later stages. Through patch clamp recordings, frequency and amplitude of excitatory postsynaptic currents (mEPSPs) were monitored in 14 DIV wild type cortical neurons, previously treated with IL-1β at 7 DIV for 20 min. Unexpectedly, given the very short treatment, neuronal physiology was affected, with a long-term effect on synaptic plasticity. As reported in **Fig. 12**, we observed a significant downregulation of mEPSP frequency (**Fig. 12C** left) and amplitude (**Fig. 12C** right), in the absence of significant effects on the decay and rising time of the current.

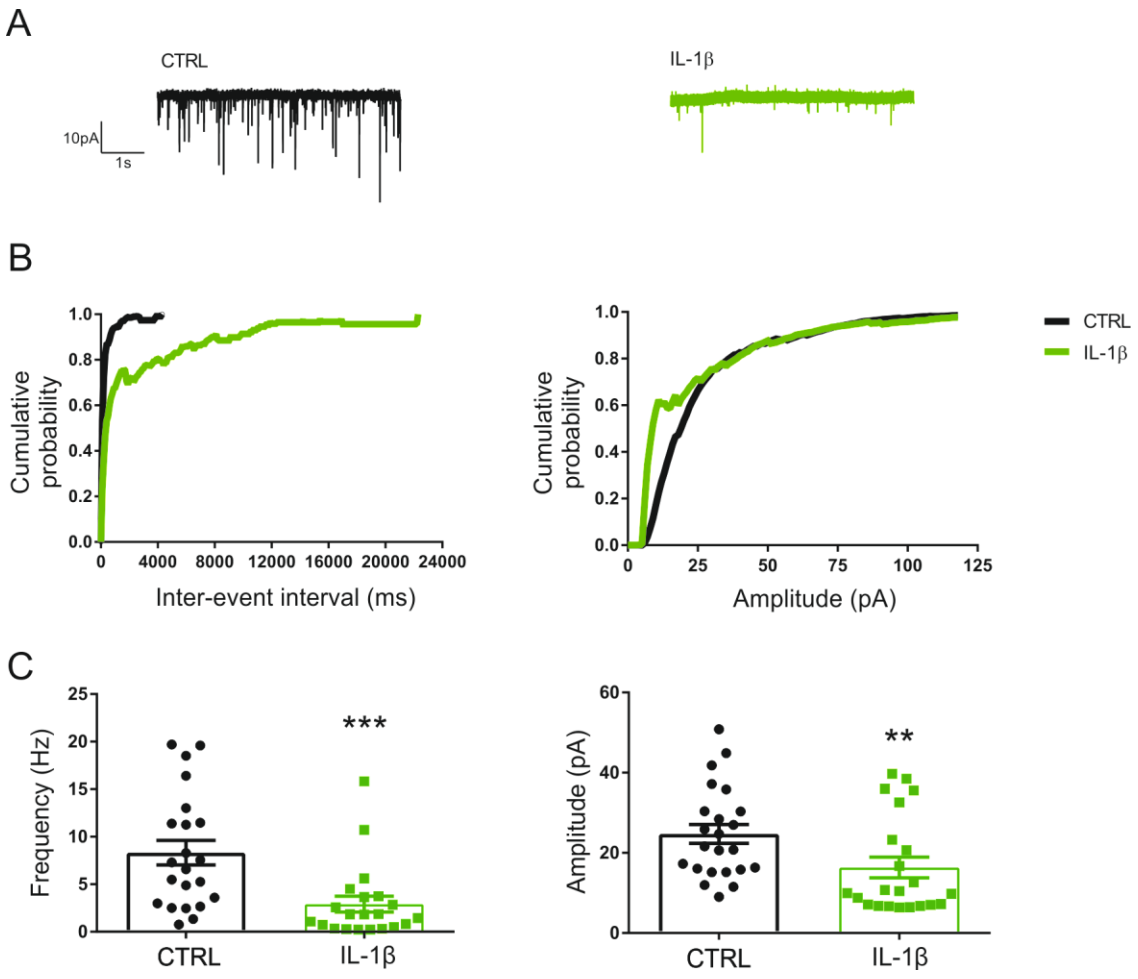


Figure 12. IL-1β treatment at 7 DIV affects spontaneous activity of wild type primary neurons at 14 DIV. Primary cortical neurons from wild type animals were treated with IL-1β (20 ng/ml) for 20 min at 7 DIV, or treated with vehicle (CTRL). Recordings were performed at 14 DIV. (**A**) Representative mEPSCs recordings and (**B**) cumulative distribution of inter-event intervals of CTRL and IL-1β-treated neurons. (**C**) mEPSC frequency (left) and amplitude (right). n = 23 cells from CTRL, n = 22 cells for IL-1β-treated samples, from 2 independent preparations. Data are expressed as means ± s.e.m. ** $p < 0.01$, *** $p < 0.001$ Mann-Whitney U-test.

In order to prove that this effect is REST-dependent, the same experiments were repeated in cortical neurons derived from REST lox/lox mice infected with lentiviruses coding for CRE recombinase, or for a truncated, inactive CRE protein (deltaCRE) (**Fig. 13**). Results showed that neurons infected with the delta-CRE lentivirus and treated with IL-1β show a decrease in mEPSP frequency and amplitude, similar to that observed in wild type neurons. REST knockdown cultures show a significant upregulation of frequency and amplitude under both control and IL-1β-treated conditions (two-way ANOVA, genotype effect $p < 0.0001$). Similarly, exposure to IL-1β caused a significant reduction in frequency and amplitude both in the presence and in the absence of REST (two-way ANOVA, treatment effect $p < 0.0001$). However, under basal conditions IL-1β caused a reduction of 64.2% and 31.1% in the frequency and amplitude, respectively, while in the absence of REST this reduction was greatly attenuated (31.5% for frequency and 13.8% for amplitude; two-way ANOVA, interaction treatment-genotype $p = 0.0004$ for frequency, $p = 0.0037$ for amplitude) compared to that observed under control conditions, indicating a significant interaction (occlusion) between treatment and genotype indicating that the observed IL-1β upregulation is, at least partly, mediated by REST (**Fig. 13C**).

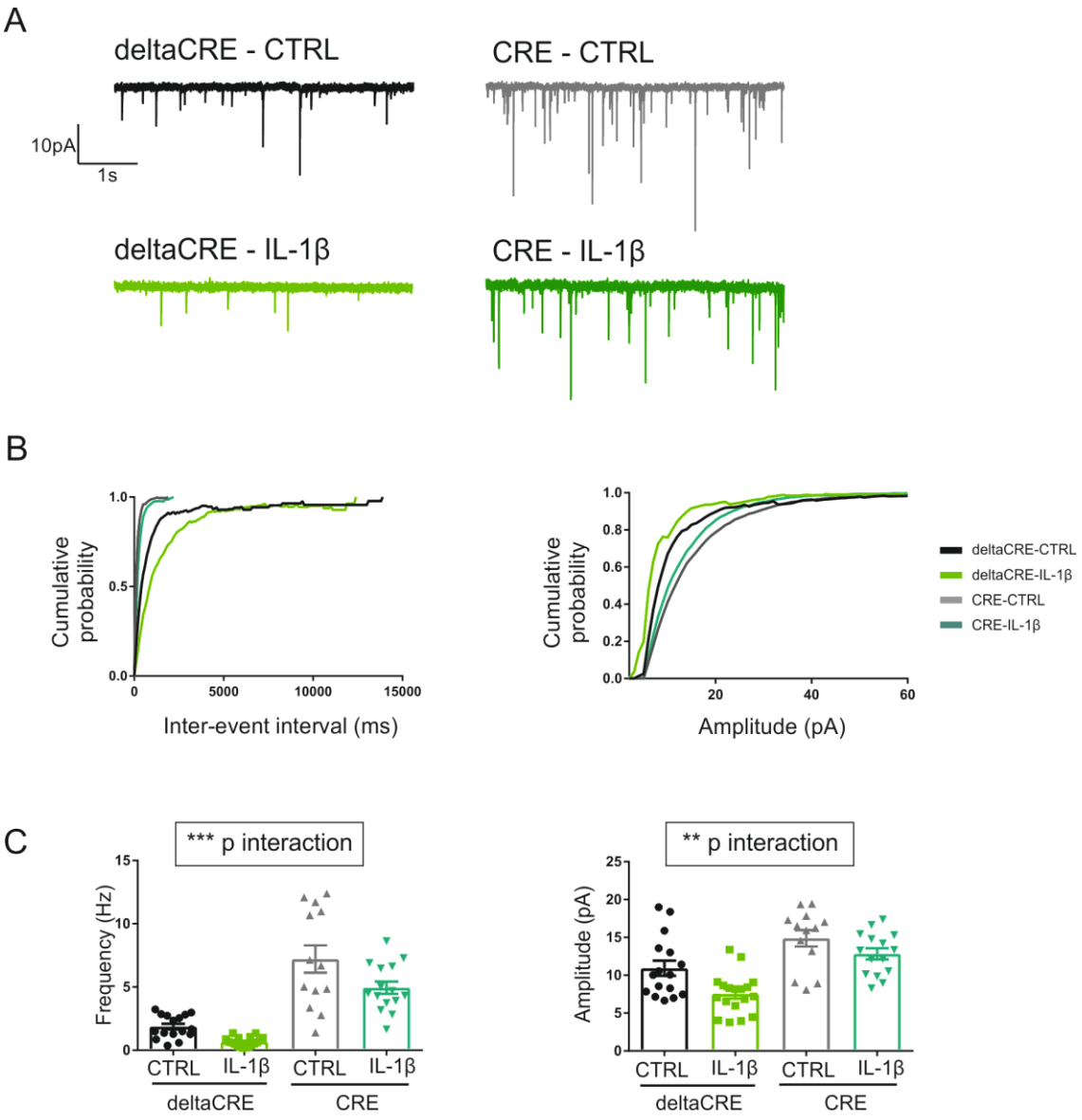


Figure 13. IL-1β treatment at 7 DIV affects spontaneous activity of REST lox/lox primary neurons at 14 DIV. Primary cortical neurons from REST lox/lox animals were infected with CRE or deltaCRE expressing lentiviral vectors, treated with IL-1β (20 ng/ml) for 20 min at 7 DIV, or treated with vehicle (CTRL). Recordings were performed at 14 DIV. (**A**) Representative mEPSCs recordings and (**B**) cumulative distribution of inter-event intervals of CTRL and IL-1β-treated neurons. (**C**) mEPSC frequency (left) and amplitude (right). n = 16 cells from deltaCRE CTRL, n = 18 cells for deltaCRE IL-1β-treated samples, n = 13 cells from CRE CTRL, n = 15 cells for CRE IL-1β-treated samples, from 3 independent preparations. Two-way ANOVA indicated a statistically

significant effect of genotype (frequency: $F = 1121$, $P < 0.0001$; amplitude: $F = 438.6$, $P < 0.0001$), treatment (frequency: $F = 146$, $P < 0.0001$; amplitude: $F = 152.5$, $P < 0.0001$) and genotype X treatment interaction (frequency: $F = 13.95$, $P = 0.0004$; amplitude: $F = 9.173$, $P = 0.0037$). ** $p < 0.01$, *** $p < 0.001$ Two-way ANOVA followed by the Bonferroni's multiple comparisons test. For clarification, it is not indicated the significance of the *post hoc* test that is the following between each condition: $p < 0.0001$. Data are expressed as means ± s.e.m.

5. DISCUSSION

Epigenetic modifications are a fundamental mechanism in the regulation of transcription and in neuronal development [114]. This is achieved through the formation of multiprotein complexes that can regulate gene expression positively or negatively. In this context, the transcription factor REST, also known as NRSF, plays a central role in the determination of the neuronal fate, as well as in the modulation of neuronal activity and plasticity [115]. In the last 20 years, the role of REST has been described in several pathologies; acting under some circumstances as an oncogene, and under other conditions as a promoter of neuronal damage. Furthermore, its expression levels have been shown to influence the outcome of several pathologies. For example, it has been described as a neuroprotective factor in Alzheimer's disease [75]. Upregulation of REST has been implicated in several neurodegenerative diseases, but whether it mediates a deleterious or protective mechanism is still debated.

The main objective of this study was to analyze the role of REST in an inflammatory scenario. To our knowledge, this is the first time the transcriptional repressor factor REST has been characterized specifically in an inflammatory context and in a model of multiple sclerosis.

REST is overexpressed in the spinal cord of EAE-affected mice.

The first part of this thesis describes the expression of REST and REST4 mRNA and protein levels in distinct regions of the spinal cord of EAE mice. We observed that REST is overexpressed in the lower region of the spinal cord of EAE mice, while its expression level decreases in the upper spinal cord, with respect to naïve animals at 4 dpo. Transcriptional analysis of REST and non-REST target genes confirmed the overall downregulation of REST target genes associated with the upregulation of REST in the spinal cord. REST upregulation therefore follows the ascendant trend of the pathology, characterized by an inflammatory burst in the lower part of the body at this stage of the disease. In line with these observations, a recent paper reported a downregulation of the REST target L1 adhesion molecule in the same experimental mouse model that was potentially attributed to REST upregulation [116]. REST expression appears to be higher where the pathological state is more severe. On the contrary, REST4 tends to have the opposite pattern of expression, in line with its proposed role as competitor of REST in the binding to RE-1 sites, where REST4 is thought to act as a derepressor.

Thanks to the immunofluorescence analysis, it has been possible to ascertain the source of REST overexpression. It is well established from literature that in mature neurons REST expression is not completely absent, and neurons can modulate REST expression in response to several environmental stimuli and pathological insults. What is evident from this study is that, in the presence of an inflammatory stimulus, REST expression levels increase in neurons, as well as

in astrocytes and microglia. The role of REST in glial cell populations has never been investigated. It is known to be involved in gliosecretion [117] and in the regulation of several genes related to astrocytes' specification and maturation [118], [119]. A high expression of REST has been described in astrocyte and microglia nuclei in the human brain cortex [117]. On the basis of our data, we propose that REST dysregulation occurs in the EAE model as a consequence of a global overexpression in neurons, microglia cells and astrocytes.

Characterization of REST in an in vitro neuroinflammation experimental system

To determine whether and how the complex EAE environment affects neuronal physiology through the modulation of REST and its targets, in the second part of this work we have analyzed REST expression in primary neuron cultures under experimental conditions mimicking a neuro-inflammatory environment. Our results show that 24 h incubation with a relatively high (20 ng/ml) concentration of IL-1β, selectively upregulates REST both at the mRNA and protein level. IL-1β is a potent and pleiotropic proinflammatory cytokine that activates several cell-specific signaling pathways, and is involved in several disorders [112]. Having seen an upregulation of REST in the microglia of EAE mice, it is not surprising to observe the same effect upon IL-1β treatment *in vitro*, since microglia is the primary source of this cytokine *in vivo*.

CREB is a well-known downstream mediator of IL-1β, especially at shorter times [120], [121], and the activation of CREB signaling has been closely associated with autoimmune inflammation in a rat model of EAE [122]. On the

other side, the consensus sequence for CREB was identified in the REST promoter [83], suggesting the presence of a regulatory feedback between them [18]. Indeed, especially in the context of synaptic plasticity and neuroprotection, there are several evidences of a CREB-REST correlation [18] [77], and a REST-dependent increase of p-GSK3β (Ser9) was described, which in turn induces CREB activation [123]. Taken together, our findings thus suggest that a direct correlation exists between IL-1β-mediated REST upregulation and the activation of the CREB signaling pathway.

Our results show that a short exposure to IL-1β treatment at a precocious stage in neuronal culture, before the establishment of a mature neuronal network, is sufficient to cause a REST-dependent long-lasting effect at both the pre- and post- synaptic levels in the mature neuronal network. The effect of IL-1β on neuronal cultures has been already described and results in synapse loss [124]. Moreover, IL-1β modulates glutamate release, enhances NMDA receptor function, [125], [126], with an inhibition of NMDA outward currents [127], induces tau phosphorylation through p38-MAPK pathway, and decreases synaptophysin, a well-known REST target gene, in cortical neurons [128]. In line with the work of Yang and colleagues [129], we observed a downregulation of the mEPSC frequency in neurons treated with IL-1β in both wild type and REST lox/lox cortical neurons infected with lentiviruses encoding a defective Cre recombinase (deltaCre). IL-1β is thus confirmed to act at the presynaptic level, influencing neurotransmitter release. On the contrary, Yang and colleagues [129] did not observe any difference in the mEPSC amplitude, although they did

not completely exclude the presence of a postsynaptic effect. Indeed, in our system IL-1β mainly elicited postsynaptic effects, with a significant downregulation in mEPSC amplitude, which is in line with recent evidence showing the downregulation of AMPA receptor expression mediated by IL-1β [130].

The role of REST in the physiology of a mature neuronal network is well described. In recent years, our group demonstrated its involvement in hyperactivity-induced intrinsic homeostasis [27], and in the homeostatic regulation of presynaptic machinery [131]. The Cre-mediated deletion of REST (our observations) causes the upregulation of mEPSCs frequency in untreated cells, which fits well with what observed by Pecoraro-Bisogni and colleagues, since this event could be due to the expression of presynaptic proteins, involved in transmitter release. The effect on the amplitude, not observed before, opens the possibility that REST modulation acts also at the postsynaptic level, hypothesis that will be addressed by further molecular investigations through transcriptomic and proteomic analysis of REST-target postsynaptic proteins, such as AMPA receptor genes [34].

Finally, when REST is knocked down, the downregulation of mEPSC amplitude and frequency is much less evident than in control cells, suggesting an occlusion effect. Thus, the IL-1β-induced changes are at least partly mediated by REST, while the residual decrease in both frequency and amplitude upon IL-1β treatment could be mediated by other signaling pathways. A comprehensive analysis of inhibitory spontaneous currents and cellular excitability will give us

more details on the role played by REST in the IL-1β-mediated alterations of network functionality.

6. FUTURE DIRECTIONS

The results presented in this thesis propose REST as a molecular target in the inflammation process, both *in vivo* and *in vitro*.

In the future, we will evaluate EAE severity and analyze the expression profile of REST target genes in conditional REST KO mice. If REST overexpression is a deleterious process in EAE, the induction of the disease in conditional REST KO mice will help us to understand whether REST depletion can significantly ameliorate disease course. Ongoing experiments are being performed through the CRE-inducible deletion of REST in REST lox/lox mice through an intra-*cisterna magna* (ICM) injection of AAV2/9 vectors, characterized by a high tropism for neurons, and expressing Cre under the strong CMV promoter. EAE will be induced in injected and control mice following the standard protocol. We will analyze the progression of the disease in REST-deficient and wild type EAE animals, and histological analysis of CNS samples will be conducted to define the causal role of REST in EAE and windows of opportunity whereby inhibition of REST can be therapeutic.

The *in vitro* part will continue with further histochemical and biochemical studies, in order to identify the molecular mechanisms underlying the observed phenotypes. In this way, we will try to understand better the molecular and functional interaction between REST and IL-1β.

Our final aim will be to understand whether REST indeed represents a pathogenic response contributing directly to neurodegeneration, or plays an endogenous neuroprotective role in the context of neuro-inflammation.

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