Study of the master neuronal regulator REST in adult brain.

By

Karin Andrea Müllendorff

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D, dissertation of

KARIN MÜLLENDORFF

Has been approved on May 23, 2014

____

Advisor, Gail Mandel, Ph.D.

____

Member and Chair, Philip Stork, Ph.D.

____

Member, Gary Westbrook, M.D.

____

Member, Richard Goodman, M.D., Ph.D.

____

Member, Anthony Barnes, Ph.D.

____

Member, John Adelman, Ph.D.

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LIST OF ABBREVIATIONS

4AP	4-aminopyridine
5AzaC	5’-aza-cytidine
AD	Alzheimer’s disease
ATP2B2	ATPase, Ca++ Transporting, Plasma Membrane 2
BDNF	Brain-derived neurotrophic factor
bp	Base pairs
CAMK	Ca2+/calmodulin-dependent protein kinase
Cdkn2b	Cyclin-Dependent Kinase Inhibitor 2B
cDNA	Complementary DNA
CHAT	Chloramphenicol transferase
CHIP	Chromatin immunoprecipitation
CKO	Conditional knockout
DAPI	4’, 6-diamidino-2-phenylindole dyhydrochloride
DME	Dulbecco's Modified Eagle's Medium
DNMT3a	DNA-methyltransferase 3a
DRAQ5	(1.5-bis{[2-(di-methylamino)ethyl]amino}-4,8-dihydroxyanthracene-9, 10-dione
DTT	Dithiothreitol
DYRK1	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
ELISA	Enzyme-linked immune-absorbant assay
EMSA	Electrophoretic mobility shift assays

FGF	Fibroblast growth factor
GABAβ3	Gamma-aminobutyric acid receptor subunit beta-3
GFAP	Glial fibrillary acidic protein
GluR2	AMPA glutamate receptor 2
GlyR	Glycine receptor
GRIN1	Glutamate receptor, ionotropic, N-Methyl D-Aspartate 1
Grp78	Glucose-regulated protein 78
GST	Glutathione S-Transferase
GT	Gene trap
GTi	Gene trap inverted
HCN1	Hyperpolarization-activated cyclic nucleotide gated channel 1
HD	Huntington's disease
HDACs	Histone deacetylases
HEK293	Human embryonic kidney 293 cells
iMEF	Irradiated mouse embryonic fibroblasts
KA	Kainic acid
L1CAM	L1 cell adhesion molecule
LIF	Leukemia inhibitor factor
LSD1	Lysine (K)-specific demethylase 1A
MeCP2	Methyl CpG binding protein 2
mESC	Mouse stem cells
MOR1	Mu opioid receptor 1
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NBT/BCIP	4-nitroblue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl-phosphate
NeuN	Neuronal Nuclei
NPC	Neuroprogenitor cells
NOVA2	Neuro-oncological ventral antigen 2
NRSF	Neural restrictive silencing factor
NSC	Neural stem cells
OGD	Oxygen glucose deprivation
PD	Parkinson's disease
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
RbFox3	RNA binding protein fox 1 homolog 3
RD	Repressor domain
RE1	Repressor element
REST	RE1 Silencing Transcription Factor
RT-PCR	Real time polymerase chain reaction
SCG	Secretogranin
SCLC	Small cell lung carcinoma
shRNA	Small hairpin RNA
SNAP25	Synaptosomal-associated protein, 25kDa
SSC	Standard sodium citrate
TrkB	Tropomyosin related kinase B

TSA	Tricostatin A
UTR	Untranslated region
XLMR	X-linked mental retardation

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ABSTRACT

Nervous system development involves complex extrinsic and intrinsic signaling pathways that precisely coordinate the maintenance of a progenitor pool and the orderly acquisition of a neuronal fate by systematically regulating the expression of genes. This process is mediated by transcription factors. Among the ones that play an essential role in this process is REST, the RE1 silencing transcription factor. REST regulates a large number of genes encoding proteins that are fundamental for neuronal traits by binding to a conserved 23bp element present in its target genes. REST is highly expressed in stem cells where it impedes the expression of those neuronal genes. As cells start to differentiate, REST is downregulated and therefore repression is lifted and neurons acquire their identity. Early studies suggested this downregulation occurs at embryonic day 10.5, and after that, REST was no longer present. However, a few papers have challenged this view and showed that REST may be expressed in adult brain.

In this thesis work, I sought to confirm and expand the notion that REST is expressed in the adult brain. In doing so, I developed rat monoclonal and polyclonal antibodies to confidently detect mouse REST protein in frozen brain tissue. I also used biochemical approaches, confocal microscopy, and a knockout mouse model to demonstrate that REST mRNA, protein, and endogenous promoter activity are found in several areas of the brain, including cortex, cerebellum, hippocampus, olfactory bulb, brain stem, among others. Importantly, REST binds to the chromatin of target genes and also regulates their expression in a brain-area specific manner in the adult brain. Lastly, a great deal of recent

attention has been placed on the potential role of REST in Alzheimer's disease. In this thesis, I provide evidence REST levels maybe regulated in an age-dependent manner in both mouse and human brains.

The major conclusion of this dissertation is that the RE1 silencing transcription factor, REST, is indeed expressed in adult brain. Although we do not know the precise function REST plays, this study opens up an entirely new avenue that should lead to a better understanding of its role in mature neurons, and ultimately, an understanding of neurological disorders and potential therapeutics.

INTRODUCTION

An important question in brain development has been to determine the mechanisms by which specific genes are expressed in space and time. That is, how their gene products become spatially restricted to particular regions and cells of the brain, and at a particular time during development. An understanding of this process has been provided by the study of neurogenesis. Here, neurons are generated from neural progenitor cells (NPCs), neuronal circuits are formed, and connections between neurons undergo remodeling as they develop into a mature and functional brain. For this to happen in a proper manner, it is paramount that extrinsic and intrinsic signals relay information to the nucleus. This integration, at the genomic level, is accomplished by transcription factors. For example, the signaling pathways mediated by Wnt, Notch, Sonic hedgehog, and FGF have been implicated in regulating NPCs maintenance and self-renewal (Wen et al., 2008). These signals are transduced by the Hes1 and Hes5 transcription factors, which recruit a co-repressor complex to proneural genes (Hatakeyama et al., 2004). With each successive stage, the progenitors become more restricted to the neuronal lineage, and the addition of proneural transcriptional activators, such Mash1 and Ngn come in to play (Sun et al., 2001). This is followed by a new wave of transcriptional activators such as NeuroD, which precedes terminal differentiation.

Comparatively, little was known about the terminal neuronal differentiation step until the identification in 1995 of the transcriptional repressor, RE1 Silencing Transcription Factor, REST, also called Neural Restrictive Silencing Factor (NRSF) (Shoenherr et al.,

1995; Anderson, et al., 1995). REST acts as a master regulator, an umbrella that oversees and regulates the expression of pan neuronal genes required for the terminally differentiated phenotype of mature neurons. This includes the regulation of genes such as voltage-dependent ion channels, synaptic proteins involved in neurotransmitter release, growth factors, etc. (Chong et al., 1995; Mori et al., 1990; Bruce et al., 2004; Timmusk et al., 1999). The current model is that REST suppresses the neurogenic competence of neural progenitor cells by maintaining their ability to self renew while restricting the generation and maturation of neurons during early neuronal development (Covey et al., 2012). Although the importance of transcriptional regulation and epigenetics regulation mediated by REST has long been recognized in stem cells and during neural development, the role of REST in adult brain has received little attention until very recently. For example, REST is expressed in a quiescent stem cell population within the hippocampus, it is downregulated as cells undergo neuronal differentiation, and then it reappears in mature granule cells (Gao et al., 2012). REST is also upregulated during ischemia to repress the expression of the AMPA receptor subunit GluR2, and therefore, provide neuroprotection (Tanaka et al., 2002; Noh et al., 2012). Interestingly, REST also appears to play an important role during aging in human brains, and its expression correlates with cognitive function and longevity (Lu et al., 2014). Therefore, a detailed characterization of REST in adult brain is needed. This may ultimately help understand how the adult brain dynamically changes in response to external stimuli all throughout the life span of an organism, and how it responds to disease.

My thesis work specifically addressed the question of whether REST is expressed in adult mouse brain, and if so, in what cell populations. To examine this issue, I characterized a new anti-REST antibody to determine REST protein localization by histology because most previous studies primarily examined RNA transcripts. I used a conditional REST knockout mouse model to validate my findings and finally demonstrate that REST is indeed expressed in adult mouse and human brain.

During the remaining of this introduction, I describe the experiments leading to the discovery of REST, followed by information on its structure and its mechanisms of repression, the role of REST in neuronal development, and lastly, the existent evidence with respect of REST in adult brain. **CHAPTER 2** will describe the characterization of rat monoclonal and rabbit polyclonal antibodies that recognize mouse REST. **CHAPTER 3** will provide the evidence that REST is expressed and functional in adult brain. **CHAPTER 4** summarizes the findings and proposes conclusions and future studies.

THE DISCOVERY OF REST

It wasn't until 1990 that Maue et al. proposed an unprecedented new mechanism for gene regulation that would probe critical for neuronal function. They isolated genomic DNA for the rat sodium channel Nav1.2 gene, which by then was known to be one of four α subunits of the voltage-dependent sodium channel expressed in the central nervous system (Maue et al., 1990). The genomic region corresponded to 177 bp of 5' untranslated region (UTR) plus 1051 bp of 5' flanking region. Using RNA protection

analysis, the authors compared the isolated upstream sequence with peripherin and neurofilament, both of which have been demonstrated to be transcriptionally affected by NGF treatment in PC12 cells (Thompson et al., 1992). Interestingly, they found a highly homologous element that shared approximately 86% identity with a 14bp sequence in the sodium channel Nav1.2 gene and also a 5bp CCAGG motif common to all three genes.

This fragment was fused to a chloramphenicol transferase (CAT) reporter and transfected into both a neuronal and a non-neuronal cell line. They showed that neuronal cells had 10 to 100 folds higher reporter activity than non-neuronal cells, indicating the presence of a negatively regulating factor that repressed sodium channel expression in the non-neuronal cells. Further deletion studies converged on a sequence between -1051 to -983 that contained the repressive activity in non-neuronal cells (Kraner et al., 1992). From this point on, the sequence was named Repressive Element 1 (RE1). Dnase I foot printing studies revealed that a 28bp sequence protected from digestion was the one conferring the silencing activity. In addition, non-neuronal cells transiently transfected with a fusion gene containing a single copy of RE1 showed little CAT activity whereas CAT activity under the minimal promoter was rather robust. Similarly, when the minimal promoter was co-transfected with 40 molar excess of the 28bp repressor binding sequence, there was a dramatic derepression of the sodium channel Nav1.2 promoter activity (Kraner et al., 1992). Because the difference in promoter activity observed depended on whether the reporter was transfected in PC12 cells, which do have sodium channels, or L6 muscle cells, which do not, it was speculated that there was an active cell-specific repressor

protein in non-neuronal cells that maintained the neuronal specificity of the sodium channel Nav1.2 gene.

As the field progressed, studies of two additional molecules that expanded the role of this silencer on gene regulation: one, secretogranin (SCG), and two, synapsin. SCG is a membrane protein that accumulates in growth cones of developing neurons (Stein et al., 1988b). In 1990 Mori et al. suggested that the SCG promoter contains an enhancer unit that maintains the gene active in both neuronal and non-neuronal cells. Unexpectedly however, analysis of a sequence further upstream revealed that there must be a silencing element that represses the activity of the SCG promoter in non-neuronal cells, which was absent in neuronal cells just like for the sodium channel Nav1.2 (Vanderbergh et al., 1989; Wuenschell et al., 1990). Sequencing of the SCG upstream region and deletion studies converged on a 62bp domain sequence that conferred the repressive activity (Mori et al., 1992). Furthermore, visual comparison of this 62bp sequence with the sodium channel sequence narrowed the repressive activity to 21bp, which proved to be both necessary and sufficient for silencing. Similarly, synapsin I, a membrane protein of small synaptic vesicles, seems to also contain both positive and negative regulatory regions (Sauerwald et al., 1990; Thiel et al., 1991; Howland et al., 1991). Interestingly, its repressive region also contains a sequence highly homologous with sequences in the sodium channel and SCG (Li et al. 1993). Therefore, there seems to be a common theme to the sodium channel, SCG, and synapsin: they all show cell type specificity for the silencing activity that is capable of reducing their expression in non-neuronal cells. This

is likely to be achieved by the presence of a binding factor more abundantly expressed in non-neuronal cells.

With all these data at hand, there were two laboratories that, by using different approaches, simultaneously cloned the protein with repressive activity. The Mandel laboratory named it the RE1 silencing transcription factor (REST), and the Anderson laboratory named it the neuron-restrictive silencer factor (NRSF) (Chong et al., 1995; Schoenherr et al., 1995). By using a yeast strain containing wild type and mutated RE1 sites upstream of a yeast promoter, the Mandel lab found three colonies in the HeLa cells complementary DNA (cDNA) library that showed strong reporter response in the screening. Further screening revealed an open reading frame that encoded the putative silencer protein, REST. Use of a REST specific antibody and northern blot analysis demonstrated that REST was highly expressed in muscle cells and hardly detectable in PC12 cells (Chong et al., 1995). The Anderson lab used a probe containing three copies of the Nav1.2 RE1 site and screened binding against a HeLa cell cDNA expression library. The one cDNA identified bound to the RE1 probe and showed similar sequence specificity to the endogenous REST. The authors further validated cDNA by using a monoclonal antibody and electrophoretic mobility shift assays (EMSA) to show similarly sized complexes bound to the RE1 compared to endogenous REST (Schoenherr et al., 1995). All these findings formally introduced REST as the protein with the silencing activity that prevents the expression of neuronal genes in non-neuronal tissue.

To show the importance of RE1 for repression, Thiel et al. (1998) placed two consensus RE1 sequences common to SCG, synapsin, the M4 muscarinic acetylcholine receptor, and the neuronal nicotinic acetylcholine receptor β2 upstream of a reporter system containing the glucose-regulated protein 78 (grp78) enhancer followed by the β globin reporter. Gene activity, measured upon cotransfection with or without REST, showed that the presence of RE1 binding sites and REST caused a significant decrease in transcription. An additional question that remained was to determine whether REST could act on an RE1 site located in an intron or at 5' enhancer. In order to answer this question, the synapsin I RE1 site was inserted in two orientations 2.3kb downstream of the transcription start site of the grp78 reporter (Kallunki et al., 1995; Kallunki et al., 1997). The authors showed that regardless of the RE1 orientation, REST was able to promote the repression of the grp78 activity. Likewise, if they added an enhancer element downstream of the open reading frame and a RE1 site upstream of the transcription start site, they observed a reduced β globin mRNA synthesis thus indicating that REST is active even when the RE1 site are located distantly from the promoter.

In summary, REST is a transcription factor that binds to a DNA element located near promoters, enhancers, and introns to repress the transcription of target genes in non-neuronal tissue. Because neural induction during development occurs in part by a default pathway, it may be that it is not the presence of an activator, but instead, the presence of a repressor that prevents the ectopic expression of neuronal genes at the wrong time during the development. This idea puts REST as a master regulator of neuronal expression due to its demonstrated activity and reciprocal expression.

REST STRUCTURAL DOMAINS AND INTERACTIONS

Analysis of the REST coding sequence showed that it contains eight Zn finger elements in the N-terminal domain plus an additional one at the C-terminus. The presence of these elements qualifies REST as a C2H2 type Zn fingers, similar to the Krüppel repressor protein found in Drosophila (Licht et al., 1990). Interestingly, homologous Zn fingers occur in proteins induced by differentiation and growth signals, in proto-oncogenes, and in genes that regulate development (Pabo et al., 1992; Marin et al., 1997). REST also contains a proline rich region, a basic amino acid rich region, and not surprisingly, a signal that confers its nuclear localization (Shimojo et al., 2006).

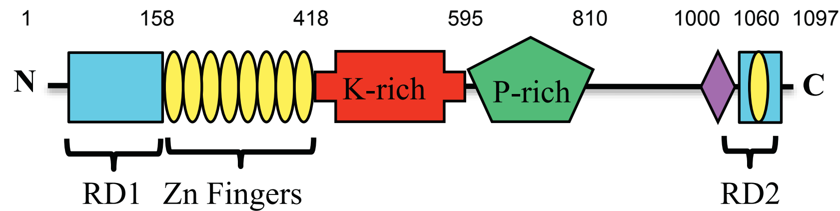


Figure 1. Schematic representation of REST domain structure. Mouse REST domain structure. RD1, repressor domain 1; K-rich, lysine rich domain; P-rich, proline rich domain; purple diamond, acidic region; RD2, repressor domain 2; all nine Zn finger domains are depicted in yellow ovals.

Structural analysis of REST amino acid sequence has provided insight into several other interesting observations. Chong et al. demonstrated that expression of just the DNA-binding domain of REST resulted in derepression of the cotransfected sodium channel Nav1.2 reporter gene (Chong el al., 1995). This suggests the eight Zn finger domains not only allow for the binding to the RE1 site, but also contain all the elements necessary for repression. Because there are no repressor consensus sequences deduced from the REST primary sequence, it was evident that basis of repressive activity was elsewhere. Competition studies later concluded that there were two repressor domains in REST that were likely to interact with distinct nuclear factors to repress transcription. One is within the first 152aa of REST, and the second was found in the C-terminal domain (Thiel, et al., 1998; Leichter et al., 1999).

Consequently, the next question was: what are the REST binding partners? Based on the nature of the REST protein sequence, two known repressor domains with well-characterized binding partners were studied. However, Leichter et al. (1999) found that REST requires different cofactors than those necessary for the biological activity of repressor proteins containing KRAB domain or the thyroid hormone receptor. Through a different approach, Andres et al. (1999) used the C-terminal half of REST as bait in a yeast two-hybrid genetic screen with a HeLa cell–Gal4 activation-domain cDNA library. They identified a cDNA with prior unknown function that they designated as CoREST. This 66KDa protein bound to a specific C-terminal REST fragment that contains the Zn finger motif known to mediate repressor activity. Of note, CoREST does not interact with the N-terminal domain of REST suggesting once again that both domains bind to a

different set of corepressors (Andres et al., 1999). As a binding partner arose for the C-terminal repressor domain, the quest to find partners for the N-terminal repressor domain continued. In a set of similar experiments, Grimes et al. also performed yeast two-hybrid screening by using the co-repressor mSin3a as bait. They found two cDNAs both encoding amino acids 32 to 122 of REST. This suggested the interaction is specific to the REST N-terminal domain. Importantly, they showed that full length REST, CoREST, and mSin3a immunoprecipitate together in HEK293 cells (Grimes et al., 2000). It is possible that CoREST and Sin3a function to recruit a repression complex so to stabilize the binding of REST to DNA and/or interfere with components of the transcription machinery to block transcription.

Another clue to follow was that other silencing factors bind to corepressors, which in turn recruit histone deacetylases (HDACs) (Alland et al., 1997; Hassig et al., 1997; Laherty et al., 1997). Treatment of C6 muscle cells with Tricostatin A (TSA), a histone deacetylase inhibitor, resulted in an increase in the endogenous levels of two target genes, GluR2 and the sodium channel Nav1.2. This response depended on the presence of the RE1 site in these genes because its deletion significantly reduced TSA potentiation (Huang et al., 1999). Because HDAC1 binds the corepressor mSin3a, the authors tested whether this was a possible binding partner for REST as well. Immunoprecipitation studies showed that REST, Sin3a, and HDAC1 indeed reside in the same complex. Not only that, but the C-terminal domain also showed HDAC1/2 activity because the formation of this complex deacetylated chick histones *in vitro*. In addition, Ballas and colleagues demonstrated that REST recruits HDACs via one of CoREST binding domains as opposed to a direct

interaction with REST (Ballas et al., 2001). Direct immunoprecipitation studies of HDAC1 and 2 not only confirmed the presence of CoREST, but also added the Lysine (K)-specific demethylase 1A (LSD1) to the complex. LSD1 is a FAD-dependent enzyme capable of providing a repressive activity by demethylating histone H3 in lysine 4 (H3K4) (Humphrey et al., 2000; Shi et al., 2004).

Unlike LSD1, a direct interaction has been observed between REST and G9a, a histone methyltransferase (Roopra et al., 2004; Tachibana et al., 20100), based on chromatin immunoprecipitation studies using antibodies against H3K9me2 or acetylated lysine 9 (H3K9ac). Analysis of several target genes including the sodium channel Nav1.2, SCG10, and the muscarinic receptor M4 showed that they are all under the umbrella of the repressive mark H3K9m2, but not the active H3K9ac mark. The authors concluded that REST is associated with histone methylase activity at its C-terminal domain as demonstrated by immunoprecipitation, immunostaining, and robust association with RE1 sites in non-neuronal cells. Most importantly, they showed that G9a is required for REST-mediated repression of transcription (Roopra et al., 2004).

Another important member of the REST complex that was discovered in a similar way was the methyl CpG binding protein 2, MeCP2. It had been observed that the histone deacetylase inhibitor TSA failed to reduce the expression of the sodium channel Nav1.2. This led to the prediction that perhaps DNA methylation instead was a strategy to keep this gene silenced. To test this hypothesis, Lunyak et al. (2002) treated Rat-1 cells with 5'-aza-cytidine (5AzaC), a potent DNA methylation inhibitor, and showed that it induced

Nav1.2 expression by reducing the CpG methylation on its promoter. Because MeCP2 is one of many candidate proteins known to methylate DNA, they performed chromatin immunoprecipitation with a MeCP2 antibody and showed that is present in the promoter of the Nav1.2 channel. Furthermore, the authors data indicated that the complex formed between MeCP2, REST, CoREST and DNA methylation are a requirement to establish and maintain TSA-independent repression of the sodium channel Nav1.2 gene in Rat-1 cells (Lunyak et al., 2002).

Lastly, REST also binds proteins that provide non-covalent modifications and that depend on the energy provided by ATP to exert their function. One example of this is the SWI-SNF complex where the ATPase BRG1 serves as the catalytic unit (Battaglioli et al., 2002). By using CoREST as bait for a two-hybrid yeast screening, Battaglioti and colleagues found that CoREST binds directly to BAF57, and it is found in an immuno complex with BRG1, BAF270, and most importantly, REST. Repression of target genes such as the sodium channel Nav1.2, synapsin 1, L1 cell adhesion molecule (L1CAM), and synaptosomal-associated protein, 25kDa (SNAP25) requires the presence of components of the SWI-SNF including BAF57, BRG1, BAF270, and BRAF35 complexes to perhaps stabilize the REST-RE1 interaction on their chromatin (Battaglioti et al., 2002; Ooi et al., 2006; Hakimi et al., 2002).

In summary, REST recruits different repressor complexes to its N- and C-terminal domains, which are mediated by mSin3a and CoREST respectively. They in turn mediate active repression via recruiting specific histone deacetylases and DNA methylases.

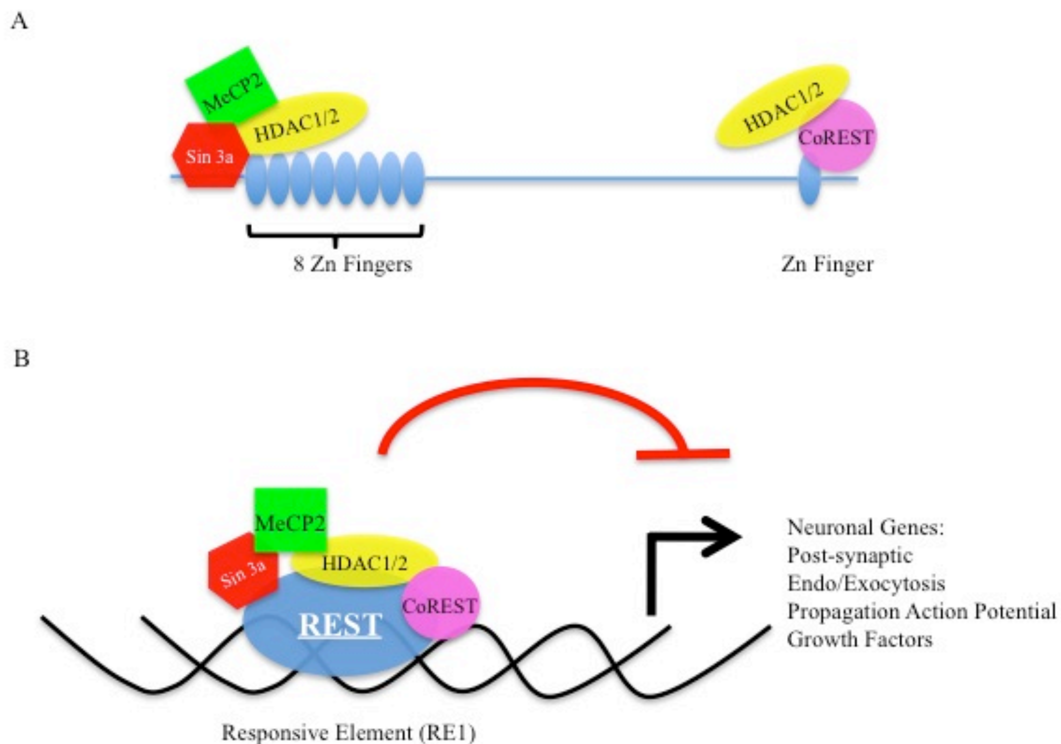


Figure 2. Schematic representation of REST and corepressors complex. A) Sin3a, MeCP2, and HDAC1/2s bind to the N-terminal domain of REST whereas CoREST and HDAC1/2s bind to the C-terminal domain. **B)** REST complex binds to the responsive element 1 to repress the expression of neuronal genes.

DEVELOPMENTAL REGULATION OF REST

REST, through the negative regulation of transcription is important for neuron-specific gene expression. Chen and colleagues first described the quintessential developmental importance of REST in 1998. For that, they used gene targeting in mouse embryonic stem

cells to generate a complete REST knock out mice. Surprisingly, this model resulted in lethality by embryonic day (E) 11.5 (Chen et al., 1998). There were two things learned from this study. First, REST is ubiquitously expressed up until embryonic day 9.5. Second, by E10.5 the REST -/- embryos showed growth retardation, significant cellular disorganization, and widespread apoptotic cell death. Further insight on developmental regulation of REST was gained years later in a seminal study published in the laboratory of Gail Mandel (Ballas et al., 2005). The authors demonstrated that REST protein is present at its highest level in the nuclei of pluripotent mouse embryonic stem cells (mESCs) as an active repression mechanism that allows a very low basal level of neuronal gene expression. As stem cells differentiate to progenitors, REST mRNA levels stay relatively constant, but REST protein is post-translationally downregulated to minimal levels. This downregulation of REST seems to be mediated by the recognition of a phosphodegron sequence at the C-terminal domain of REST that targets it to degradation mediated by the proteasome machinery (Westbrook et al., 2005; Nesti et al., *In preparation*). The final downregulation of REST results in its dissociation from the chromatin, derepression of neuronal genes, and the final transition towards mature neurons.

Further understanding of the role of REST during early neuronal development was examined by Mandel et al. in 2011. Here, the authors manipulated REST levels by using *in utero* electroporation in developing cortex. Overexpression of REST resulted in the arrest of progenitor cells at the boundary of the ventricular zone and intermediate zone. Because these cells eventually migrated and became neurons, the authors speculated that

REST is not critical for the neuronal fate decision, but instead acts as a timer for terminal differentiation. Additional studies in the Mandel laboratory are currently expanding this idea by using a conditional gene trap mouse (Schnütgen et al., 2005) by specifically knocking down REST in neural progenitor cells. Tamilla Nechiporuk, a postdoctoral fellow in our lab, showed these mice have significantly smaller brains. This phenomenon is explained by a premature terminal differentiation, which is accompanied by reduction in the apical progenitor pool, cell death, and premature cell cycle exit. The element that links REST to this phenotype is the cell cycle inhibitor protein cdkn2b, a new REST target.

ROLE OF REST IN THE PATHOPHYSIOLOGY OF DIVERSE DISORDERS

REST acts as a molecular platform recruiting a series of factors that dynamically modify histone and DNA to ultimately promote chromatin remodeling and gene repression. Many of its targets genes including ion channels, neurotransmitter receptors, growth factors, and synaptic vesicles proteins are responsible for a broad range of developmental and homeostatic functions. The deregulation of these proteins are suspected to be important in the pathophysiology of several disorders of the nervous system raising the question of whether REST plays a role in any of the molecular processes leading or preceding some these disorders. Available evidence suggests that REST may be involved in ischemia, seizures, cancer, and neurodegenerative and neurodevelopmental disorders.

Role of REST in ischemia and epileptogenesis. Ischemic events are characterized by the sudden lack of oxygen due the disruption of blood supply that result in cell death particularly in CA1 pyramidal neurons of the hippocampus. The AMPA receptor GluR2, which is a REST target, appears to be important in this process (Tanaka et al. 2002; Myers et al. 1998). Calderon et al. (2003) demonstrated that global ischemia triggers an increase in REST mRNA and protein expression that is mostly circumscribed to CA1 and CA3 pyramidal neurons of the hippocampus. This increase in REST resulted in repression of GluR2 both determined by the acetylation of associated core histones proteins, and also, a reduction of GluR2 mRNA as measured by *in situ* hybridization. Most importantly however, REST antisense blocked GluR2 expression and provided protection to CA1 neurons from oxygen glucose deprivation (OGD) in organotypic hippocamapal slices, thus implicating REST in ischemia-induced cell death (Calderone et al., 2003). A similar effect has been observed with another REST target, the mu opioid receptor 1 (MOR1), which is down regulated in CA1 after global ischemia. This seems to be a result of a stronger association of REST to its promoter and the concomitant deacetylation of histone 3 and 4 (Kim et al., 2004).

Seizures induce alteration in neuronal circuitry including synaptic reorganization and persistent hyper-excitability. Brain-derived neurotrophic factor (BDNF) and the tropomyosin related kinaseB (Trkb) are two genes whose expression is downregulated by epileptogenesis (He et al., 2004; Kokaia 1995). BDNF and TrkB, just like GluR2, are also regulated by REST (Zuccato et al., 2003; Garriga-Canut et al., 2006) thus putting REST at center stage in the regulation of the process that leads to epilepsy. Further, Palm

et al. in 1998 showed for the first time that kainic acid (KA)-induced seizure increases REST mRNA levels in hippocampus. Consistent with this observation, REST mRNA levels were also increased in kindling- and pilocarpine-induced epileptogenesis (Hu et al., 2011; Garriga-Canut 2006; Spencer et al., 2006). Knock down of REST in excitatory Ca2+/calmodulin-dependent protein kinase (CAMK) positive post-mitotic neurons resulted in an accelerated epilepsy progression compared to wild type mice. This was partly due to the selective upregulation of BDNF and FGF14 but not other REST target genes (Hu et al., 2011). REST might also be involved in dowregulating target genes that affect network activity like the hyperpolarization-activated cyclic nucleotide gated channel 1 (HCN1), which has a low expression level upon KA-induced seizure (McClelland et al. 2011)

Role of REST in cancer. Evidence suggests that REST can function as either a tumor suppressor or an oncogene depending on the context. For example, in non-neural tissue the diminished expression of REST has been associated with colon cancer and transformation of human epithelial cells (Westbrook et al., 2005). Full length REST transcript expression was absent from a subset of small cell lung carcinoma (SCLC) cell lines, and instead, it was replaced with a truncated REST isoform with a novel 13-amino acid not present in wild-type REST (Neumann et al., 2004; Coulson et al., 2000). Similarly, REST expression in breast cancer cell was significantly lower compared to normal and benign breast samples. By using a shRNA approach to knock down REST in the human breast cancer MCF-7 cells, Lv et al. (2010) demonstrated that absence of REST increases cell proliferation, suppression of apoptosis, and reduced sensitivity to

anti cancer drugs (Lv et al., 2010). Furthermore, the lack of full length REST correlates with poor outcome. More specifically, there was a 20% reduction in disease free survival when compared to their REST full-length counterpart over 10 years period. Also, less than 20% of patients with full length REST showed an early recurrence happening within three years (Wagoner et al., 2010). Thus REST may be targeted for inactivation during non-neuronal cancer progression, or might be replaced by truncated forms such to act as dominant negative, which would ultimately result in the activation of neuronal programs. This type of phenomenon has been described as paraneoplastic neurological degeneration, where tumors originating in non-neuronal tissues activate the expression of neural peptides, like vasopressin, to elicit an immune response (Coulson et al. 1999).

In contrast to non-neuronal tissue, REST plays an oncogenic function in brain tumors. One of the first pieces of evidence suggesting this idea came from very simple studies in neuroblastoma cells that demonstrated not only high levels of expression of full length REST, but also, high levels of its spliced forms (Palm et al., 1999). In addition, REST also showed very high levels of repression of a reporter construct thus indicating unusual endogenous activity in these neuroblastoma cells compared to PC12 cells and neuronal progenitors (Lawinger et al., 2000). Interestingly, the absence of REST repression alone was not sufficient to activate a significant amount of target genes. Therefore such a process may require the additional presence of positive activators. To demonstrate this idea, the authors fused REST to the activation domain of the viral activator VP16 (REST-VP16) and showed that it activates neuronal differentiation markers such glutamate receptors, acetylcholine receptors, and synapsin, but did not affect expression

of stem cell genes like nestin (Lawinger et al., 2000; Immaneni et al., 2000). *In vivo* studies have found that subcutaneous xenografts of medulloblastoma cells into the flanks of nude mice cause tumors (He et al., 1989). However, infection with an adenoviral version REST-VP16 blocked the tumorigenic potential of these cells and induced apoptosis rather than promoting differentiation into mature neurons (Lawinger et al., 2000). Similarly, inoculations of REST-VP16-infected medulloblastoma cells into the brains of nude mice resulted in no visible tumors compared to the large ones observed with the inoculation of vector alone. Furthermore, REST immunoreactivity in human medulloblastoma showed positive signal in 17 out of the 21 samples (Fuller et al., 2005).

REST, however, is not sufficient to cause tumorigenesis. It is possible REST works with other oncogenic pathways induced by genetic or epigenetic alteration to push cancer progression. For instance, many human medulloblastoma samples and cell lines show a significant positive correlation between the expression of the oncogenic myc and REST, an association that is more common than what would be predicted by chance (Su et al., 2006). Because the cerebellum is the major site of medulloblastoma occurrence, the same authors assessed the properties of cerebellum-derived neural stem cells (NSCs) that had been stably transfected with activated c-myc and or REST. Upon injection into the cerebellum and through histological analysis, REST in conjunction with c-myc induced large cerebellar tumors with morphology similar to human medulloblastoma. To the contrary, NSCs containing vector alone or c-myc alone had normal cerebellar architecture, and thus no cerebellar tumors. They concluded that the presence of REST and Myc promotes tumorigenesis by increasing myc-induced proliferation and REST-

induced persistence of self-renewal. This oncogenic role of REST has been further supported in studies of glioblastoma cell lines where the degree of tumor invasiveness in mouse brain is also dependent on the amount of REST expression (Kamal et al., 2012). All in all, REST has both a tumor-suppressor and tumorigenic effect depending on cellular context, stage of development, and the cohort of binding partners in the respective cell lines.

REST in neurodevelopmental disorders. REST plays a role in neurodevelopmental disorders such as the Down syndrome. Down Syndrome is one of the most common forms of mental retardation whose animal models have shown a significant deregulation of genes that are targeted by REST (Lepagnol-Bestel et al., 2009). In this case, REST complexes with the dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1a) and SWI/SNF to regulate gene expression. For example, overexpression of DYRK1 results in higher levels of REST protein, reduced levels of L1CAM and Elmo, and ultimately, shorter neurites and less dendritic arbor complexity. The later of which are phenotypic traits of Down syndrome. REST has also been demonstrated to play a role in X-linked mental retardation (XLMR) (Tahiliani et al., 2007; Ding et al., 2007). One of the proteins that connect REST to this disorder is SMCX. SMCX is a demethylase whose more than twenty mutations have been linked to patients with mild to severe mental retardation (Tzschach et al., 2006). Although part of its activity is compromised with these mutations, the bulk of its repressive function is accomplished by its association with other repressive complexes, namely the E2F6 complex or the REST complex (Tahiliani et al., 2007). In addition, this study showed that the SMCX-REST complex sits in RE1

sites of genes like SCN2A, BDNF, and SCG10, all of which have been implicated in mental retardation. Through a similar mechanism, MED12, a member of a "dissociable repressive module", acts as a mediator that links REST to G9a-dependent histone H3K9 dimethylation to suppress neuronal genes. Mutations of MED12 disrupt association with REST, and therefore, the restriction for neuronal expression is lifted (Ding et al., 2008). This misregulation of REST target genes could conceivably affect neuronal differentiation and possibly contribute to XLMR.

REST in neurodegenerative disorders. In neurodegenerative disorders such as Parkinson's disease (PD), REST is responsible of maintaining homeostasis of neurotransmitters like dopamine and serotonin. A conditional REST knockout model showed that animals lacking REST are more sensitive to the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that causes permanent loss of dopamine neurons (Yu et al., 2013). The mechanism explaining these effects reside in the misregulation of genes such BDNF, parkin, and tyrosine hydroxylase, but also in a marked increase in the number of astrocytes that perhaps render serotonergic and dopaminergic neurons in to a more sensitive state upon insult.

Huntington's disease (HD) is another neurological disorder that has questioned the role of REST. It is characterized by the successive CAG expansion in the 5' coding region of the huntingtin protein that results in progressive neuronal death of striatal and cortical neurons. In the context of a misfolded huntingtin, it has been suggested that neurotrophins like BDNF are downregulated thus perhaps preventing a trophic support to

the striatum (Zuccato et al., 2001). Because BDNF contains a RE1 site and the levels of BDNF are dependent on the levels of misfolded protein, it was hypothesized that perhaps REST and huntingtin interplay at the genomic level. In order to test this hypothesis, Zuccato el al. transfected ST14A striatal cells that express wild type huntingtin or mutant huntingtin with CAT reporter containing 300bp of the BDNF promoter II encompassing the RE1 site. They showed that mutant huntingtin reduced reporter activity compared to wild type huntingtin (Zuccato et al., 2001). Analysis of human brains also demonstrated a significant difference in BDNF expression between healthy and HD patients. More specifically, they showed that the BDNF mRNA and protein levels were reduced in the cortex of patients with HD, and this difference was more clearly observed at the early stages of the disease. In addition, EMSA studies demonstrated the observed increase in BDNF expression was orchestrated by the huntingtin-mediated sequestration of the REST complex to the cytoplasm. The mutant protein, on the other hand, retained REST in the nucleus to continue to repress the expression of BDNF and another selective group of genes under pathological conditions (Zuccato et al., 2003). This suggests that perhaps REST is regulating their expression in cortex, and therefore, preventing their transport to the striatum where most of the detrimental effects of huntingtin occur (Zuccato et al., 2008). Although the expression and localization of REST in human brain remains controversial, a recent paper showed that both huntingtin and REST colocalize in neurons of the caudate nucleus, brain stem, and cerebellum, and this is in both, healthy controls and HD samples. Thus opening up the possibility for a new potential therapeutic target for HD (Schiffer et al., 2014).

Lastly, a new report describes a role for REST in stress resistance during normal aging and Alzheimer's disease. Although contentious, this study proposes that REST may coordinate a neuroprotective response against detrimental factors such as reactive oxygen species and β amyloid secretion by regulating the expression of anti-apoptotic genes such as bcl-2 or cell death genes during normal ageing and pathological conditions in human brain (Lu et al., 2014).

Collectively, there is significant evidence to propose that REST plays a crucial role in pathophysiology of diverse disorders. Therefore, the understanding of the exact mechanism of REST regulation is paramount to perhaps evaluate the potential for therapeutics.

CHAPTER 2

Characterization of rat monoclonal and rabbit polyclonal antibodies that recognize mouse REST

Karin A. Müllendorff, Glen Corson, Gail Mandel

ABSTRACT

The transcriptional repressor, REST, is a master regulator of genes that confer neuronal identity. Because detection of this protein *in vitro* and *in vivo* is fundamental for understanding its function, many antibodies have been developed by different laboratories throughout the years. While some of these antibodies work well for analysis of *in vivo* binding of REST to chromatin and Western blot analysis, tissue histology has traditionally been problematic due to non-specific staining and the lack of comparison to REST knock out mouse tissue. Thus, a primary goal of my thesis work has been to develop and characterize an antibody that is appropriate for histology, in tandem with REST knock out analysis. In the present chapter, I report a number of both polyclonal and monoclonal antibodies targeting two different epitopes from the deduced REST primary sequence. Although several efficacious antibodies were found to recognize the right molecular size protein in Western blot analysis, the rat monoclonal antibody clone 4A9 was the best for brain histology based upon nuclear staining and lack of labeling in our REST conditional knock out mice model. Therefore, I used this antibody to determine REST localization in both mouse adult brain and REST-expressing murine stem cells.

INTRODUCTION

The establishment and the maintenance of neuronal identity is a key event during neuronal development. For this process to occur in an ordered manner, a series of pan and specific neuronal transcriptional regulators are needed. Early work showed that repression rather than activation was a primary event in this process. A classic example of this type of regulation was shown with the voltage-dependent sodium channel Nav1.2 (Grubman et al., 1988), whose expression is largely restricted to the central nervous systems and not to non-neuronal tissues (Beckh et al., 1989). It was demonstrated that the RE1 Silencing Transcription Factor, REST, was the master regulator of neuronal gene expression in non-neuronal tissue (Maue et al., 1990; Kraner et al., 1992; Chong et al., 1995; Schoenherr et al., 1995). It does this by forming a silencing complex with a series of corepressors that concertedly bind to a 23bp responsive element to block the expression of its target genes. Thus, there has been a great deal of interest in trying to understand the nature of the repression and when the repression is needed.

Studies from our laboratory determined that REST is highly expressed in stem cells, but then, its expression declines as cells acquire a more differentiated phenotype (Ballas et al., 2005). That is, REST expression decreases as cells become neural progenitors, and it almost disappears as they become mature neurons. It is known that post transcriptional and post transcriptional mechanisms has been involved in this regulation. The former is mediated by a RNA-binding factor that recognizes an AU-rich element in the 3' UTR of REST mRNA that results in its destabilization (Cargnin et al., 2014). The latter is

mediated by the specific phosphorylation of two residues in the C-terminal domain of REST that results in the proteasome-mediated degradation (Ballas et al., 2005; Westbrook et al., 2008; Nesti et al., *In preparation*) Therefore, it has been long hypothesized that REST protein is poorly expressed or not expressed at all in the brains of adult mammalians relative to stem cells and non-neuronal tissue. In contrast to this however, several pieces of evidence have suggested that REST is also expressed in adult brain. For example, early work by Palm et al. showed that REST mRNA is present in areas of the brain such as hippocampus and cortex (Palm et al., 1998). Later, Gao et al. showed that REST is expressed in quiescent neural stem cells of the hippocampus, it is downregulated as these cells progress into differentiation, and then reappears in immature and mature granules neurons of the hippocampus (Gao et al., 2012). In general however, there are three central problems to these studies. First, they mostly demonstrate REST mRNA levels by in situ hybridization, leaving it open-ended as to whether the transcripts are translated into protein. Second, there is a disconcerting lack of consensus with regard to the size of the REST protein that is being studied, thus casting doubts on the validity of the REST antibodies that were used. Studies in our laboratory comparing wild type and REST knock out models have consistently shown that its right molecular weight is found at a migrating band of approximately 180KDa. Third, the antibodies available must lack both in sensitivity and specificity when looking at low REST expressing cells because almost 30 years after REST discovery only a handful of studies have shown REST expression in those cells.

Recently, a breakthrough study has shown that REST protein levels increase in healthy ageing brains, but they are significantly downregulated in patients with Alzheimer's disease (Lu et al., 2014). Because of the potential for therapeutics, my efforts have focused onto developing and characterizing antibodies against mouse REST. This will allow us to better understand the role of REST in adult murine brain and perhaps gain a closer insight into the REST-mediated processes that occur in human brain.

By using immunobloting and immunofluorescence, we characterized both rat monoclonal and rabbit polyclonal antibodies that have been directed against epitopes located at the mid- to C-terminal domain of mouse REST. Most of the hybridomas tested gave a specific 180KDa band corresponding to the full length REST protein. However, only some of them showed a very specific nuclear signal in mouse stem cells. We observed that rabbit polyclonals 095 have a very strong western blot signal, however, they showed unspecific staining in mESCs. To the contrary, rat monoclonal antibodies clones 4A9 and 7D10 gave the most specific nuclear signal in mESCs as shown by immunostaining, but they were less sensitive than polyclonal when tested by immunoblotting. Altogether, this study concludes that depending on the question and the technique to be used, rabbit polyclonal antibodies are the best source to detect mREST by immunoblotting, whereas, rat monoclonals, particularly clone 4A9, was the best to detect mREST in frozen tissue and *in vitro* cultures.

MATERIAL AND METHODS

Animals

All experiments were carried out in accordance with the protocols approved by the IACUC at Oregon Health and Science University (OHSU). Six to ten week old wild type and REST conditional knockout (CKO) mice were obtained from mouse colonies at OHSU. Generation and genotyping of the REST CKO mice has been recently described (Nechiporuk, et al. *In preparation*). In brief, REST GT (D047E11) mutant mice were generated by blastocyst injection of the D047E11 gene trap clone (GenBank Acc#: DU821609). REST GTi (D047E11) carrying the inverted gene trap vector was generated by crossing to Fliper deleter mice (Rodriguez et al., 2000). To provide specific knock down of REST in neural progenitors, REST GTi mice were crossed to Nestin:CRE #0036771 mice (Jackson Laboratories). Strains were backcrossed to C57BL/6J background for at least 10 generations. Mice genotyping was performed as described (Nechiporuk et al., *In Preparation*).

Generation of rat monoclonal and rabbit polyclonal anti-mREST antibodies. Glen Corson in our laboratory cloned the peptides to be used for antibodies generation. In addition, Dan Cowley at the core facility of Oregon Vaccine and Gene Therapy Institute generated the monoclonal antibodies described below. The full-length mouse REST is 1024 amino acids long. The partial REST coding sequences encoding amino acids 412 to 548 and amino acids 889-1035 were cloned separately into pGEX-3X vector (GE Healthcare) and introduced into bacterial host strain BL21(DE3) (Agilent). GST-REST

fusion peptides were induced by treating the cultures with 1mM IPTG followed by glutathione-agarose affinity purification. Hundred mg of the purified peptides were used to immunize rats twice and hybridomas were derived using established procedures (Goding, 1980) (Contributed by Dan Cowley at the core facility of Oregon Vaccine and Gene Therapy Institute). Supernatants from the growing hybridomas were screened by enzyme-linked immune-absorbant assay (ELISA). The positive wells were clonally expanded by limited dilution and tested again by indirect ELISA. Antibodies were purified from the supernatant of cloned cell lines using mercaptoethylpyridine chromatography (Pall Biosciences) followed by thiophilic chromatography (Pierce Chemical) using protocols recommended by the manufacturers. To generate polyclonal antibodies against the mouse REST, one hundred mg of the C-terminal purified peptide encompassing amino acids 889 to 1035 were used to immunize two New Zealand white rabbits (Covance). The antiserum was affinity purified against its antigen after the test bleed. The specificity of antibody was tested by comparing REST protein levels between $REST^{-/-}$ (Jørgensen et al., 2009) and wild type embryonic stem cells by Western blot analysis and immunofluorescence.

Mouse Embryonic Stem Cell (mESC) Culture

Mouse embryonic fibroblasts (iMEFs), irradiated for mitotic inactivation, were first plated in 0.1% porcine gelatin plates. They were grown for 24h with DME medium plus 10% fetal bovine serum and antibiotics. At this point, mESCs were thawed and propagated on top of the iMEFs and maintained in mESC media containing: 15% fetal bovine serum, 1x nucleosides, 2mM glutamine, 0.1mM Non-Essential amino acids,

50μM β-mercaptoethanol, DME medium supplemented with 1000U/ml of leukemia inhibitor factor (LIF) (Bain et al., 1995).

Western Blotting

Wild type and REST knockout mouse stem cells were rinsed twice with cold PBS and lysed with RIPA buffer containing 50mM Tris HCl, pH 7.4, 150mM NaCl, 1% NP-40, 0.25% sodium deoxicholate, 1mM NaF, 1mM Na_3VO_4, and protease inhibitors containing EDTA (Roche Diagnostics). Samples were then homogenized for one minute with a motorized mini rotor and centrifuged at 14,000 rpm for 15 min at 4C. Supernatant was collected and protein concentration was measured by the Lowry method following the manufacturers instructions (BioRad). Five micrograms of total lysate were subjected to SDS-PAGE by loading into 3-8% Tris-Acetate gels (Invitrogen) and ran at a constant 100V. Gel was transferred to 0.45μm nitrocellulose membranes at 100V for 2h. Blots were incubated for 1h at room temperature with blocking solution: 5% non-fat milk, 1% BSA, 0.1% Tween-20 and sodium azide. This was followed by incubation with purified monoclonal or polyclonal primary antibodies diluted in blocking solution at 4C overnight. The membranes were washed three times with phosphate buffer saline (PBS)-0.1% Tween (PBS-T) and incubated with goat anti-rat or goat anti-rabbit IgG conjugated to horseradish peroxidase respectively at room temperature for 2h. Following there washes with PBS-0.1% Tween (PBS-T) the reactive bands were visualized using enhanced chemiluminescence (Pierce).

Immunocytochemical staining

Mouse stem cells were grown on top of gelatin-coated cover glasses, and one day after plating, they were gently rinsed with cold PBS and fixed in 4% freshly prepared paraformaldehyde (PFA). After rinsing three times with PBS, they were permeabilized with PBS-0.1% Triton X-100 for 15 minutes at room temperature followed by incubation with blocking solution consisting of 10% normal serum (Jackson Laboratories) in PBS-0.1% Tween for 1h at RT. Primary monoclonal and polyclonal antibodies were diluted in blocking solution and incubated overnight at 4C. Cover glasses were rinsed with PBS-0.1% Tween and then incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies, Eugene, OR) diluted in 10% normal serum in PBS-T for 2h at room temperature. Depending on the number of channels used for each staining, we either used DRAQ5 (1.5-bis{[2-(di-methylamino)ethyl]amino}-4,8-dihydroxyanthracene-9, 10-dione (Cell Signaling Technology, Danvers, MA) or DAPI (4', 6-diamidino-2-phenylindole dyhydrochloride) (MP Biochemicals, LLC, Solon, OH) for counterstaining of cell nuclei. In the case of the former, it was added in conjunction with the secondary antibody incubation at a concentration of 1:1,000. In the case of the later, the secondary cocktail was removed from the slide, replaced with DAPI at 1:10,000 and incubated for 10 minutes at room temperature. The slides were then washed with PBS-T three times, mounted with ProLong Gold antifade reagent (Life Technologies, Eugene, OR), and cover slipped with #1 ½ micro glasses (Fisher Scientific). Images were acquired with a Zeiss confocal laser scanning LSM 510 microscope, using a 25X or a 40X objective with an acquisition setting at a resolution of 1024x1024 pixels.

For immunocytochemical studies in adult brain tissue, mice were deeply anesthetized with isofluorane and transcardially perfused with PBS followed by freshly prepared 4% w/v PFA in PBS at pH 7.4. After perfusion, the brains were removed from skull and immersed in 4% w/v PFA in PBS, pH 7.4 for 3h at 4C. Brains were immersed overnight in 30% sucrose at 4C, embedded in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, PA) and stored at -80C. The frozen tissue was sectioned into 20µm sections and placed on Superfrost Plus Slides (VWR International, LLC, Radnor, PA). For REST staining with monoclonal and polyclonal antibodies, slides were fixed and permeabilized for 10’ with cold acetone. The remainder of the procedure follows the protocol described above.

RESULTS

Antigen design and production

We chose to develop both a monoclonal and polyclonal antibodies to detect REST *in vivo*. Monoclonal antibodies recognize only one epitope, and therefore, are more specific and have less background than polyclonals. However, because monoclonal antibodies are sensitive to the accessibility of that epitope, we also chose to generate a polyclonal antibody. The use of polyclonal also maximizes the signal obtained from a target protein, especially low expressing ones, because they bind to multiple epitopes. I began by selecting two amino acid sequences within the mouse REST protein, the domains of which are shown in (Figure 1A). The first fragment encodes amino acids 412 to 548 (mREST-H), which encompasses a lysine rich domain of REST (Figure 1B). We chose this fragment because it has a low homology with its counterpart in humans, it is specific of REST because it does not show high homology with other proteins, and because it is highly charged, which allows for the epitope to be exposed and recognized by the antibodies. The second fragment encodes amino acids 889 to 1035 (mREST-J), which encompasses a poorly conserved region with mixed composition and a highly conserved acidic region that includes REST's phosphodegron (Nesti et al., *In preparation*) (Figure 1C). We chose this fragment also because it is a highly charged region, and low homology, but most importantly because our lab has produced antibodies in the past, namely the C-REST antibody (Figure 1D), that has successfully detected this most C-terminal region of human REST. Both of these sequences were run against the whole genome database to exclude the existence of other proteins with significant sequence

homology. These peptides were then fused to Glutathione S-transferase (GST) and expressed in *Escherichia coli.* Upon purification through affinity chromatography, the immunogen mREST-H was injected in rat whereas mREST-J was injected both in rat and rabbit.

Antibody characterization

The first step towards characterizing the rat monoclonal antibodies was to screen the supernatant of at least two dozen hybridomas by immuno-labeling in two independent biological systems: mouse embryonic stem cells (mESCs) and human embryonic kidney 293 cells (HEK293). The relative advantage of using mESCs is that they express high levels of endogenous REST compared to other cell lines such as fibroblasts and HEK293 cells. Although HEK293 express very low levels of REST, they are very amenable to transfection and have high efficiency of transfection and protein synthesis, thus making them a useful tool to overexpress mouse REST (studies performed by Glen Corson, not shown). The immunostaining analysis revealed three labeling patterns. One pattern was exemplified by rat monoclonal clone 4A9, which showed a very specific nuclear staining that matched the pattern depicted by the nuclear dye DRAQ5 (Figure 2A). This is the expected localization because REST is a transcriptional factor that binds DNA (Chong et al. 1995). I also observed that REST is absent from nucleolus and distributes inside the nucleus with a speckle-like pattern characteristic of heterochromatin. The rat monoclonal clone 7D10 also showed a similar specific signal in these cells (Figure 2B). Interestingly, we observed cells undergoing mitosis as depicted by their particular chromosomal alignments. In this case, REST was dispersed throughout the cell (Figure 2B, right panel

arrowheads) indicating it was ejected from mitotic chromatin as it has been described for other sequence-specific transcription factors and co-factors during mitosis (Gottesfeld et al., 1997; Kadauke et al., 2013). In addition, clones 9H9, 15H6, and 6B3 (Figure 2C-E) showed nuclear staining but also showed a significant proportion of staining in the cytoplasm and plasma membrane. Based on these results, I used clones 4A9 and 7D10 for further clonal expansion and purification because they show the most clear nuclear localization.

We analyzed the rabbit polyclonal antibodies in the same manner. Our results showed that unlike some of the rat monoclonals, the two rabbit antisera tested had both a nuclear and cytoplasmic staining (Figure 3A and 3B). Nonetheless, we chose the rabbit 095 antibody for further studies because it showed the least amount of background. For comparison, we used a rabbit polyclonal anti-human REST antibody, which recognizes the C-terminal domain of human REST from amino acid 801 to 1097 (Figure 1D) and has been reliably used for the purposes of immunoblotting and chromatin immunoprecipitation in mouse stem cells (Ballas et al., 2005). We observed that similar to the polyclonal 095 and 092, the C-REST 719 antibody also shows a nuclear and some cytoplasmic staining (Figure 3C). Therefore, we conclude these antibodies are not necessarily suitable for immunostaining, but instead might have a better use in immunoblotting and immunoprecipitation.

Purified monoclonal and polyclonal antibodies recognize mouse REST epitopes in Western blot analysis of mESCs.

In order to assess the specificity of the purified monoclonal and polyclonal antibodies by immunoblotting, I generated whole cell protein lysates from mESCs from both wild type and REST -/- cells (Jørgensen et al., 2009). The fractions were processed and equal protein concentrations were loaded into acrylamide gels. I first analyzed the C-REST antibodies 719 and 720 (Figure 1D). I found that they detected a band migrating at approximately 180KDa in wild type stem cells, and this band was absent in our REST -/- cells (Figure 4A and Figure 4B). However, we detected a 135KDa migrating band, which we believe is a truncated form of REST product of the presence of a cryptic methionine within the exon2. The existence of this truncated form explains why we observe signal in these cells when performing immunostaining with all these antibodies. In addition, I detected several cross-reactive bands that varied in intensity along the gel, and that appeared in both wild type and REST -/- cells. We assume they are non-specific bands, and they constitute only a minor component. I also compared blots using a polyclonal antibody that recognizes the N-terminal domain of human REST from amino acid 73 to 543 (Figure 1E). I found that it also recognizes a band at approximately 180KDa in wild type stem cells. However, I did not observe the 135KDa in the REST-/- cells (Figure 4B). This is consistent with the idea that the truncated form observed with the other polyclonal antibodies is a result of a cryptic initiation methionine that is found in exon 2 of REST. This would give rise to a truncated form that lacks the most N-terminal domain of the protein. The analysis of polyclonal antibody 095 raised against the mouse REST revealed a pattern similar to the one observed with the other antibodies raised against human

REST. That is, I observe both a strong 180KDa band and the truncated form at a lower molecular weight. It also appears to be slightly more specific because there are not many cross-reactive bands (Figure 4D). Lastly, the study of our newly purified rat monoclonal antibodies 7D10 and 4A9 showed they both recognize full length REST in the wild type stem cells. However, the signal of the 7D10 antibody was significantly lower than the other rabbit polyclonals at the same exposure time (Figure 4E). In addition, it showed a lot less cross-reactivity because the blot did not show other bands, which could be attributed to the lower sensitivity. Surprisingly, the rat monoclonal antibody 4A9 was barely noticeable at the exposure time the other antibodies were revealed. For that reason, we loaded different protein concentrations: 5µg, 25µg, and 50µg per lane respectively. This showed that only at twenty-five micro grams of protein, we could more clearly detect REST (Figure 4D). Altogether, we conclude that the polyclonal antibodies constitute the best tools to analyze REST protein expression by immunoblotting. The rat monoclonals on the other hand require a lot more protein to be loaded in order to get a significant signal. Most importantly however, all these antibodies recognize REST because the REST -/- failed to detect the 180KDa protein.

Monoclonal and polyclonal antibodies detect REST in mouse brain tissue.

A handful of studies to date have shown the expression of REST protein in brain tissue under basal conditions (Palm et al., 1998; Gao et al., 2011; Noh et al., 2012; Lu et al., 2014). However, it still remains controversial as to whether the signal being detected is specific, given the very low levels of REST in tissue. Therefore, my main goal was to optimize the conditions that could allow me to use our monoclonal and polyclonal

antibodies to resolve the question of whether or not REST is expressed in adult brain and where in the brain it was present. The optimization methods involved assessing several fixation approaches, antigen retrieval, detergents types, and blocking conditions to optimize the signal to noise. The first observation we made is that optimal perfusion with freshly made paraformaldehyde is fundamental for a good staining. With poor fixation, the REST signal was greatly diminished and could not be recovered by post-fixation attempts (data not shown). Also, no perfusion at all resulted in a very strong nuclear signal that is likely to be unspecific because not only it was observed all over the brain, but also, it was observed with the same intensity in our REST -/- animals (data not shown). We also compared no post-fixation versus post-fixation with acetone or paraformaldehyde. We found that incubation with cold acetone for 10 minutes gave us the best signal, followed by PFA, and no post-fixation. When it came to assessing whether antigen retrieval would improve our signal, we observed no signal difference by steaming the frozen tissue with sodium citrate buffer. Similarly, comparing blocking with 2.5% BSA versus fish gelatin and normal serum did not reveal any differences as it did the use of detergents such as Triton X-100 or saponin. However, it did seem that 0.3% Triton showed slight improvement in signal compared to 0.1% (data not shown). In summary, we showed that the best conditions to detect REST in frozen mouse brains are to perfuse with freshly made PFA and post-fixed with 100% acetone for 10minutes. This should be followed by blocking with 10% normal serum with 0.3% Triton X-100.

Having established fixation conditions, I went on to determine the best antibody concentration to be used in adult frozen brains, and tested the antibodies at different

dilutions in mouse cerebellum. We observed that the absence of primary antibody, as expected, resulted in no immunoreactivity whereas the 1:300 dilution gave the best signal among the additional two tested (1:100 and 1:500). This held true for the rat monoclonal clone 7D10 (Figure 5A) and the rat monoclonal 4A9 (Figure 5B). Interestingly, we observed the staining pattern was localized to large cells in the molecular layer of the cerebellum likely to correspond to Purkinje cells. In the case of the rabbit polyclonal 095, we determined that 1:500 was the best dilution from the 1mg/ml stock (Figure 5C). Its staining pattern matched the ones observed with the monoclonal antibodies, however, we observed more unspecific staining resembling processes located in the molecular layer of the cerebellum (Figure 5C, right panel arrowhead). Because our studies points towards the rat monoclonal antibody being the most specific to recognize mouse REST among them all the ones tested, I compared the pattern of expression between wild type and our gene trap REST knockout mice. I showed that that REST is expressed in large pyramidal cells of the piriform cortex and this is specific because there is no signal in our REST deficient mice (Figure 5D).

DISCUSSION

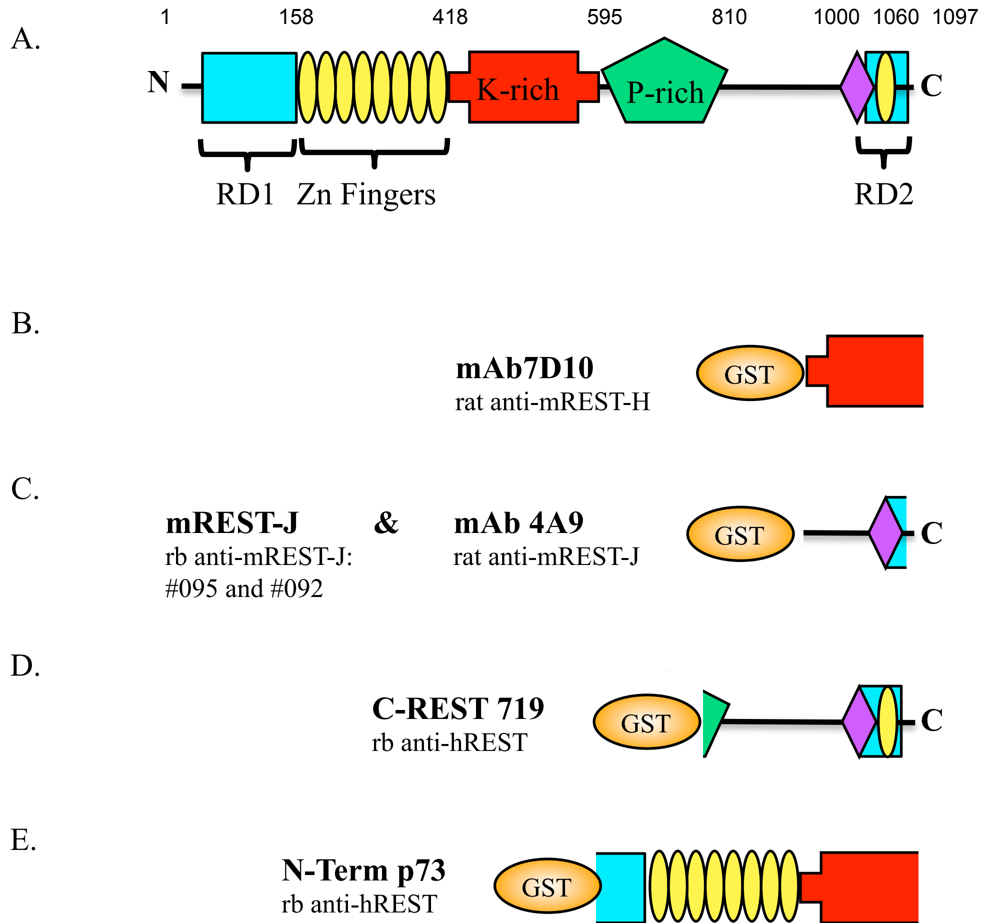
In the present study, I have characterized a series of antibodies raised against two different domains of mouse REST with the ultimate purpose to develop a robust and reliable protocol for immunoblotting, and most importantly, for the immunocytochemical localization of REST in rodent brain tissue. These antibodies were raised in either rabbit or rat and were directed towards either the most C-terminal domain of REST (mREST-H) or the most central region of it (mREST-J). As expected, most of the hybridomas initially tested by immunostaining showed nuclear signal. However, most of them lacked in specificity because they showed reactivity within the cytoplasm. Based on these studies and initial studies in HEK293 (data not shown), we selected three different antibodies for further purification and analysis: the rabbit polyclonal 095 and the rat monoclonal 4A9 both of which were directed against the C-terminal of REST and the rat monoclonal 7D10 directed against the central portion of REST.

There was no single antibody identified that was suitable for every technique tested. More specifically, we found that REST monoclonal antibodies failed to show strong signal in immunoblotting compared to the rabbit polyclonals. We believe this resides on the nature of monoclonal antibodies in that they only recognize one epitope in the REST protein sequence. It is possible the protein extraction induces conformational changes in the REST protein that hinder the REST epitope from being detected by the monoclonal antibodies. In contrast, all our polyclonal antibodies both directed against human REST and a newly generated against mouse REST detected a very strong REST signal in mouse

stem cells. It is worth noting, however, that even though we observe the right molecular weight band under 180KDa and its disappearance in our REST -/-, we also observe an aberrant form of REST in the REST -/- stem cells. I believe it may be a result of the transcription from a cryptic methionine present within the exon 2. In addition, we observe series of cross-reactive bands that may confound future studies and may explain why other publications have shown the wrong molecular weight signals. We cannot discard there are other REST isoforms that are been detected (Palm et al., 1998) as is the case for the hypothesized truncated form in our REST -/-. Therefore, it is fundamental to have the appropriate negative controls.

The most important aspect of this study was to characterize these antibodies in their ability to detect REST protein by immunofluorescence. We show that all three purified antibodies detect REST both in mouse stem cells and frozen mouse brain tissue. In addition, they all recognize a nuclear protein and show overlap in their localization pattern in adult brain. However, both 7D10 and the rabbit polyclonal 95 showed signal in processes and cytoplasm, which prompt me to discard this antibody for further immunological studies. The remainder antibody, the rat monoclonal 4A9, was the antibody that showed the clearest and most specific signal. I also found it is fundamental that the perfusion of mouse brains with PBS and freshly prepared paraformaldehyde is made correctly so to not risk a poor immunofluorescence down the line. In addition, great care must be taken in to storing the antibodies in the right location and conditions to also prevent the loss of antigenicity.

In summary, I presented data supporting the use of several monoclonal and polyclonals to detect mouse REST. These new tools should provide a new avenue for the study of REST expression in low expressing tissues such as the mouse brain. I ultimately hope to unravel the controversial idea that REST is expressed in adult brain, and that it may perhaps play a new role outside early neuronal development.


A.
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N
K-rich
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RD1
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B.
mAb7D10
rat anti-mREST-H
GST
C.
mREST-J
rb anti-mREST-J:
#095 and #092
&
mAb 4A9
rat anti-mREST-J
GST
C
D.
C-REST 719
rb anti-hREST
GST
C
E.
N-Term p73
rb anti-hREST
GST

Figure 1. Schematic representation of mouse REST protein domain structure and the approximate epitope sites for rat and rabbit polyclonal antibodies. A. Mouse REST domain structure. RD1, repressor domain 1; K-rich, lysine rich domain; P-rich, proline rich domain; purple diamond, acidic region; RD2, repressor domain 2; all nine Zn finger domains are depicted in yellow ovals. **B.** Rat monoclonal antibody raised against amino acids 412 to 548 of mouse REST (mREST-H) clone 7D10. **C.** Rabbit polyclonal and rat monoclonal antibodies raised against amino acids 889 to 1035 of mouse REST (mREST-J) specimen 095/092 and clone 4A9 respectively. **D.** Rabbit polyclonal antibody raised against the C-terminal domain of human REST between amino acids 801 to 1097. **E.** Rabbit polyclonal antibody raised against the N-terminal domain of human REST between amino acids 73 and 543.

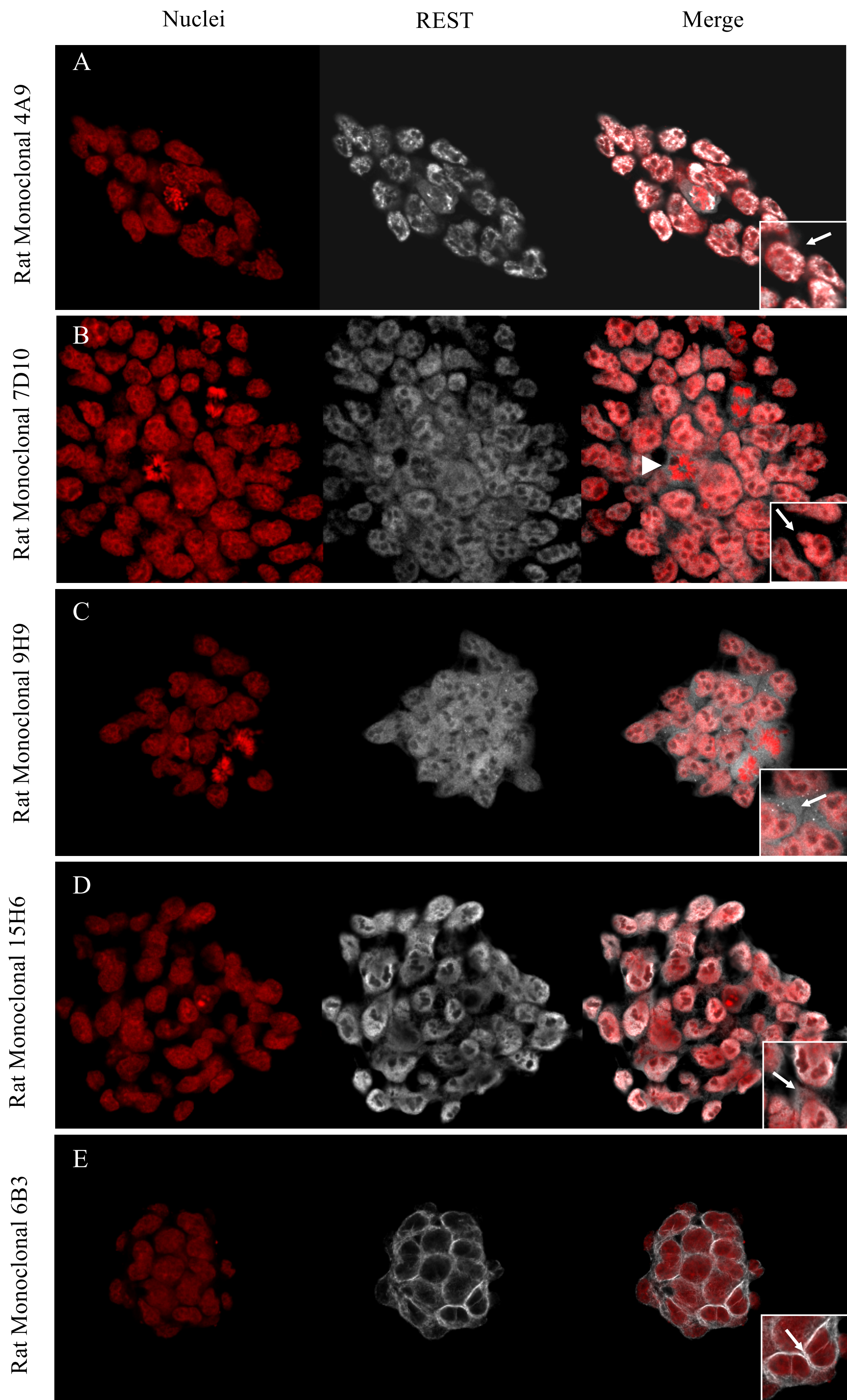
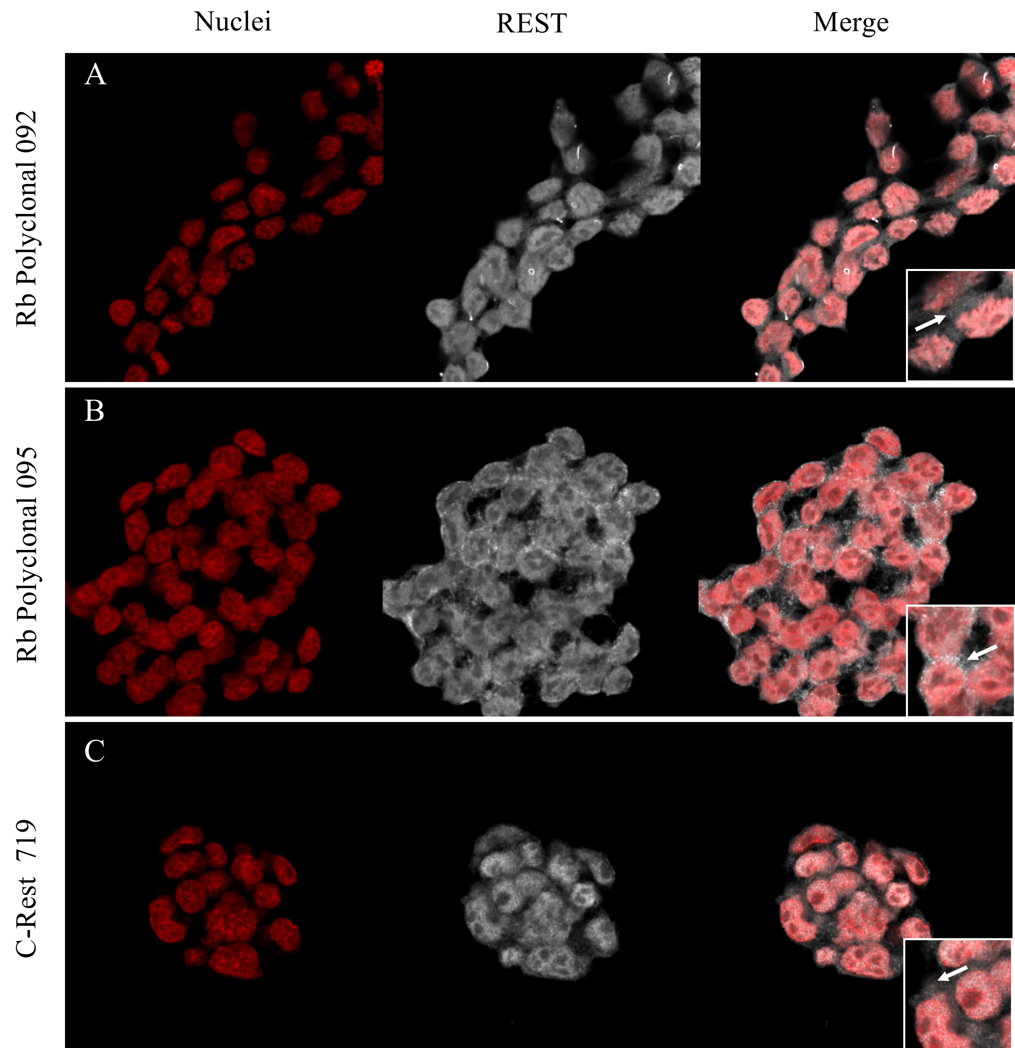

Nuclei
REST
Merge
A
Rat Monoclonal 4A9
B
Rat Monoclonal 7D10
C
Rat Monoclonal 9H9
D
Rat Monoclonal 15H6
E
Rat Monoclonal 6B3

Figure 2. Rat monoclonal antibodies immunoreactivity against REST. Two dozen rat hybridomas supernatants were tested for immunoreactivity against mouse REST in mouse stem cells. These supernatants encompass epitopes against two different domains of REST. After fixation and double immunolabeling cells were examined by confocal microscopy. *Red* and *grey scales* represent nuclei and REST staining, respectively. The *far right panels* show red and gray images merged to give a composite. *Insets* in right panels indicate higher magnification of an area of the merged image. *Arrows* inside inset indicate areas of unspecific staining**. A.** Rat monoclonal clone 4A9 recognizes amino acids 889 to 1035 of REST protein and shows specific staining only in the nucleus. **B.** Rat monoclonal clone 7D10 recognizes amino acids 412 to 548 of REST protein and shows specific staining only in the nucleus. *Arrowhead* shows mitotic cell. **C.** Rat monoclonal clone 9H9 directed against amino acids 801 to 1097 of REST protein shows nuclear staining, however, it also shows unspecific signal in the cytoplasm as shown by the arrow. **D.** Rat monoclonal clone 15H6 directed against amino acids 801 to 1097 of REST protein shows nuclear staining but also shows unspecific signal in the cytoplasm. **E.** Rat monoclonal clone 6B3 directed against amino acids 801 to 1097 of REST protein shows no nuclear localization at all. Because it seems to label cytoskeleton, it was discarded from further processing.



Nuclei
REST
Merge
A
B
C
Rb Polyclonal 092
Rb Polyclonal 095
C-Rest 719

Figure 3. Rabbit polyclonal antibodies recognize endogenous mouse REST. Mouse stem cells were tested with three different rabbit polyclonal antibodies recognizing two different epitopes in the C-terminal domain of REST. After fixation and double immunolabeling cells were examined by confocal microscopy. *Red* and *grey scales* represent nuclei and REST staining, respectively. The *far right panels* show red and gray images merged to give a composite. *Insets* in right panels indicate higher magnification of an area of the merged image. *Arrows* inside inset indicate areas of unspecific staining**.** **A.** Rabbit polyclonal 092 recognizes amino acids 889 to 1035 of REST protein and shows nuclear staining in addition to some unspecific signal in the cytoplasm. **B.** Rabbit polyclonal 095 also recognizes amino acids 889 to 1035 of REST protein and shows nuclear staining in addition to some unspecific signal in the cytoplasm. **C.** Rabbit polyclonal C-REST 719 directed against amino acids 801 to 1097 of REST protein shows nuclear staining in addition to some unspecific signal in the cytoplasm.

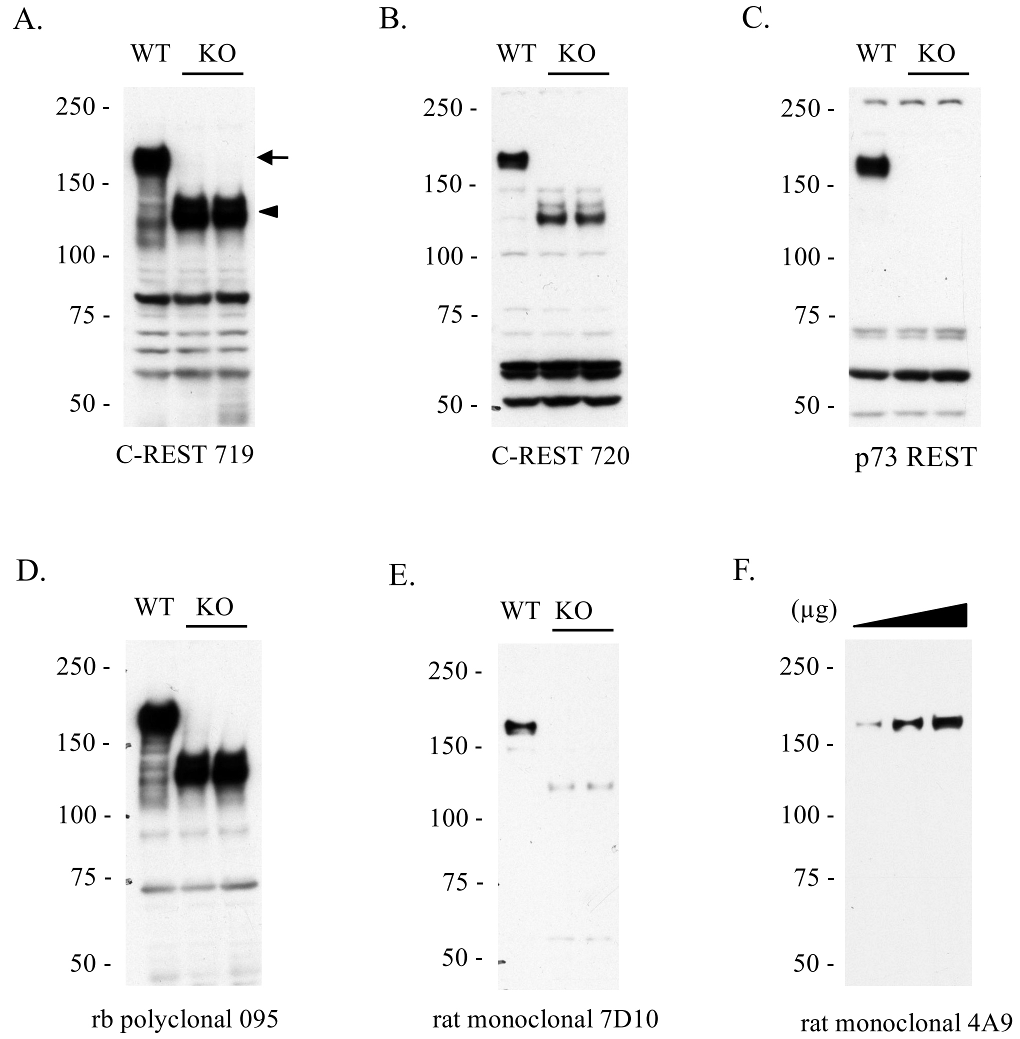
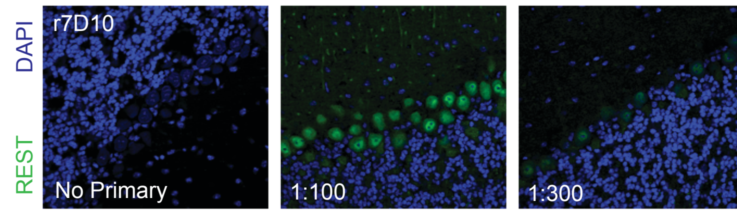
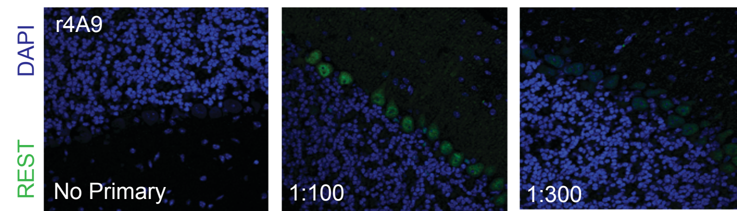

A.
WT
KO
250 -
150 -
100 -
75 -
50 -
C-REST 719
B.
WT
KO
250 -
150 -
100 -
75 -
50 -
C-REST 720
C.
WT
KO
250 -
150 -
100 -
75 -
50 -
p73 REST
D.
WT
KO
250 -
150 -
100 -
75 -
50 -
rb polyclonal 095
E.
WT
KO
250 -
150 -
100 -
75 -
50 -
rat monoclonal 7D10
F.
(µg)
250 -
150 -
100 -
75 -
50 -
rat monoclonal 4A9

Figure 4. **Anti-REST antibodies immunoreactivity in mouse stem cells.** Mouse stem cells both wild type and REST knockout cells were used to test rat monoclonal and polyclonal immunoreactivity against endogenous mouse REST. All blots are shown at the same exposure time except for figure 4F. *Arrow* indicates full-length REST. *Arrowhead* indicates truncated REST form. **A.** Western blot analysis by using classic rabbit polyclonal C-REST 719. **B.** Western blot analysis by using polyclonal C-REST rabbit 720. **C.** Western blot analysis by using rabbit polyclonal REST p73 antibody raised against N-terminal domain of REST. **D.** Western blot analysis by using newly generated rabbit polyclonal mREST-J 095 raised against the C-terminal domain of REST. **E.** Western blot analysis by using newly generated rat monoclonal mREST-H clone 7D10 raised against the more central regions of REST. **F.** Western blot analysis by using newly generated rat monoclonal mREST-J clone 4A9 raised against the C-terminal domain of REST did not show immunoreactivity at the exposure of the other blots. Therefore, five, twenty, and fifty micrograms of wild type stem cells were loaded for detection. n=3

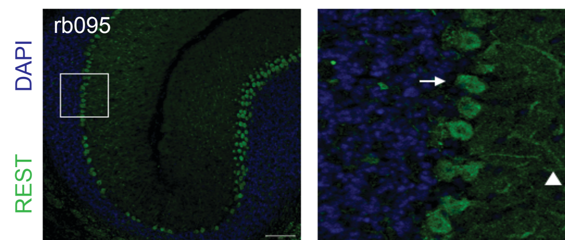
A



B



C



D

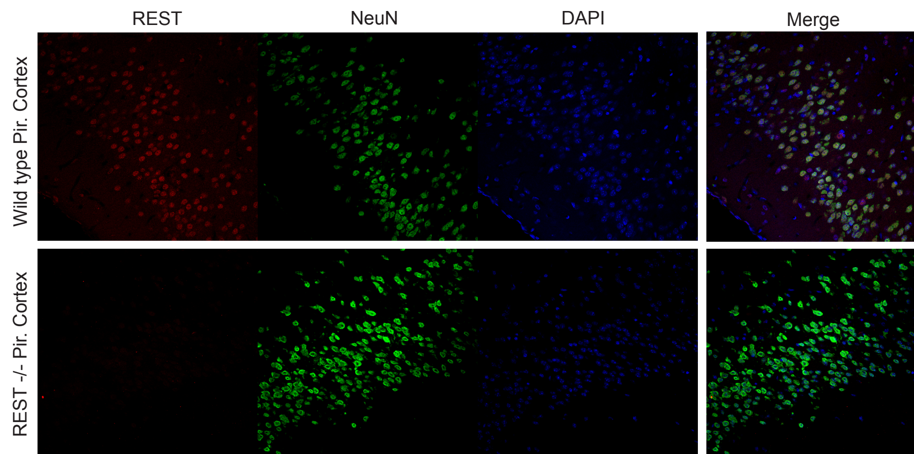


Figure 5. REST protein is detected by all monoclonal and polyclonal antibodies in mouse adult brain. Wild type adult cerebellum was used to test the monoclonal and polyclonal antibodies at different dilutions. Brains were fixed with paraformaldehyde, post-fixed with cold acetone and double stained with a nuclear dye and REST antibodies. Slides were examined by confocal microscopy. *Blue* and *green* represent nuclei and REST staining, respectively **A**. Immunostaining of REST with rat monoclonal clone 7D10 at different dilutions. *Arrowhead* indicates unspecific staining of processes. **B**. Immunostaining of REST with rat monoclonal clone 4A9 at different dilutions. **C**. Immunostaining of REST with rabbit polyclonal 095 at 1:500 dilution. *Inset* indicates area of higher magnification for the following image. *Arrow* indicates nuclear localization. *Arrowhead* indicates unspecific staining of processes. **D**. Wild type and REST -/- piriform cortex from mouse adult brain was stained with rat monoclonal 4A9 antibody. Signal was absent in brains of REST -/-. *Red,* REST; *Green*, NeuN; *Blue*, DAPI. n=3

CHAPTER 3

Study of REST expression in the brains of adult mice

Karin A. Müllendorff, Gail Mandel

ABSTRACT

Despite the fact that many studies have focused on REST function in pluripotent stem cells and neural progenitors, little is known about whether REST is present and functional in adult brain. In this chapter, I demonstrate that REST is expressed in 6-8 weeks old mouse brain. Furthermore, I show REST expression pattern is restricted to only certain areas of the brain and neuronal cell types such as Purkinje cells of the cerebellum, hippocampus, mitral cells of the olfactory bulb, brain stem, and piriform cortex. I show that REST retains its ability to bind to chromatin and its loss results in the up-regulation of several of its target genes compared to wild type. These results provide firm evidence that REST is not only important in early embryogenesis but expands to a new role in adult brain. Because REST regulates the expression of genes crucial to synaptic function and a newly discovered set of splicing factors, I speculate that REST may play role on maintaining intrinsic neuronal homeostasis.

INTRODUCTION

For almost 20 years, REST has been known as a master regulator of neuronal gene expression. Historically, the evidence has pointed to a transcriptional repressor that has the ability to bind 21 to 23bp repressor elements (RE1) motifs in the SCG10 and sodium channel Nav1.2 neuronal genes (Chong et al., 1995; Schoenherr et al., 1995). However, genome-wide chromatin occupancy analysis has shown that REST binds to over a 1,000 genes, which include but are not limited to ion channels, neurotransmitter receptors, growth factors, and synaptic vesicle proteins (Bruce et al., 2004; Schoenherr et al., 1995; Johnson et al., 2006; Otto et al., 2007). The structural domains found in REST allows for the recruitment of a series of cofactors and enzymatic activities that provide a dynamic interplay between gene regulation during particular developmental stage and cell context (Ballas et al., 2005). For example, the N-terminal domain of REST recruits Sin3a and histone deacetylases 1 and 2 (HDACs1/2) whereas the C-terminal domain is known to recruit CoREST, Sin3a, HDACs1/2, G9a, and LSD1 (Lunyak et al., 2002; Andres et al., 1999; Grimes et al., 2000; Huang et al., 1999; Humphrey et al., 2000; Shi et al., 2004).

The importance of REST in development was first established when targeted deletion of REST resulted in embryonic lethality (Chen et al., 1998). This later lead to the understanding that REST is important for maintaining the repression of neuronal genes not only in non-neuronal tissue, but also, in stem cells (Ballas et al., 2012). The current dogma establishes that REST is highly expressed in mice up until embryonic day 10.5 where it is found in neural progenitor cells and then downregulated by a mechanism

involving posttranslational modifications and degradation via the proteasome machinery (Ballas et al., 2005) and also transcriptional repression (Cargnin et al., 2014).

Paradoxically however, several studies have shown that REST is expressed in adult brain although mostly in the context of disease. For example, from basal love levels REST mRNA has been shown to increase in response to seizures and ischemic insults to regulate the expression of genes such as GluR2 (Calderon et al., 2003; Hu et al., 2011; Tanaka et al. 2002; Myers et al. 1998). Similarly, other studies have implicated REST in Huntington's disease and also with some types of brain cancer (Zuccato et al., 2001; Zuccato et al., 2003; Schiffer et al., 2014; Lawinger et al. 2000; Fuller et al., 2005; Su et al., 2006; Kamal et al., 2012). There are only a few studies that have addressed the presence of REST in the adult brain under basal conditions (Palm et al., 1998; Gao et al., 2011; Sun et al., 2005). Palm et al. gave the first clues that REST was expressed in adult brain by *in situ* hybridization, whereas Gao et al. demonstrated that REST is expressed in a biphasic manner in the hippocampus in a way that parallels the sequential stages of adult hippocampal neurogenesis. More specifically, Gao et al. showed REST is present in the quiescent neuroprogenitor cell (NPCs) pool, it is downregulated as neuronal differentiation occurs, and then expression is restored in immature and mature neurons. A recent study however has evoked a great deal of attention because it uncovered for the first time that REST is expressed in adult human brains, and that its expression is regulated through adulthood (Lu et al., 2014). They demonstrated not only that REST expression increases with ageing, but also that is markedly reduced in the Alzheimer's disease cohort studied, suggesting that REST might be neuroprotective. These studies

were performed while I was developing better reagents to examine where and how is REST expressed in the adult brain.

In the present study, I employed wild type and conditional REST knockout (REST -/-) mice to investigate whether REST is expressed in adult mouse brain. I found that both mRNA and protein is present in cortex, hippocampus, brain stem, and Purkinje cells of the cerebellum and mitral cells of the olfactory bulb. In addition, REST protein is downregulated in an age-dependent manner, and most importantly, REST is bound to chromatin of known target genes in six to eight week old mice. This in turn results in the regulation of the expression of some of these genes, but interestingly, in a brain area-dependent manner. In sum, my results conclusively demonstrate that REST is expressed in adult brain thus raising a potential repressive role of REST in mature neurons.

MATERIALS ANS METHODS

Animals

All experiments were carried out in accordance with the protocols approved by the IACUC at Oregon Health and Science University (OHSU). Six to ten week old wild type and REST CKO mice were obtained from mouse colonies at OHSU generate by Tamilla Nechiporuk, a postdoc in the lab. Generation and genotyping of the REST CKO mice has been recently described (Nechiporuk, et al, *In Preparation).* In brief, REST GT (D047E11) mutant mice were generate by blastocyst injection of the D047E11 gene trap clone (GenBank Acc#: DU821609). REST GTi (D047E11) carrying the inverted gene trap vector was generated by crossing to Flpe deleter mice (Rodriguez et al., 2000). To provide specific knock down of REST in neural progenitors, REST GTi mice were crossed to Nestin:CRE #0036771 mice (Jackson Laboratories). Strains were backcrossed to C57BL/6J background for at least 10 generations.

For immunohistochemical studies, mice were deeply anesthetized with isofluorane and transcardially perfused with PBS, followed by freshly prepared 4% w/v PFA in PBS at pH 7.4. After perfusion, the brains were removed from skull and immersed in 4% w/v PFA in PBS, pH 7.4 for 3h at 4C. Brains were immersed overnight in 30% sucrose at 4C, embedded in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, PA) and stored at -80C. The frozen tissue was sectioned into 20μm sections and placed on Superfrost Plus Slides (VWR International, LLC, Radnor, PA). For REST staining, slides were fixed and permeabilized for 10’ with cold acetone. For all other stainings, slides

were place on a heating plate at 65C for 2h and post-fixated with 4% PFA in PBS for 15’ at room temperature (RT). This was followed with permeabilization with 0.3% TX-100 in PBS for 15’. For all staining purposes, slides were blocked for 1h at RT with a 10% normal serum in 1X PBS. Sections were then incubated overnight at 4C with primary antibodies diluted in blocking buffer (Table 1). Primary antibodies used are listed in Table 1. Slides were then washed three times with PBS-T and incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies, Eugene, OR) diluted in 10% normal serum in PBS-T for 2h at room temperature. Depending on the number of channels used for each staining, we either used DRAQ5 (1.5-bis{[2-(di-methylamino)ethyl]amino}-4,8-dihydroxyanthracene-9, 10-dione (Cell Signaling Technology, Danvers, MA) for DNA staining or 4’, 6-diamidino-2-phenylindole dyhydrochloride (DAPI) (MP Biochemicals, LLC, Solon, OH). In the case of the former, it was added in conjunction with the secondary antibody incubation at a concentration of 1:1,000. In the case of the later, the secondary cocktail was removed from the slide, replaced with DAPI at 1;10,000 and incubated for 10 minutes at room temperature. The slides were then washed with PBS-T three times, mounted with ProLong Gold antifade reagent (Life Technologies, Eugene, OR) and cover slipped with #1 ½ micro cover glasses (Electron Microscopy Sciences, Hatfield, PA).

X-gal Staining

Frozen 20µm sections were left at room temperature for 30min and fixed with 0.5% glutaraldehyde in 1x PBS for 2min. After several rinses with PBS they were incubated with X-Gal solution: 1.3mM $MgCl_2$, 3mM $K_3(CN)_6$, 3mM $K_4Fe(CN)_6$, and 1mg/ml X-Gal and placed in an incubator at 37C overnight. The following day they were rinsed with distilled water followed by dehydration washes with 75% ethanol 5min, 95% ethanol 5min, 100% ethanol twice for 5min, and two xylene washes for 5min each.

Immunohistochemistry

Glial-fibrillary acidic protein (GFAP) was examined using immunohistochemistry (Table1). First, peroxidase activity was quenched with 0.3% H_2O_2 for 30min at room temperature. Slides were blocked with 10% normal serum in 0.3% TX-100 for 1h at room temperature, followed by incubation with primary antibody diluted in blocking buffer overnight at 4C. After PBS washes, sections were incubated for 1h at room temperature with biotynilated anti-mouse or anti-rabbit secondary antibody at 1:250 dilution (Vector Laboratories, Burlingame, CA). The avidin and biotinylated horseradish peroxidase Vecstatin ABC reagent (Vector Laboratories, Burlingame, CA) was prepared 30 min prior to use and then added to the tissue samples for another 30min. Slides were then washed with PBS and incubated with the DAB peroxidase substrate (Vector laboratories, Burlingame, CA) between 2 to 10 min. Water rinses were followed by dehydration with 75% ethanol 5min, 95% ethanol 5min, 100% ethanol twice for 5min, and two xylene washes for 5min each. Permount was added to seal the slides

Chromatin Immunoprecipitation (CHIP)

CHIP assay was performed according to the following protocol (Ballas et al., 2001): Mouse cortices were minced in to small pieces and 1% formaldehyde solution was added for 10min at room temperature. Cross-linking was stopped with glycine at a final concentration of 0.125M at room temperature for 5min. Cells were lysed and nuclei were isolated with buffer containing 5mM Hepes (pH 8.0), 85mM KCl, and 0.5% Triton X-100. The pelleted nuclei were resuspended in lysis buffer containing 50mM Tris-HCl pH 8.0, 10mM EDTA, and 1% SDS. Samples were sonicated (Branson Ultrasonics, Danbury, CT) for 10 rounds of 12 pulses at 90% duty cycle each. Sheared chromatin fragments were between 300-400bp. The chromatin suspension (150μl) was diluted 10-fold with CHIP dilution buffer containing 16.7mM Tris-HCl pH 8.0, 1.2mM EDTA, 167mM NaCl, 0.01% SDS and 1.1% Triton X-100 and incubated with 4μg of polyclonal C-REST antibody #720 or anti-rabbit IgG overnight at 4C. Dynabeads Protein A (Novex, Oslo, Norway) were blocked with 0.5% BSA for 1h at 4C and then added to the immunoprecipitated suspension for 3h with continual rotation. After washes and elution, adding a final concentration of 200mM NaCl reversed the cross-linking, and the samples were then incubated at 65C overnight. DNA was treated with RNase A and Proteinase K and then purified with QIAQuick PCR Purification kit (Qiagen, Valencia, CA). Stem cell and cortex samples were eluted with 200μl of TE buffer.

Real-time quantitative PCR of ChIP DNA

Two µl of CHIP elute from a total of two hundred microliters total volume and approximately 800mg of brain cortex were used for each SYBR Green PCR reaction. The mixture contained 7.5µl SYBR Green PCR Mix (Applied Biosystems, Warrington, UK), 0.5µM of each primer, and 4.5µl of dH_2O. The reactions were incubated at 95C for 10min followed by 40 cycles at 95C for 15sec and 60C for 1min then 15min at 95C, 15min at 65, and a final 15min for 95C in an ABI PRISM 7700 Sequence Detector. The abundance of the immunoprecipitated DNA was expressed as a percent of the input DNA.

Western Blotting

Brain tissue was micro dissected and either stored at -80C or was directly homogenized. To separate nuclear from cytosolic proteins, extracts were prepared by using a modified Dignam method. First, tissue was minced in small pieces and then resuspended in cold cytosolic buffer containing 20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors with EDTA (Roche Diagnostics, Mannheim, Germany). Samples were then lysed with a dounce homogenizer with 15 strokes and then kept once ice for at least 30min. Tissue was then centrifuged at 3,000g for 10minutes at 4C, supernatant was discarded, and the pellet was resuspended in nuclear lysis buffer containing: 20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, and protease inhibitors with EDTA. Samples were left on ice for another 30 minutes and then centrifuged at 14,000rpm for 15 min at 4C.

***In Situ* hybridization**

Probe preparation and in situ hybridization were performed as described previously (Yamaguchi-Shima and Yuri, 2007; Yamaguchi and Yuri, 2012). Briefly, a 1133bp cRNA probe complementary to bases 501 to 1634 (an EcoRI-XbaI fragment) of the mouse REST cDNA was used for hybridization. Linearized DNA was incubated in transcription buffer containing digoxigenin (DIG)-UTP and followed the manufacturer's instructions to synthesize RNA. Frozen brain sections placed on slides were fixed in fresh 4% paraformaldehyde solution for 15 min at room temperature. The sections were then washed with PBS-T followed by bleaching with 6% H_2O_2 in PBS-T for 5min at RT. After washing with PBS-T threes times, brains were permeabilized with 10 µg/ml of proteinase K in PBS-T for 10 min at RT. The treatment was stopped with fresh 2mg/ml glycine in PBT for 10min at RT. After two sashes with PBS-T, slides were post fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS-T for 15min at RT. Add pre-hybridization buffer containing 50% formamide, 5X Standard Sodium Citrate [(SSC) 1×SSC: NaCl, 150 mM; sodium citrate, 15 mM)], 1% SDS, 50 µg/ml baker's yeast tRNA (Boehringer, Mannheim, Germany), 50µg/ml heparin and incubate for 1h at 65C. Hybridization was conducted in a humid chamber by incubating overnight with hybridization buffer containing 1ng/µl of antisense or sense probe in an oven at 65C. Following hybridization, the slides were washed three times with 4X SSC pH 4.5 containing 50% formamide and 1% SDS for 15 min each at 70C. This was followed by a second wash with 2X SSC pH 4.5 and 50% formamide three times at 65C for 15min each. The slides were incubated with blocking solution for 1 h and with anti-DIG-AP Fab

fragment (1:500, Boehringer, Mannheim, Germany) for 30 min at room temperature. The slides were then washed and signal was detected by incubating the slides in AP-substrate solution 4-nitroblue tetrazolium chloride and 175μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP)(Roche Diagnostics, Mannheim, Germany) for 15 h at room temperature. Sections were then dehydrated and mounted with Permount solution (Fisher Scientific, Fair Lawn, NJ)

RESULTS

β-Gal activity in conditional REST knockout mice shows REST promoter activity in mouse adult brain.

A recent study in our laboratory has characterized a novel conditional REST knock mouse (Nechiporuk et al., *In Preparation*). This line was originally generated by the insertion of the gene trap (GT) allele in the *Rest* genomic locus upstream of the initiation methionine and between exons 1c and 2. The GT cassette contains not only a splice acceptor site and polyadenylation signal but also the promoterless *β*-galactosidase sequence. Therefore, we are able to follow the endogenous REST promoter activity based on the *β*-gal localization in tissue samples. In addition, to more specifically look at the effects of REST in the central nervous system, we crossed the gene trap mice with mice carrying the Nestin Cre transgene to knock out REST in neural progenitors. Six to ten weeks old mice brains were studied. Wild type and REST -/- mice brains were compared, and we observed REST -/- mice have smaller brains than wild type (Figure 1A). Studies in our laboratory suggest that this is due to an increase in cell death and premature differentiation that leads to fewer progenitors able to further proliferate and later differentiate into neurons (Nechiporuk et al., *In Preparation).*

Tracking of *β*-gal expression should correlate with REST promoter activity and transcription. Therefore, I performed *β*-gal staining in WT and REST -/- frozen brains. As predicted, I did not observe signal in the wild type animals because they do not contain the gene trap cassette (Figure 2A). However, I did observe *β*-gal staining in

several areas of sagittal brain sections (Figure 2B, *top panel*). These areas correspond to cortex, hippocampus, brains stem, cerebellum, olfactory bulb, midbrain, etc. Although REST promoter activity is detected throughout the brain, it is limited to very particular cell populations within the different brain regions. For example, there is a very specific signal in Purkinje neurons of the cerebellum (Figure 2B, bottom panel, left corner), hippocampus (Figure 2B, bottom panel, middle picture). This is also true for mitral cells and periglomerular cells of

the olfactory bulb (Figure 2B, bottom panel, right corner). These results suggest that endogenous REST promoter is active in six to eight week mouse adult brain.

REST mRNA and protein are found in mouse adult brain.

To determine whether REST promoter activity correlates with mRNA expression, I performed *in situ* hybridization using specific antisense and sense probes against nucleotide 501 to 1604 of mouse REST. The signal obtained shows a very similar pattern to what was observed with β-gal staining. That is, REST mRNA is expressed throughout the brain, and also strongly expressed in Purkinje cells of the cerebellum, mitral cells of the olfactory bulb, and hippocampus (Figure 3, left panels). I did not observe signal with the sense probes, thus demonstrating our pattern was specific to REST transcripts.

To determine whether REST mRNA levels parallel REST protein expression, I performed immunofluorescence studies with the rat 4A9 monoclonal antibody described in chapter 1. Similar to β-gal reporter activity and the mRNA expression profile, I observed REST protein in Purkinje cells of the cerebellum and mitral cells of the

olfactory bulb by immunofluorescence (Figure 4A, right panels). To further confirm these results, I micro dissected several areas of the adult brain and enriched REST by separating nucleus from cytoplasm. Equal amounts of nuclear protein extract were loaded, and Western blot analysis was performed by using the anti-human REST polyclonal antibody. I show a migrating band of approximately 180KDa in cerebellum, olfactory, and hippocampus. I conclude this band corresponds to endogenous REST because it was dramatically reduced in their counterparts in the REST knockout mice (Figure 4B). Because our mice are not a complete knockout, I speculate that the remaining signal we observe in both immunofluorescence and Western blot is likely due to leakage of the transgene. These results suggest both REST mRNA and protein are found in mouse adult brain.

REST binds to the chromatin of target genes in adult mice brain.

To determine whether REST binds DNA in adult brain, I performed chromatin immunoprecipitation studies with dissected cortices from mouse adult brain because it provides a significant amount of starting material. I first show that REST binds to the REI sequences of several of its established target genes such as the glycine receptor (GlyR), the ATPase, Ca++ Transporting, Plasma Membrane 2 (ATP2B2), the glutamate receptor, ionotropic, N-Methyl D-Aspartate 1 (GRIN1), and secretogranin 3 (Scg3) (Figure 5A). In addition, I tested the binding to a new target found in the CHIP seq database from mESC that shows the third strongest repression by REST. I determined that the RNA binding protein fox 1 homolog 3 (RbFox3), a splicing factor of the nervous system better known as NeuN is a strong REST target (Figure 5A). I demonstrated that REST also binds to the

responsive element of SNAP25, L1CAM, and another new target: the DNA-methyltransferase 3a (DNMT3a), an enzyme that catalyzes the transfer of methyl groups to CpG structures (Figure 5B). In addition, I observed that the REST -/- mice showed small background binding probably resulting from the small amount of REST remaining in these mice (Figure 5A and B). Altogether, this data indicates that REST does bind to the chromatin of known target genes and two newly discovered ones: RbFox3 and DNMT3a in mouse adult cortex.

Absence of REST results in the upregulation of some of its target genes in specific areas of the mouse adult brain. To determine whether the lack of REST results on the predicted upregulation of some of its targets genes, I dissected several areas of the brain and analyzed protein expression by Western blot and immunofluorescence. Interestingly, I found the target gene L1CAM is upregulated only in cortex but not in cerebellum of REST -/- mice, whereas to the contrary, the glycine receptor remained unchanged in cortex but increased in cerebellum (Figure 6A). I also observed a large upregulation of the splicing factor neuro-oncological ventral antigen 2 (NOVA2) in cortex of REST -/- mice than in any other brain region studied (Figure 6B) (Ule et al., 2006). Classic target genes such as the sodium channel Nav1.2 remained unchanged between wild type and REST -/- in cerebellum (Figure 6C). However, the expression of the P-type calcium channel Cav2.1 and the gamma-aminobutyric acid receptor subunit beta-3 (GABAβ3) increased in the cerebellum of REST deficient mice. These results indicate that REST target genes are not regulated equally, thus suggesting there might be more than one mechanism to explain why REST target genes are not increased.

REST expression decreases with age in mouse adult brain.

A recent study in adult human brains has reported that REST expression is dynamically regulated throughout adulthood (Lu et al., 2014). This paper was published while I was studying REST expression in cerebellum and hippocampus of wild type mice at different ages. In my hands, REST is expressed at high levels in young mice between two to four weeks old to then decrease as it progresses through adulthood. More specifically, REST lowest expression is observed at 18 months old in both cerebellum and hippocampus (Figure 7A). This was found to be the case for the 2 mice per group studied so far. To determine whether REST is also developmentally regulated in adult human brain, I obtained post-mortem hippocampus from the Oregon Brain Bank at Oregon Health and Science University (OHSU). These control subjects were selected based on how many hours post-mortem the brains were collected and the presence of plaques. We chose brains that had been collected no longer than 24h post-mortem and contained a low number of plaques as determined by pathological analysis. This is with the purpose to prevent protein degradation and to discard the presence of early stage neurodegenerative disorders, respectively. Western blot analysis of the male cohort between middle age men at 44 years old (yo) and the oldest old at 100yo showed no significant changes in expression except for a couple of individuals with low REST protein level at 52yo and 79yo respectively (Figure 7B). The female cohort, on the other hand, showed a great variability among samples even though protein concentration was measured and loaded equally for Western blot analysis. Grouping samples into a young (Figure 6C, lanes 1, 2, 3 and 4) and old cohort (Figure 6, lanes 5 through 8) may show a trend towards a protein increase with ageing. However, the variability in the young female group still prevents us

from making a strong conclusion. Altogether, these results suggest that REST protein expression may be regulated differently amongst mouse and humans. For more conclusive results, the human samples need to be expanded and include many more young individuals.

Loss of REST results in the increase of the GFAP in mouse brains.

My previous immunofluorescence analysis showed that REST is expressed in large cells with faint nuclei, whose morphology resemble that of neurons (Figure 4A and B). I did not observe colocalization with the glial fibrillary acidic protein (GFAP), a marker of intermediate filaments in astrocytes, thus indicating REST may not be present in these cells (data not shown). However, a serendipitous observation led me to further compare GFAP expression between our two mouse models. First, I performed an immunofluorescence study by staining both wild type and REST -/- mice cerebellum with GFAP. I observed a significant increase on GFAP reactivity in the knock out mouse cerebellum corresponding to Bergmann glia because it was interdigitated with Purkinje neurons in the molecular layer of the cerebellum (Figure 8A). A similar phenomenon was observed in cortex by immunohistochemistry. Here, wild type cortex showed a light GFAP staining of astrocytes whereas the REST deficient mice showed the classic morphology of reactive astrocytes with an apparent increase in the number and length of GFAP positive processes (Figure 8B) (Wilhelmsson, et al., 2006). This increase in GFAP staining also resulted in a protein increase in both cortex and cerebellum as demonstrated by Western blot (Figure 8C). These results indicate that the absence of REST perhaps indirectly promotes an increase on GFAP expression in mouse brain. Further studies are

needed to elucidate whether this is a result of an increase in the number of astrocytes or an increase in the number of reactive astrocytes perhaps in response to chronic inflammation or compensation for damage.

DISCUSSION

Historically, the activity of REST as a neuronal repressor has been considered fundamental for proper neuronal differentiation during embryonic development (Chen et al., 1998; Nechiporuk et al., *In preparation*). However, several studies have raised the possibility that REST might be present in adult mammalian brains as well (Gao et al., 2011; Lu et al., 2014; Palm et al., 1998). In this study, I show that REST is expressed in several areas of the brain and in specific population of cells that resemble neurons in their morphology. More specifically, I used our REST gene trap mouse model to show endogenous REST promoter activity through the β-Gal reporter staining. In addition, I performed *in situ* hybridization and immunostaining to reveal that REST is expressed in Purkinje cells of the cerebellum, mitral cells of the olfactory bulb, and neurons throughout the cortex, brain stem, hippocampus, and midbrain, etc. Although this work does not address the role of REST in adult brain, I determined through chromatin immunoprecipitation that REST binds to the chromatin of known target genes such the glycine receptor, SNAP25, L1CAM, GRIN1, SCG3 and the newly discover target genes: the splicing factors Rbfox3 (NeuN) and the methyltransferases Dnmt3a.

Because REST is a transcriptional repressor, the prediction is that in its absence there should be an increase in expression of its target genes. My studies suggest this is only the case for some of its targets and not others. A study by Immaneni et al. and others have suggested that the absence of REST alone is not sufficient to activate a significant amount of target genes (Immaneni et al., 2000). However, the fusion of REST with the

viral activator VP16 resulted in an increase in the expression of the acetylcholine and glutamate receptors. This leads me to speculate that changes in the expression of REST targets genes in the REST -/- mice is dependent upon the complement of activators and repressors stationed at a particular gene. This may also explain why these targets genes are only regulated in some areas of the brain but not other like it is case for the glycine receptor, which I showed to be upregulated in cerebellum but not cortex.

The general question that arises with this study is: what is the role of REST in adult brain? There is one element that might provide a clue: the fact that one of the most remarkable features of the adult nervous system is that it dynamically changes in response to external stimuli all throughout the life span of an organism. This regulation is believed to mediate the effects of sensory experience on site-specific gene expression, thus resulting in changes in synaptic transmission and ultimately behavior. Based on this study, I propose REST may be regulating the expression of four types of genes in adult brain. One, REST regulates the expression of neuronal genes that provide neurons their identity such as channels and pre- and post-synaptic proteins in response to activity. Calderone et al. supported this idea by demonstrating that ischemic insult increases REST expression, and this concomitantly resulted in the repression of the AMPA receptor GluR2 (Calderone et al., 2003). Two, REST regulates the expression of neural specific splicing factors such as NOVA2 (Ule et al., 2005) and Rbfox3 (NeuN). The prediction would therefore be that REST directly regulates transcription and indirectly regulates splicing of many other targets that play a critical role in brain-specific processes such as synapse assembly and axon guidance (Mikulak et al., 2012). Three, REST regulates the

expression of genes involved with epigenetic regulatory mechanisms such as the DNA methyltransferase 3a (Dnmt3a). This leads to hypothesize that REST not only regulate the epigenetic landscape in neurons by recruiting its own core of histone and DNA modifying enzymes, but also, it regulates the expression of other activities such as Dnmt3a, which has been reported to be required for learning and memory, as well as hippocampal synaptic plasticity (Feng et al., 2010). Lastly, based on my observation that REST deficient mice show an increase in GFAP expression and that REST deficiency does not change the astrocyte pool (Covey et al., 2012), I believe REST may also regulate the expression of genes in neurons that send a signal to neighboring astrocytes to respond to stress and inflammation. A better understanding of the role of REST in adult brain may be achieved by generating a conditional knock out mice that specifically knocks down REST in post-mitotic neurons as supposed to early embryonic development. For that, we could cross our gene trap mice with CAMKII-cre mice, which targets the pyramidal neurons of the hippocampus or the Pcp2-cre mice, which specifically targets Purkinje cells of the cerebellum. Alternatively, I could gain further insight on the genes that REST regulate by comparing the transcriptome of cells where REST is expressed and compared them to the REST -/-.

A recent study has underscored the role of REST in the process of ageing. The authors showed that REST is dynamically regulated in human brains by having low REST expression in young individuals to then significantly increase in the oldest ones (Lu et al., 2014). They hypothesized that REST may be neuroprotective by repressing the expression of genes that mediate cell death, stress resistance, and pathology. In contrast

to this study however, my results show that REST protein decreases in the brain of mice as they age. Although the number of animals I studied (two per group) do not allow for statistical analysis yet, I believe the existing difference may reside in the sequence homology between these two species or perhaps the post-translational modifications that this protein goes under in mouse and humans. My human studies revealed a greater variability in REST expression than the ones observed in the Lu et al. study. However, a better understanding will be gain by increasing the sampling number and including more young individuals.

Collectively, the data herein support the idea that REST is expressed in adult brain, binds chromatin, and regulates the expression of its target genes. A deeper understanding of its role and how and what genes it regulates may allow for an effective treatment for neurodegenerative disorders.

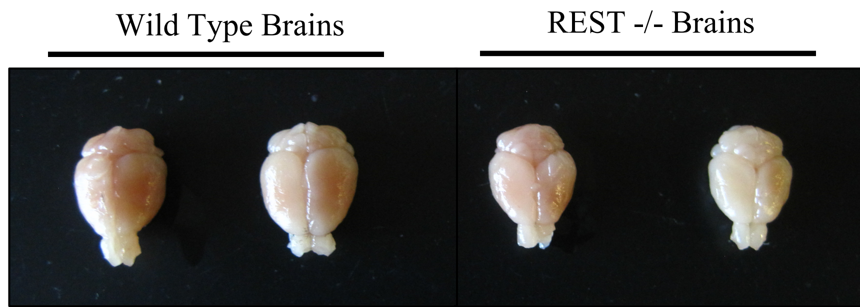
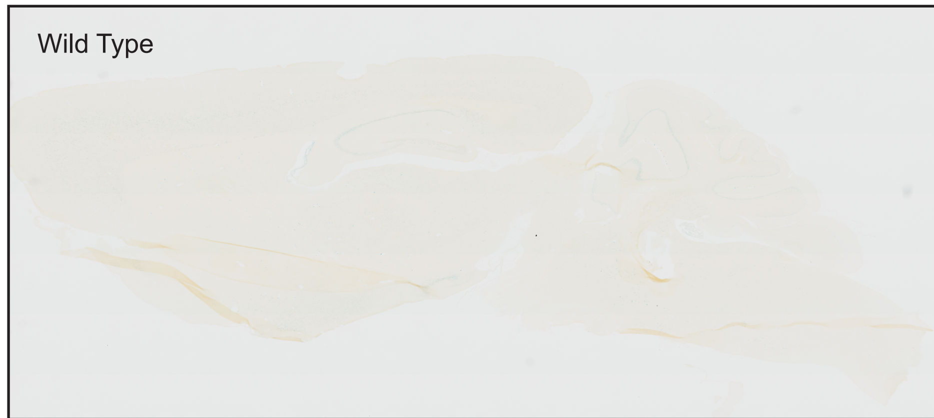


Figure 1. REST deficient adult brains are smaller than wild type adult brains. Representative brains of two wild type mice (*Left panel)* and two REST -/- mice at 8 weeks *(Right panel)*.

A



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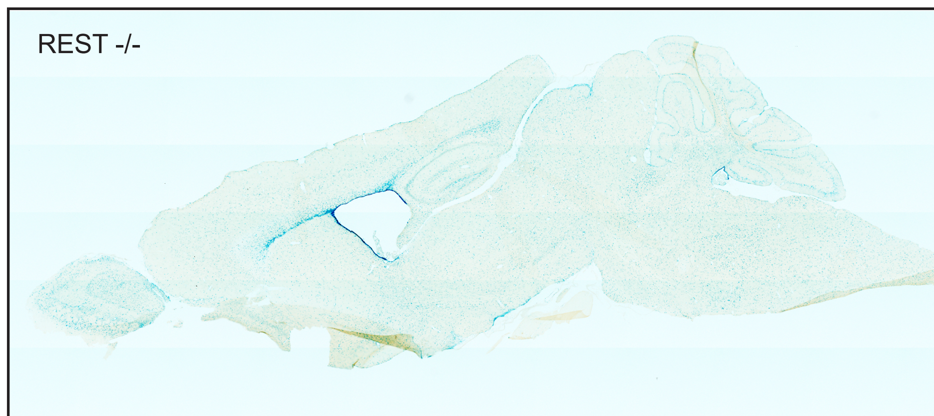
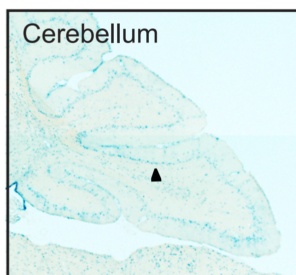
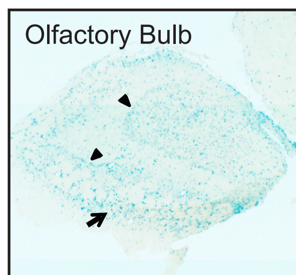






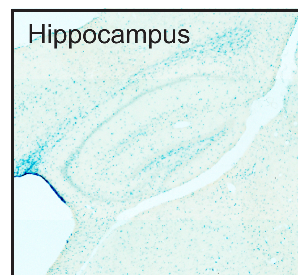
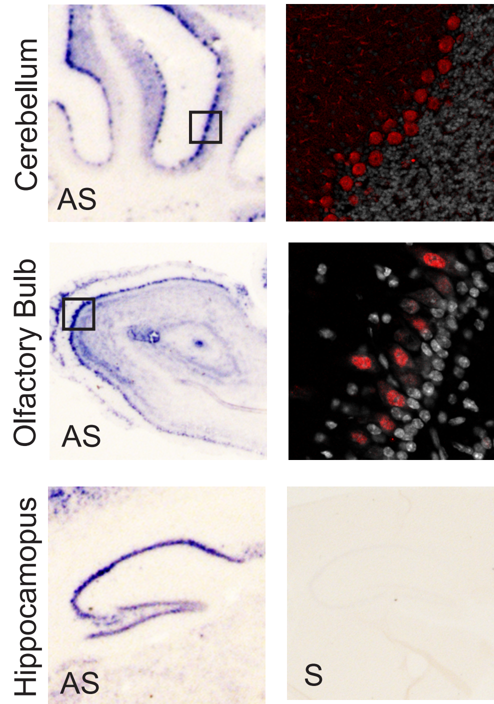


Figure 2. X-Gal staining shows REST promoter activity in mouse adult brain. Sagital sections from six to eight week old murine brains were use to perform X-gal staining. The blue signal corresponds to endogenous REST promoter activity. **A)** Wild type brains show no signal. **B)** β-geo expression in REST -/- sagital brain sections show endogenous REST promoter activity throughout the brain. Lower left panel shows higher magnification in cerebellum. *Arrowhead* shows Purkinje neurons. Middle panel shows olfactory bulb. *Arrowhead* shows mitral cells. *Arrow* shows periglomerular cells. Right panel shows hippocampus. n=6

A



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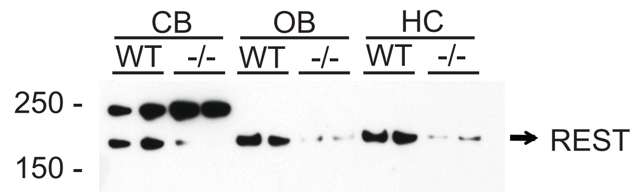
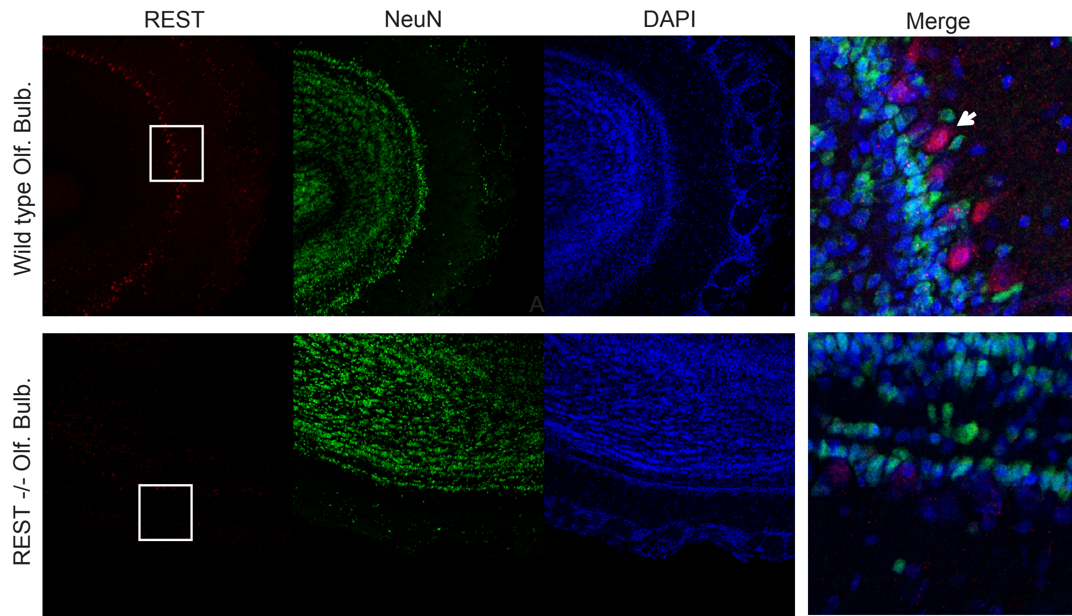


Figure 3. REST mRNA and REST protein is expressed in mouse adult brain. A) Sagital sections from six to eight week old murine brains were use to perform *in situ* hybridization. The purple digoxigenin staining corresponds to the anti sense probes recognizing REST mRNA in cerebellum, olfactory bulb, and hippocampus. *AS*, anti sense probe; *S*, sense probe. *Box* represents comparable areas of the brain being magnified in the merged immunofluorescence to the right, n=3. *Red*, REST staining with monoclonal 4A9 antibody; *Green,* Neuro N; *Blue*, DAPI. **B)** Cerebellum (CB), olfactory bulb (OB), and hippocampus (HC) from WT and REST -/- adult brain were dissected. Cytoplasm and nuclei were isolated. Nuclei were used for Western blotting by using C-REST polyclonal antibody n=6.


A
REST
NeuN
DAPI
Merge
Wild type Olf. Bulb.
REST -/- Olf. Bulb.

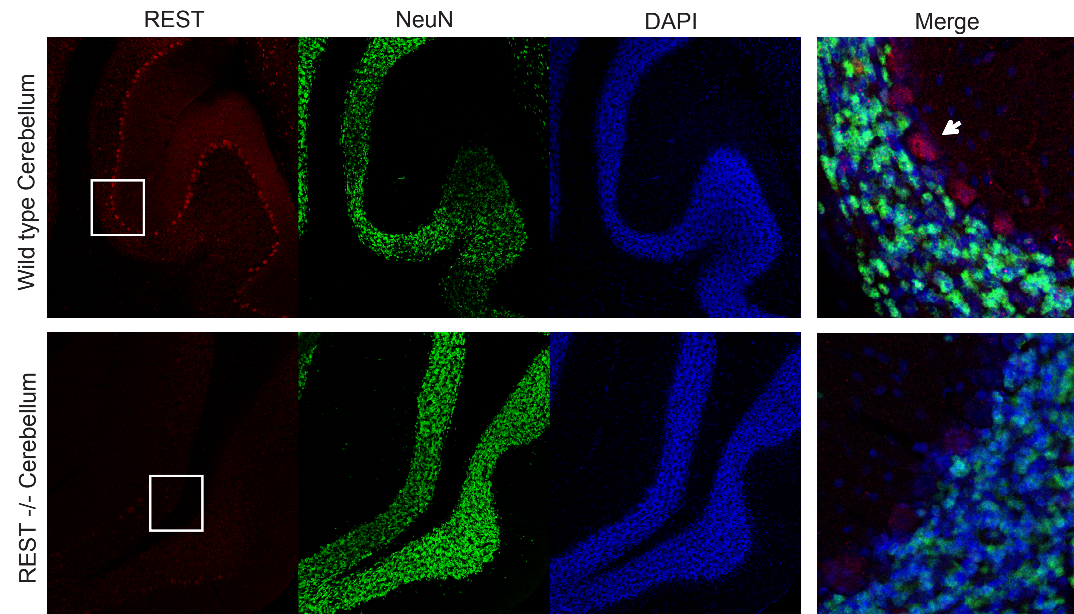
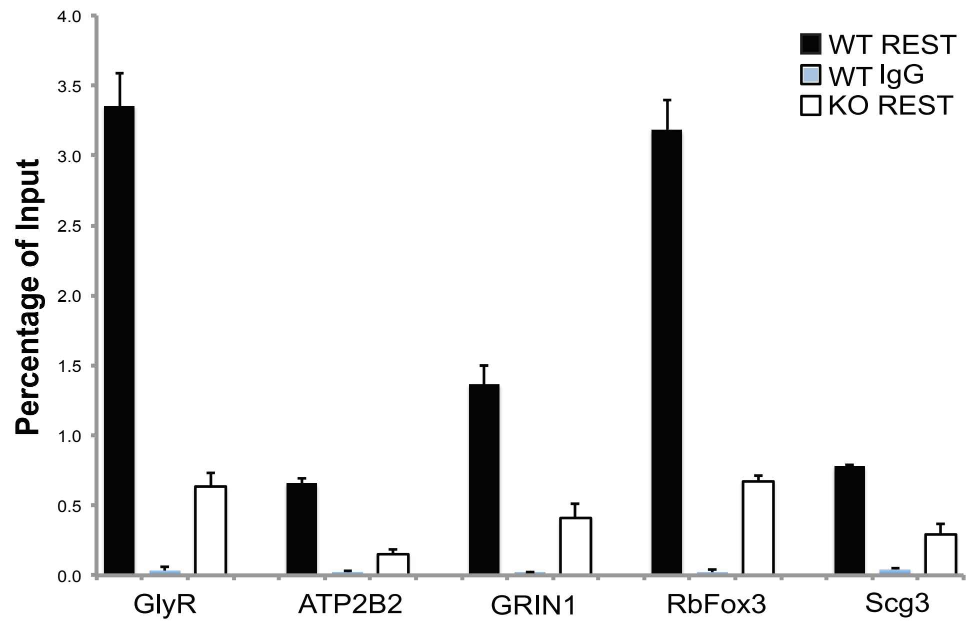

B
REST
NeuN
DAPI
Merge
Wild type Cerebellum
REST -/- Cerebellum

Figure 4. REST is expressed in specific populations of cells in the cerebellum and olfactory bulb. Six to eight week old adult murine brains from wild type and REST -/- mice were used to perform immunofluorescence with 4A9 monoclonal antibody. *Red*, REST; *Green*, NeuroN; *Blue*, Nuclei. *Box* shows area of higher magnification used at the far right merged images. REST. **A)** Staining of olfactory bulb showing immunofluorescence signal in the wild type mice (upper panels) and the almost complete absence of REST in the REST-/- animals. *Arrow* indicates cells expressing REST protein in mitral cells **B)** Staining of the cerebellum showing immunofluorescence signal in the wild type mice (upper panels) and the almost complete absence of REST in the REST-/- animals. *Arrow* indicates cells expressing REST protein in Purkinje cells n=4

A



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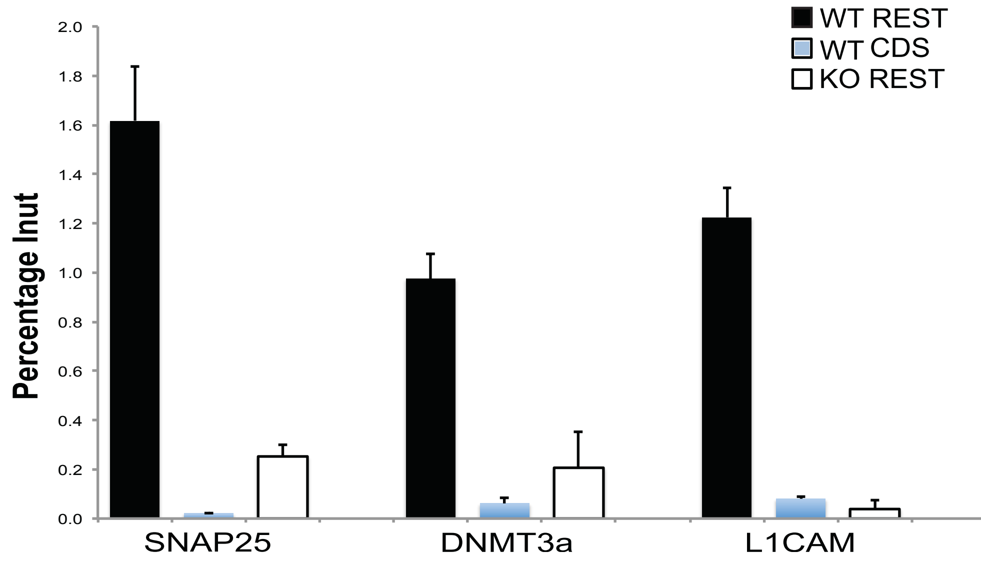
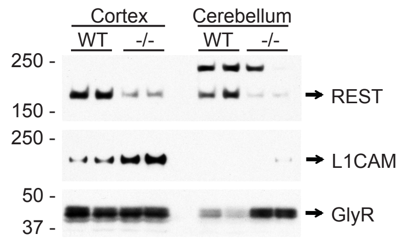


Figure 5. REST bind to the chromatin of target genes. Chromatin immunoprecipitation (CHIP) analysis of six to eight week old murine cortex in wild type and REST -/- mice by using the rabbit polyclonal C-REST antibody. **A)** REST is bound to the RE1-containing target genes such as the glycine receptor (GlyR), ATPase, Ca++ Transporting, Plasma Membrane 2 (ATP2B2), Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 1 (GRIN1), RNA binding protein, fox-1 homolog 3 (Rbfox3), secretogranin 3 (Scg3). REST -/- mice showed a small background binding, whereas rabbit IgG served as negative control. **B)** REST is also bound to the RE1-containing target genes such as the synaptosomal-associated protein, 25kDa (SNAP25), DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3a), L1 cell adhesion molecule (L1CAM). REST -/- mice showed a small background binding whereas primers designed several kilo bases away from RE1 site (primarily in the coding sequence, CDS) were used as negative control. Error bars are the mean $\pm$ s.e.m. based on three mice assessed.

A



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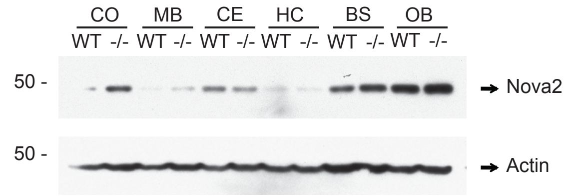

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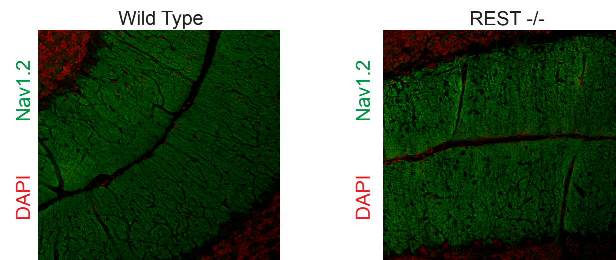

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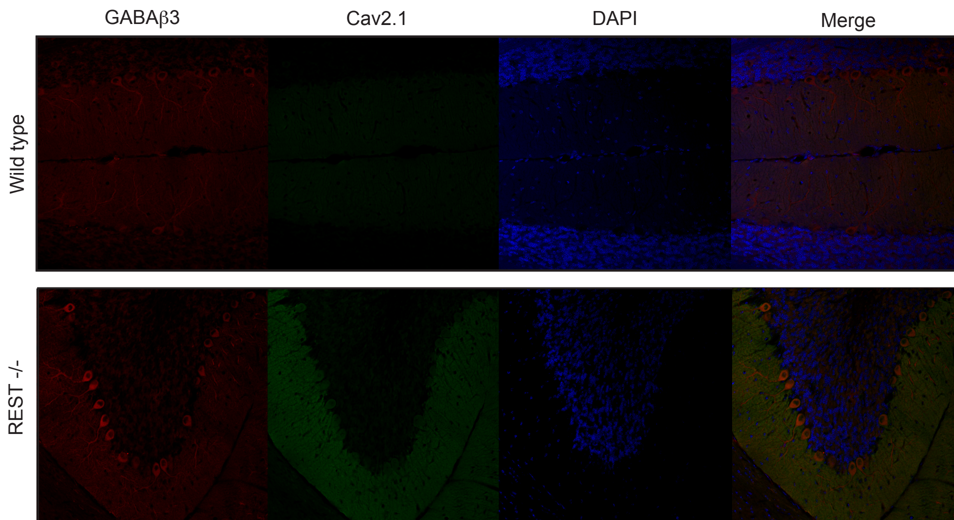
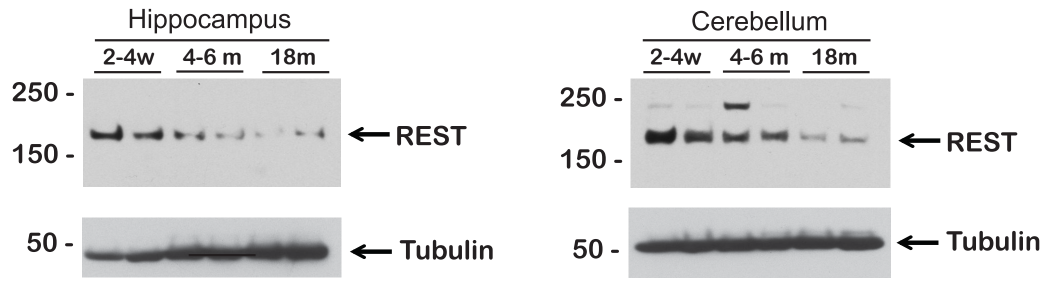
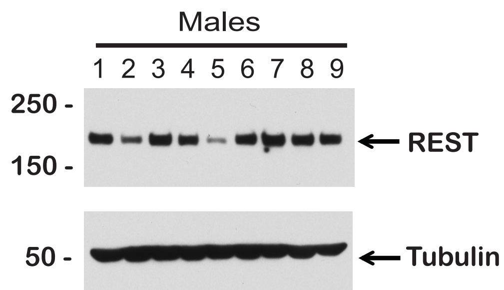
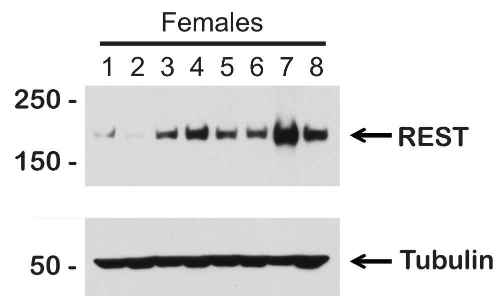


Figure 6. Absence of REST results in selective upregulation of target genes.

Six to eight weeks old mouse WT and REST -/- brains were used for Western blot and immunofluorescence analysis. **A)** Cortex and cerebellum were isolated and equal protein concentration were used for Western blot analysis. *Upper* panel shows high REST expression in WT and significantly diminished signal in REST -/- mice. *Middle* panel shows increase in L1CAM protein expression only in cortex of REST -/- mice. *Lower* panel shows increase in glycine receptor (GlyR) only cerebellum on REST -/- mice. n=3. **B)** Six brains areas were micro dissected and probed against the splicing factor neuro-oncological ventral antigen 2 (NOVA2). Levels of expression varied among areas, but increased in cortex and brain stem. *CO*, cortex; *MB*, midbrain; *CE*, cerebellum; *HC*, hippocampus; BS, brain stem; *OB*, olfactory bulb. n=2. **C)** Immunofluorescence analysis of the voltage-gated sodium channel Nav1.2 in cerebellum remains unchanged between wild type and REST -/-. *Red*, nuclei; *Green*, Nav1.2. n=4. **D)** Immunofluorescence analysis of the REST target proteins gamma-aminobutyric acid receptor subunit beta-3 (GABAβ3) and voltage gated P-type calcium channel (Cav2.1) show increased expression in the cerebellum of REST -/- mice. n=3





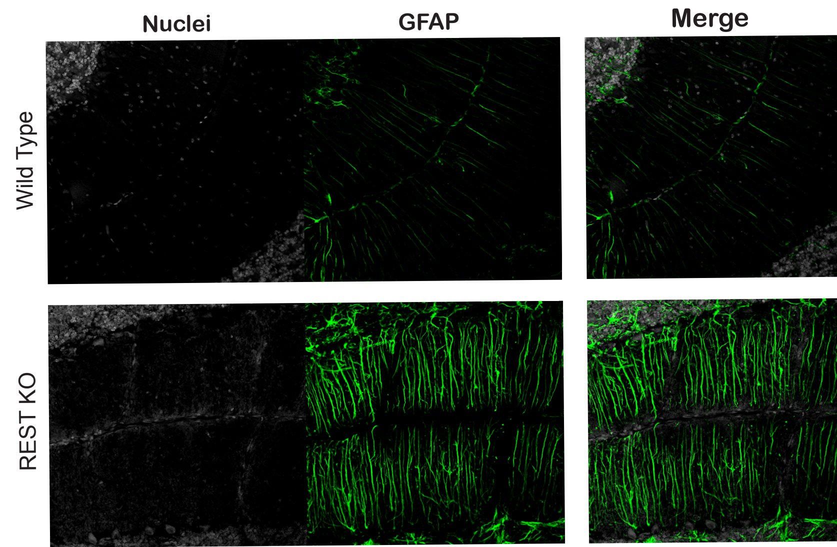


Lane	Males(yo)
1	49
2	52
3	54
4	56
5	79
6	94
7	94
8	100
9	100

Lane	Females(yo)
1	22
2	28
3	29
4	42
5	69
6	70
7	89
8	90

Figure 7. REST expression changes with ageing. A) Cerebellum and hippocampus from wild type mice between 2-4 weeks old, 6-8 weeks old, and 18 months old were used to isolate nuclei and cytoplasm. Equal concentrations of nuclear extracts were run on a SDS-PAGE and immunoblotted by using the polyclonal C-REST antibody. Alpha tubulin was used as a loading control. **B)** Hippocampi from post-mortem male brains between the ages of 49 and 100 were run on a SDS-PAGE and immunoblotted by using the polyclonal C-REST antibody. Alpha tubulin was used as a loading control. Bottom table shows the ages in years old (yo) of individuals from lane 1 through 9. **C)** Hippocampi from post-mortem female brains between the ages of 22 and 90 were run on a SDS-PAGE and immunoblotted by using the polyclonal C-REST antibody. Alpha tubulin was used as a loading control. Bottom table shows the ages in years old (yo) of the individuals from lane 1 through 8.

A



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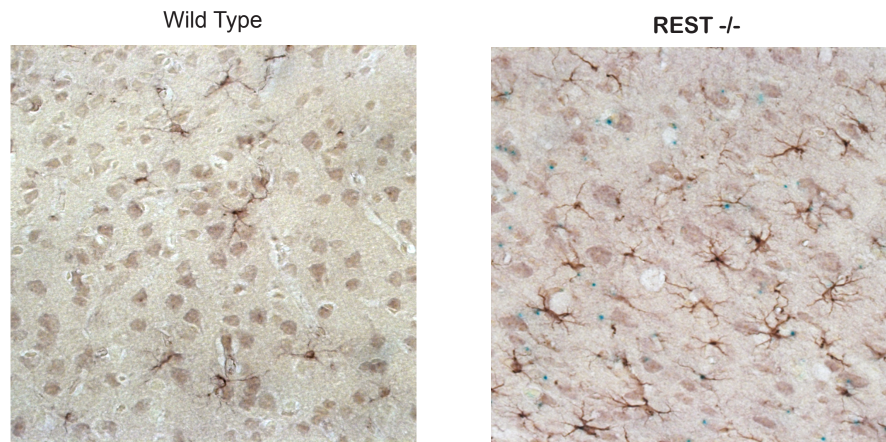

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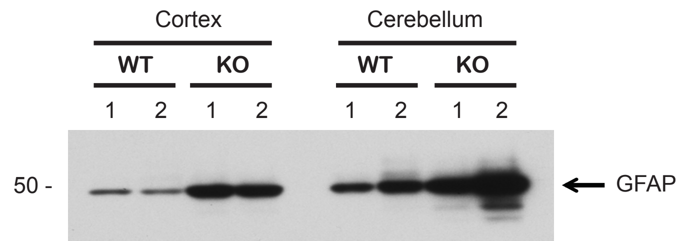


Figure 8. Loss of REST results in an increase in GFAP expression. Analysis was performed in six to eight week old sagital sections of wild type and REST -/- brains. **A)** Immunostaining shows an increase in GFAP staining in cerebellum of the REST -/- presumably corresponding to Bergman glia. *Blue*, nuclei; *GFAP*, grey, *Nav1.2* green. n=3. **B)** Immunohistochemical analysis shows an increase in the number of GFAP positive cells in the REST -/- cortex. n=2. **C)** Western blot analysis shows an increase in GFAP expression in extracts of REST -/- cortex and hippocampus. n=2

Antibody	Host	Source (Catalog No.)	Application	Dilution
C-REST #719/720	Rabbit	In House	WB	1:1,000
REST 4A9	Rat	In House	IHC	1:500
NeuN-Alexa 488	Mouse	Millipore (MAB377X)	IHC	1:1,000
GFAP	Mouse	Millipore (MAB360)	WB/IHC	1:4,000/1:1,000
GlyR	Rabbit	Thermo Scientific (PA1-4661)	WB	1:2,000
Nova2	Rabbit	Novus Biologicals (NBP1-92196)	WB	1:1,000
Voltage gated Ca^{2+} Channel (P/Q type)	Rabbit	Synaptic Systems-SYSY (152 203)	IHC/WB	1:600/1:1,000
Gabab3	Mouse	Neuromab (75-149)	IHC	1:500
α-tubulin	Mouse	Antibody list - Developmental Studies Hybridoma Bank (AA4.3)	WB	1:4,000
L1CAM	Rabbit	Abcam (ab123990)	WB	1:1,000
Actin	Mouse	Antibody list - Developmental Studies Hybridoma Bank (JLA20)	WB	1:4,000
Sodium Channel Nav1.2	Mouse	Neuromab (75-024)	IHC	1:500

Table 1. Primary antibodies used

CHAPTER 4

CONCLUSIONS

The main goal of my thesis work has been to resolve the question of whether the neuronal repressor REST is expressed in adult mammalian brain. Previous studies had focused on early developmental roles of REST where levels of REST are much higher than later in development. Two aspects of this question have been addressed in this thesis. First, I characterized a series of new antibodies against mouse REST for their abilities to detect REST epitopes in brain tissue by histology. Second, I used molecular and biochemical techniques as independent approaches to directly ask the question of whether REST is expressed in adult brain.

My results have important implication for future research directions. Therefore, in the following sections I will address the significance of the specific REST expression pattern observed, discuss the role that REST plays under basal conditions, and also its role in aging and neurodegenerative disorders. In parallel, I will propose future studies that may provide a deeper understanding of the unprecedented function of REST in adult mammalian brain.

REST expression in adult brain

In Chapter 2, I characterized a panel of antibodies that made it possible to visualize the expression of REST in adult murine brains. In Chapter 3, I demonstrated by using these antibodies that REST is indeed expressed in adult mouse brain. Furthermore, the visualization through immunofluorescence allowed me to determine that REST is not expressed everywhere in the brain, but instead is limited to particular regions of the brain. In general, the localization of REST by immunolabeling matched well with my *in situ* hybridization results and the β-Gal reporter activity using our REST gene trap mouse, thus providing more confidence in the immunolabeling studies. In addition, my results are consistent with a previous finding by Palm et al. in 1998 that gave the first hints of a possible REST expression in adult brain through *in situ* hybridization studies. Because we observe REST expression in Purkinje cells of the cerebellum and the mitral cells of the olfactory bulb, we wonder what is the role of REST in this specific population of cells. In order to get an answer to this question, future studies should aim to take a closer look at the transcriptome of these cells. A method that holds potential to resolving this question is perhaps single cells transcriptomics between singly isolated Purkinje cells from both wild type and REST -/- mice. The RNA expression profiles and gene ontology analysis obtained between these two lines could provide a lead as to what neuronal properties are being affected, and then allude a specific function of REST in these cells.

Because REST acts as a repressor, one would predict that REST expression should be inversely related to the expression of its target genes. A test of this idea is the case of voltage-dependent sodium channel, Nav1.2, which is expressed in cerebellar granule cells

but not in Purkinje cells (Shaller et al., 2003). Because I detected REST only in the Purkinje cells, the prediction is that sodium channel Nav1.2 gene expression should be higher (de-repressed) in Purkinje cells of REST -/- mice compared to control mice. However, this is not what I observed. Immunostaining analysis of the REST knock out mice showed no changes in the sodium channel protein levels in the Purkinje or granule cells where the sodium channel was originally expressed. This observation was not an exception. Indeed, several genes analyzed remained unchanged in the knockouts even though I found that REST is bound to responsive elements in their wild-type counterparts in vivo. In contrast to the sodium channel, genes encoding the glycine receptor or the L1CAM did show a significant increase in expression but only in the cerebellum and cortex respectively. Yet other regions of the brain including the hippocampus and brain stem did not show changes in glycine receptor or L1CAM expression. This leads me to conclude that REST-mediated gene regulation is more complex than expected. This complexity might be attributed to the lack of activators able to prompt the transcription of these targets, or a stringent regulation mediated by other repressors with the ability to bind DNA elements in the same genes when REST is gone. Alternatively, it is possible that a homeostatic mechanism is set in place where cells sense an excess of mRNA or protein and immediately downregulate them by a post-transcriptional mechanism or the proteasome machinery, respectively. A feasible study to discriminate between these alternatives is to transfect primary cortical or hippocampal neurons from wild type and REST -/- mice with REST fused to the activation domain of the viral activator REST-VP16. The prediction would be that if other activators are needed to promote full upregulation of REST target genes, then I should observe an increase in the

mRNA levels of targets in the REST deficient cells that have been transfected with the REST-VP16 constructs. Alternatively, to test whether the proteasome machinery keeps REST upregulated genes at a basal level, I could treat the same primary cultures with a protease inhibitor and assess whether their levels are upregulated in the REST -/- cells in response to this treatment.

GFAP upregulation in REST deficient brains

REST is mostly expressed in large cells with weakly stained nuclei, a characteristic that is proper of neurons. Interestingly, although glia have been reported to express functional REST (Gao et al., 2011), I only sporadically observed REST expression in cells labeled with the glial fibrillary acidic protein (GFAP), an astrocyte marker. Because I cannot discard a lack of REST antibody sensitivity, further experiments need to be performed. For example, Okada et al. in 2011 developed a direct fluorescence-activated cell sorting (FACS) strategy to isolate neuron and glial nuclei from intact brain. It could be possible, therefore, to directly isolate these cells from our wild-type brains and resolve the question of whether REST is also expressed in the astrocytes nuclei.

I also observed that our REST knockout mice showed an increased expression of GFAP as demonstrated by Western Blot and immunofluorescence analysis even though I do not observe REST in astrocytes and GFAP is itself not a REST target. This raises the question of whether this is a result of an increase in the number of astrocytes in the REST knockout mice per se or an indirect product of an increase in reactive astrocytes

(Sofroniew et al., 2009). Studies by Covey et al. suggested already that the former is not the case because loss of REST enhances the generation of neurons and oligodendrocytes but does not affect the generation of astrocytes (Covey et al., 2012). This result leads us to follow up the second option. Therefore, to confirm this is the result of an increase in reactive astrocytes, western blots should be performed with additional antibodies that label astrocytes such as S100β and glutamine synthetase between wild type and REST -/- mice. The prediction would be that the expression of these two proteins should remain the same across the mouse models if GFAP is only increased due to reactive astrocytosis.

REST function in mature neurons

May REST regulate gene expression in an activity-dependent manner? The results shown in Chapter 3 raise the question of what is the role of REST in adult mature neurons. Because many of the REST targets encode proteins required for proper neuronal function, such as voltage-gated sodium channel Nav1.2, synapsin, secretogranin, and L1CAM, etc. (Chong et al., 1995; Mori et al., 1990; Li et al., 1993; Kallunki et al., 1997; Bruce et al., 2004), it does not seem plausible that REST is in the adult brain to block their expression. Therefore, an obvious idea based on the role of REST during early neuronal differentiation is that REST may repress genes involved in stem cell maintenance and differentiation such Ascl1, Mash1, and NeuroD1 to ensure the proper maintenance of a fully mature phenotype all throughout adulthood. If this were the case, one would expect at least some degree of de-differentiation of the mature phenotype towards a more neural progenitor-like state in REST knockout animals, and potentially

cell death. Indeed a striking result I have observed, in collaboration with a postdoc in the lab, Tamilla Nechiporuk by using a gene trap mouse she generated, was a modest precocious differentiation leading to a reduced progenitor pool, and ultimately smaller brains (Nechiporuk et al. *In preparation)*. This phenomenon early in development was also accompanied by cell death. So, it is possible that REST is in the adult brain to repress the expression of cell death-related genes as well. A way to test this idea is by simply determining the levels of active caspase 3 in adult brains or to look for the binding of REST to the chromatin of genes involved in apoptosis. Because the neuronal specific REST target genes happen to be expressed in adult brain, it is possible that REST is loosely bound to the chromatin to only maintain the DNA poised for future activity-dependent regulation. The rationale for this idea stems from the fact that neuronal activity has been shown to alter the expression of many genes in adult neurons, some of which are REST targets. These alterations are thought to promote long-lasting or adaptive changes leading to synaptic reorganization or persisting hyperexcitability associated with long-term potentiation, seizures, etc. (West et al., 2002). Some of the first evidence supporting this idea derives from the studies of Palm et al. in 1998. These authors studied the effect of kainate-induced seizures on the expression of REST and found that mRNA levels were increased as early as 4h and stayed up by 24h after treatment. This upregulation might be a response to regulate the expression of genes encoding proteins such as the AMPA glutamate receptor 2 (GluR2) (Jia et al., 2006), a subunit that governs AMPAR Ca2+ permeability, single channel conductance, etc. (Swanson et al., 1997; Hollman et al., 1991). A similar effect has been observed upon ischemic insults. REST is upregulated, and it is believed to suppress the expression of Glur2 in specific population

of cells in the hippocampus (Calderone et al., 2003). Furthermore, but a complex assembly was observed between REST and CoREST, mSin3A, HDAC1/2, G9a, and MeCP2 at the promoters of other target genes such as *grin1, chrnb2, nefh*, *trpv1*, *chrm4, syt6* under similar circumstances (Noh et al., 2012). A more recent study has shown that treatment of hippocampal neuronal cultures with 4-aminopyridine (4AP), a stimulatory agent that blocks K^+ channels, results in an early upregulation of REST that lasts up until 48h after treatment. One of the consequences of this increase is the parallel decrease in one of the first REST targets ever discovered: the sodium channel Nav1.2 (Chong et al., 1995; Schoenherr and Anderson, 1995). The authors speculate that the upregulation of REST exists to establish a negative homeostatic response at both the single-neuron and the network level to ameliorate the hyperexcitability-induced by 4AP and thus acts as a protective mechanism to control intrinsic excitability (Pozzi et al., 2013). Studies to investigate this question should make use of REST knockout models. More specifically, I can make use of our conditional Nestin-Cre REST knockout mouse and ask the question of whether the absence of REST results in preponderance to epileptic seizures. Preliminary studies in our laboratory suggested this is the case because peritoneal injection of kainic acid resulted in accelerated progression into epileptogenesis as measured by the time taken to reach generalized motor convulsions (data not shown). Although this preliminary result is confounded by the fact REST is knocked down at the progenitor cell stage, a study by Hu et al. demonstrated that knocking down REST in mature excitatory neurons by crossing floxed REST alleles with a CAMKIIα-Cre mouse resulted in the same phenotype. Mice entered epileptogenesis not only more quickly, but also, showed increased expression of target genes such as *Fgf14* and *Bdnf*. Another set of

useful future studies that may address the question of REST's role in adult brain is to perform electrophysiological studies in acute slices of both wild type and REST -/- mice. The purpose of this would be to determine whether there is any difference in classic parameters such conductance, resting potential, and I/V curves at resting levels. I speculate the results might be comparable between wild type and REST -/- mice because I do not observe evident phenotypic differences between these two lines. That leads me to the next step, which would be to block specific channels and measure the response in both of these mouse strains. My prediction would be that the absence of REST results in significant changes in channel conductivity. I hypothesize that this would be likely due to an increase in the expression of channels that alter the subunit composition of an entire receptor, thus ultimately resulting in changes in channel conductivity.

May REST affect alternative splicing? Recent studies have shown a new role for REST in the regulation of alternative splicing. This new mechanism has come to light because REST has been found to regulate the expression of Nova2, a brain-specific splicing factor (Mikulak et al., 2012). The authors showed that low REST expressing cells have high levels of Nova2, and this concomitantly results in the incorporation of the full length L1CAM in hippocampal cultures. Nova2 also regulates the splicing of the P-type CaV2.1 channel that may result in changes on its conductance (Allen et al., 2010). More importantly, this channel also happens to be a REST target. Altogether, this data leads to the idea that REST acts in two ways: directly by repressing the transcription of a gene, and indirectly by repressing the transcription of the splicing factor that also regulates it.

This may turn out to be a general mechanism of REST function because in this thesis I demonstrate that REST also binds very strongly to a response element in the Rbfox3 gene. Rbfox3, better known as Neuronal Nuclei (NeuN), is a new member of the Fox-1 gene family of splicing factors (Kim et al., 2009). It is reasonable to speculate then, that the REST-dependence of splicing is likely a widely important process, not limited to L1CAM but extended to a whole panel of Nova2, NeuN, and REST targets that play a role in brain-specific processes such as synapse assembly and axon guidance. Therefore, future studies should include an analysis of alternative splicing of genes by RT-PCR and immunoblotting of wild type and REST knock out mice brains.

REST in aging

Along with demonstrating that REST is expressed in mouse adult brain, I showed in Chapter 3 that REST is expressed in an age-dependent manner in male mice. Specifically, I observed that REST is expressed as early as two weeks old postnatally, and that its expression decreases as mice age. These results are in accordance with previous studies in rat that demonstrated through RNA protection assays and northern blot assays that REST mRNA is lower in adult cortex than post natal day 7 rat cortices (Palm et al., 1998). Kohyama et al. (2010) demonstrated that BMP-induced REST expression regulates the establishment and maintenance of astrocytic identity. Therefore, it is possible to speculate that in addition to a role in mature neurons, REST may be expressed in the progenitor cells that give rise to astrocytes at the early postnatal stage to repress the expression of neuronal genes as the neural progenitors differentiate into mature

astrocytes. A first crucial study however, is to determine when is that REST reappears after its down regulation at embryonic day 10.5. This could be done by western blotting and RT-PCR analysis of mouse embryos brains at embryonic days 17 and 19 followed by brains obtained at postnatal day 1, 7, and 14. This analysis can be done with cortex, hippocampus, and cerebellum because as early as embryonic day 17 these structures are already formed or are in the processes of maturation.

REST in disease

A recent study has emerged that has placed REST at the center stage of the public arena and the neuroscience field. This study reported that REST is instrumental in protecting the aging brain (Lu et al., 2014). More specifically, by utilizing tissue from brain banks and dementia trials, the authors sampled prefrontal cortex from human brains and determined that those of young adults (ages of 20 to 35) expressed low levels of REST whereas healthy aging adult brains (ages of 73 and 106) had significantly higher amounts. Interestingly, they also observed a downregulation of REST in patients with Alzheimer's disease (AD) and a correlation between REST levels and cognitive function. They hypothesize that high REST levels are there to repress the expression of genes that are involved in oxidative stress and inflammation during ageing. They further extended their studies by analyzing primary cortical neurons of REST conditional knockout mice and assessed their vulnerability to oxidative stress (H_2O_2 treatment) and oligomeric Aβ. They showed that REST-deficient neurons showed a markedly increased degeneration and cell

death relative to wild type culture, thus suggesting REST is neuroprotective in a wild type animal.

My preliminary observations seem at odds with this study because I observed a decrease rather than an increase in REST protein levels with age in male mice. However, there are three reasons why the results of Lu et al. require further confirmation. In chapter 1, I raised the issue that there is a disconcerting lack of consensus with regard to the size of the REST protein. Having both a REST knockout mice and REST knock out stem cells for comparison, I demonstrated that mouse REST protein migrates at approximately 180KDa in denaturing SDS gel electrophoresis. This contradicts with this later paper that shows a 148KDa protein. Because they do not compare it to any REST deficient line, we can hypothesize they are showing a non-specific band. Secondly, in chapter 3 I demonstrated that REST protein expression remains remarkably unchanged between the ages of 44 and 100 years old of human male hippocampi. Interestingly, there was much more variability in REST expression from female human brains, precluding firm conclusions at this point. Future studies should therefore include collecting hippocampi from younger individuals to assess the expression of REST in that region at young ages. Lastly, Lu et al. provided results by using a Nestin-Cre REST knockout mice (Figure 1a). In my hands, that transgenic line turns out not to be a complete knock out, but instead it generates a 135KDa truncated form of REST (Figure 1B) that is likely to be a product transcribed from a cryptic methionine within exon 2. Therefore, this animal model is not truly a knock out making their results difficult to interpret.

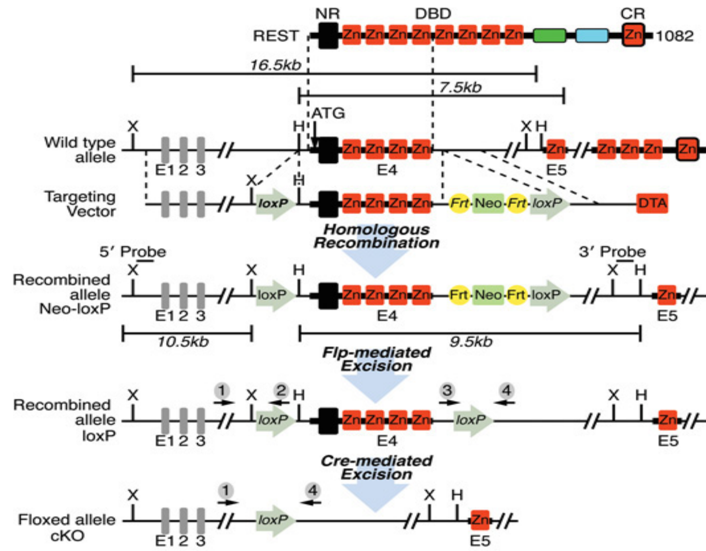
The discrepancy observed in the results with my mice and the human brains may be explained by the difference in protein sequences between the human REST and mouse REST (Figure 2). This is particular important at the C-terminal domain where only the Zn finger domain shows high conservation between the species. Therefore, their sequence divergence may result in a different posttranslational regulation as both human and mice progress in to adulthood and ageing,

In the case of the most recent Alzheimer's publication, several studies can be performed to gain further detail on the role of REST in this disorder. Because AD is mostly a disorder of the hippocampus, we can cross our gene trap mice with a CRE line under the CAMKII promoter to specifically knock down REST in post-mitotic neurons of the hippocampus. Then, we can ask the question: does the absence of REST result in more cognitive and memory impairment than the wild type counterparts? For that, we can perform classic water maze studies and compare between those two models. If a REST increase is indeed necessary during normal ageing in humans, we can predict that the complete absence of REST in our animal model should result in memory impairment and even perhaps a faster progression into the disease. In addition, we can go back and analyze the expression of REST target genes in our ageing mice colony and compare it to the young ones. Of particular interest would be to analyze the expression of pro-apoptotic genes such as Bax and Daxx, AD-related genes such as presenilin, and also genes involved in neurotransmission.

Final Conclusion

This dissertation utilized biochemical, cellular, confocal microscopy, and *in vivo* methods to ask the question of whether REST is expressed in adult mammalian brain. In Chapter 3, I determined that REST is expressed in specific areas of the brain, it binds chromatin and regulate the expression of target genes, and its expression is developmentally regulated with age. The results described herein are of wide and applicable interest to studies of the biology of neurodegenerative diseases such as Alzheimer's disease. Therefore opening a new avenue for potential therapeutics. While the data in this dissertation makes a strong case demonstrating the presence of REST in brain, future studies must determine how exactly REST carries out its function in adult brain, the exact pool of genes it regulates, and how it ultimately contributes, through epigenetic modifications to the dynamic changes that occur in the adult brain in response to external stimuli. This will aid to understand how misregulation of these processes ultimately results in disease.

A.



B

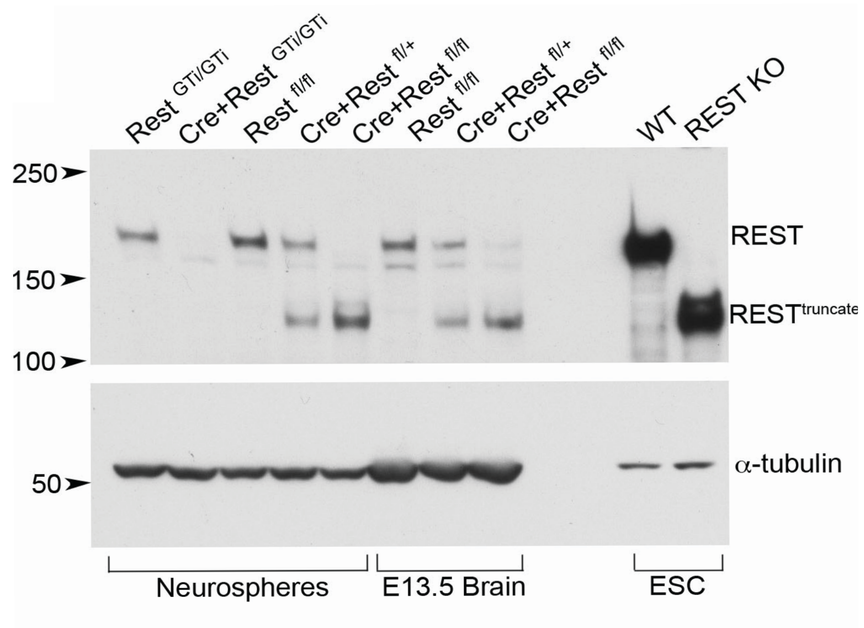


Figure 1. Gene trap knock out mice results in complete elimination of REST whereas REST knock out mice that uses flox alleles generates a truncated form. A) Strategy to generate a REST conditional knockout (cKO) allele. Protein, corresponding exonic structure, targeting vector, and targeted allele are depicted. The FRT site flanked neomycin resistance cassette was removed by crossing to transgenic animals expressing **hACTB:FLPe in the germline. H: HpaI; X: XbaI; NR: N- terminal repressor domain; CR: C-terminal repressor domain; DBD: DNA binding domain. *Modified form Gao et al., 2011*. **B)** Western blot analyses using REST C-terminal antibody of protein lysates from neurospheres from *Cre-* and *Cre+* mice, as well as protein extracts from neurospheres, E13.5 brains and ES cells with targeted exon 2, indicated as *Rest* $^{fl/fl}$, *Cre+ Rest* $^{fl/+}$, *Cre+ Rest* $^{fl/fl}$, REST KO and WT ESC cells. Note the disappearance of the full length REST protein in both REST GT knockout and exon 2 targeted REST KO cells and tissues, and the presence of an aberrant REST protein of approximately 130 KDa in *Cre+ REST* $^{fl/+}$, *Cre+ REST* $^{fl/fl}$ and REST KO mESCs in contrast to *Cre+* and *Cre-* mice, *Rest* $^{fl/fl}$, or WT mESCs.

```
Mouse      PAELAALKESARVSS-SEQNSAMPEGGASHSKCQTGSSGLCD
Human      LPGLAANINESTHISSSGQNLNTPEGETLNGKHQTDS-IVCE
           xxx   xxxxxxxx x x  xxx   xxxxx x  x xxx x

Mouse      --VDTEQKTDT-VPMKDSAAE-PVSPPTPTVD---RDAGSPAVVASPPITLAENESQEI
Human      MKMDTDQNTRENLTGINSTVEEPVSPMLPPSAVEEREAVSKTALASPPATMAANESQEI
           xxx  x x xxxxxxxx xx x    xx xxxxxx x x xxxx    x x x

Mouse      DEDEGIHSHDGSDLSDNMSEGSDDSGLHGARPTPPEATSKNGKAGLAGKVTEGE
Human      DEDEGIHSHEGSDLSDNMSEGSDDSGLHGARPVPQESSRKNAKEALAVKAAKGD
                    x      x               x x xxx  x xx  x xxx x
```

Figure 2. Comparison of C-terminal domain of human and mouse REST. Amino acids 889 to 1035 of mouse REST (*black*) were compared with its human counterpart (*red*). Line bellow human sequence indicated with an x show amino acids that diverge from mouse REST.

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