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The many faces of REST oversee epigenetic programming of neuronal genes

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Nervous system development relies on a complex signaling network to engineer the orderly transitions that lead to the acquisition of a neural cell fate. Progression from the non-neuronal pluripotent stem cell to a restricted neural lineage is characterized by distinct patterns of gene expression, particularly the restriction of neuronal gene expression to neurons. Concurrently, cells outside the nervous system acquire and maintain a non-neuronal fate that permanently excludes expression of neuronal genes. Studies of the transcriptional repressor REST, which regulates a large network of neuronal genes, provide a paradigm for elucidating the link between epigenetic mechanisms and neurogenesis. REST orchestrates a set of epigenetic modifications that are distinct between non-neuronal cells that give rise to neurons and those that are destined to remain as nervous system outsiders.

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Current Opinion in Neurobiology 2005, 15:500–506

This review comes from a themed issue on
Neuronal and glial cell biology
Edited by Fred H Gage and A Kimberley McAllister

0959-4388/\$ – see front matter
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DOI 10.1016/j.conb.2005.08.015

Introduction

Epigenetic regulation is a compelling mechanism for controlling developmental events [1,2]. In this form of regulation, distinct patterns of gene expression are inherited by chromatin modifications, such as DNA and histone methylation, that do not involve changes in DNA sequence. Neurogenesis, a process central to vertebrate development, requires the acquisition of neural cell fates within the developing nervous system and, in parallel, maintenance of non-neural cell fates outside the nervous system [3]. These two complementary events must be coordinated precisely for correct formation of the nervous system. Furthermore, neurogenesis requires that, within the developing nervous system, only post-mitotic neurons will express neuronal genes, because neural stem cells or progenitors have not yet committed to a neural lineage

[4]. These requirements raise the fundamental question of how neuronal gene chromatin is epigenetically programmed in different cellular contexts. How, for example, does neuronal gene chromatin in non-neuronal cells, where neuronal genes are never expressed, compare to that in neurons where these genes are expressed? In multipotent neural stem or progenitor cells, neuronal genes are repressed, but the cells have the capacity for subsequent expression in response to a developmental signal. Does neuronal gene chromatin in the progenitors reflect a state that is intermediate between suppression and activation, or is there a switch between a silenced and active state upon differentiation? Finally, what is the status of neuronal gene chromatin in pluripotent embryonic stem (ES) cells that have the unique capacity to differentiate into all cell lineages of the developing embryo?

For the establishment of epigenetic modifications representing distinct stages of differentiation, chromatin modifiers, such as DNA methyltransferases, histone methyltransferases and histone acetyltransferases, are recruited to specific genomic loci by DNA binding proteins, either repressors or activators [5]. A compelling candidate for orchestrating epigenetic events is the DNA binding protein, REST (RE1 silencing transcription factor; also called NRSF). REST was discovered in 1995 as a repressor of neuronal genes containing a 23 bp conserved motif, known as RE1 (repressor element 1 or NRSE) [6,7]. Several lines of evidence now point to REST as a key protein for regulating the large network of genes essential for neuronal function [8]. Here, we discuss the most recent studies on epigenetic mechanisms, orchestrated by REST, that characterize specific stages of mammalian neurogenesis.

Wiring a genetic network for permanent silencing of neuronal genes outside the nervous system

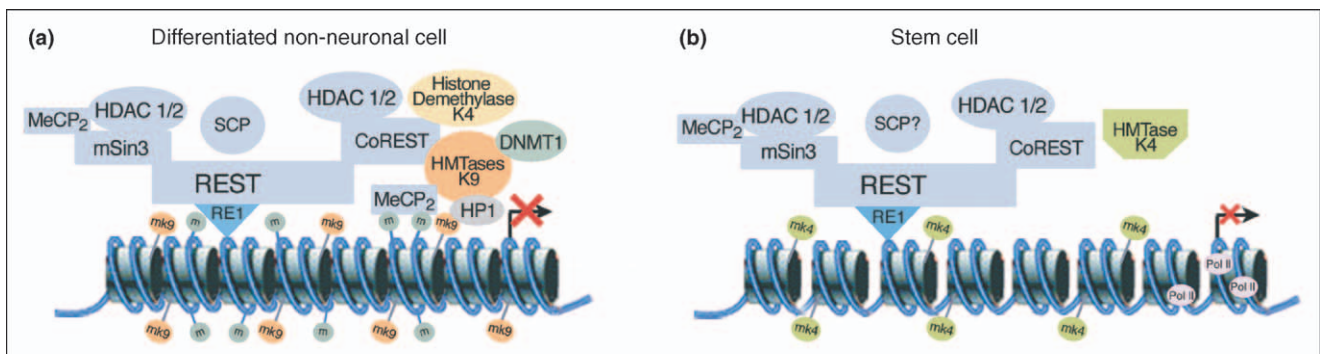
REST is obligatory for the correct development of vertebrates, because perturbation of REST expression or function in the developing embryo results in ectopic expression of neuronal genes in non-neuronal tissues and early embryonic lethality [9]. In terminally differentiated non-neuronal tissue, neuronal genes are presumably in a long-term silencing state. How does REST direct this mode of silencing? The answer lies in part with its signature functional domains. REST harbors three functional domains: a DNA binding domain containing eight zinc-finger motifs that binds to the RE1 motif, and two independent repressor domains one

located at the amino- and one at the carboxy- terminus of the protein [10]. The amino terminal repressor domain interacts with mSin3, a corepressor found in all eukaryotes that recruits histone deacetylases (HDACs) [11–14]. The mSin3–HDAC complex, however, is associated primarily with a dynamic mode of repression that can alternate between repression and activation and, therefore, by itself, would probably be inadequate for long-term silencing of neuronal genes. This conundrum was solved by the discovery of the corepressor CoREST, which interacts directly with the carboxy terminal repressor domain of REST [15,16] and, similar to mSin3, exists stably in complexes with HDACs [16–18]. Interestingly, unlike mSin3, CoREST is present only in organisms with a nervous system [19], pointing to CoREST as a more specialized corepressor. Several recent studies indicate that the REST–CoREST complex recruits chromatin modifiers for long-term silencing of neuronal genes [20–22] (Figure 1a). Specifically, CoREST can form immuno-complexes not only with HDACs but also with the histone H3 lysine 9 (H3–K9) methyltransferase G9a [23] and with the newly discovered histone H3 lysine 4 (H3–K4) demethylase LSD1 [24**] (that is also known as KIAA0601 or BHC110) [25], both of which mediate modifications associated with gene silencing. Importantly, these histone-modifying enzymes are required for REST–CoREST silencing in non-neuronal cells [22,24**]. Furthermore, CoREST recruits to the REST–RE1 site other silencing machinery, including the methyl DNA-binding protein MeCP2 and the histone

H3–K9 methyltransferase SUV39H1 [21]. Heterochromatin protein 1 (HP1), which causes compaction of chromatin and is associated with histone H3–K9 methyltransferases, is also present on the neuronal gene chromatin [21], specifically on the RE1 region [22]. The effects of these modifications are manifested in histone deacetylation, an absence of H3–K4 methylation, and presence of H3–K9 methylation, which creates binding sites for HP1 and condensation of the targeted chromatin (Figure 1a). Additionally, the recruitment of silencing machinery by REST–CoREST might result in the propagation of silencing across a large chromosomal interval containing several neuronal genes that do not have their own REST binding sites [21], suggesting a relationship between higher order chromatin structure and patterns of gene expression.

The methylation of cytosine residues in CpG dinucleotides in the genome represents an additional epigenetic modification of biological importance [26]. DNA methylation can interfere with transcription by repulsing or attracting DNA binding proteins. The REST binding site (RE1) contains a CpG dinucleotide and recent studies reveal that the RE1 and surrounding region of neuronal genes is methylated in differentiated non-neuronal cells [27**]. Furthermore, the DNA methyltransferase DNMT1, which interacts with histone H3–K9 methyltransferases [28], is associated with the RE1 region of neuronal gene chromatin (J Chenoweth and G Mandel, unpublished). Binding of REST to the RE1 site, how-

Figure 1



REST–CoREST orchestrates differential epigenetic mechanisms to inactivate neuronal genes in non-neuronal cells. **(a)** REST–CoREST recruits a silencing complex to neuronal genes in terminally differentiated non-neuronal cells. Neuronal gene chromatin is a substrate for chromatin modifying enzymes including histone deacetylases (HDAC 1,2), histone H3 lysine 4 demethylase (histone demethylase K4), and histone H3–K9 methyltransferases (HMTases K9). Methylated lysine 9 residues (mK9) are binding sites for heterochromatin protein 1 (HP1), which causes chromatin condensation. The REST binding site (RE1) and adjacent region is methylated at CpGs (m) and associated with the methyl DNA binding protein MeCP2. MeCP2 is also associated with Sin3–HDAC complexes. DNA methyltransferase 1 (DNMT1) is recruited to the methylated RE1 site. The small carboxyl terminal domain (CTD) phosphatase (SCP) might block RNA polymerase II activity. **(b)** REST–CoREST–mSin3 recruits a repressor complex to neuronal genes in embryonic stem and progenitor cells. HDAC 1 and 2 are predominant modifiers mediating repression of neuronal genes. RNA polymerase II (Pol II) is associated with neuronal gene chromatin probably because of a relatively low state of chromatin compaction (compare a with b). The RE1 sequence and adjacent region is not methylated and histones are marked by methylation of K4 (mK4). The presence of methylated K4 suggests that histone H3–K4 methyltransferase (HMTase K4) is probably present on the RE1 site. The presence of SCP is not confirmed, but functional studies suggest it is probably present on the RE1. SCP could function to keep Pol II minimally active.

ever, is independent of DNA methylation [27**]. The repressor MeCP2 (methyl-CpG-binding protein-2) binds methylated DNA [1] and recruits additional modifiers such as HDAC [29] and histone H3–K9 methyltransferase activity [30]. In some cases, a reciprocal relationship was found between DNA and histone methylation, whereby methylation of K9 in histone H3 induced DNA methylation and vice versa [31–33]. The methylation-independent binding of REST to the RE1 motif raises the question of whether REST could mediate a type of repression that, unlike the case in differentiated non-neuronal cells, does not involve histone H3–K9 methylation.

Programming a poised status for neuronal gene chromatin in pluripotent embryonic stem cells

The silencing of neuronal gene chromatin in differentiated non-neuronal cells is stable, inheritable and endures the lifetime of the animal. By contrast, embryonic stem cells, although also non-neuronal, still have the capacity for self-renewal and differentiation along all cell lineages. The question arises as to whether these two fundamentally different non-neuronal cell types utilize similar epigenetic mechanisms to suppress the same neuronal genes? If so, ES cells and, presumably, neural stem and progenitors must erase the epigenetic silencing marks and reprogram chromatin, during differentiation, to enable expression of neuronal genes in a lineage-dependent manner. Our recent studies indicate that erasure and reprogramming of chromatin does not occur. Rather, neuronal gene chromatin in ES and progenitor cells is programmed to stay in a repressed state that is nonetheless poised for expression [27**] (Figure 1b). In this state REST is bound to the RE1 motif but, surprisingly, its corepressors, CoREST, mSin3, HDAC and MeCP2, which are present on silenced neuronal gene chromatin, are also present in ES cells. Analysis of the epigenetic modifications, however, reveals that, unlike the situation in differentiated non-neuronal cells, the RE1 motif and surrounding sequences in neuronal genes are not methylated. In this case, MeCP2 is probably recruited to the RE1 by a mSin3–HDAC complex [34]. Coincident with the hypomethylated DNA is the absence of histone H3–K9 methylation in the RE1 region and greatly reduced levels of the associated methyltransferase G9a (when compared with the levels in terminally differentiated non-neuronal cells) [27**]. Moreover, the repressed neuronal gene chromatin in ES cells is instead enriched in di- and tri- methylated K4 on histone H3 [27**], modifications associated normally with actively transcribed genes [35]. In the case of ES cells, but not terminally differentiated non-neuronal cells, RNA polymerase II (Pol II) is present on RE1 sites in the 5' untranslated regions of several neuronal genes, accompanied by very low transcript levels [27**]. Thus, the epigenetic modifications associated with the RE1 sites of neuronal genes

in stem cells point to an inactive, but permissive, chromatin state that is poised for subsequent activation. Recent studies have shown that a family of small Pol II carboxyl-terminal domain phosphatases (SCPs) are probably recruited by REST to the RE1 sites of neuronal genes in P19 embryonal carcinoma stem cells [36**]. Phosphatase-inactive forms of SCP interfere with REST function and promote neural differentiation [36**]. One of the roles of the SCPs in ES cells might be to contribute to a poised state by maintaining lower levels of Pol II activity on neuronal genes. SCPs were also found in REST complexes in differentiated non-neuronal cells [36**]. Although Pol II is not associated with neuronal genes in these cells, SCPs might provide additional security for the silenced state.

Taken together, these findings suggest that the core REST complex establishes a distinct set of epigenetic marks by recruiting different chromatin modifying proteins in differentiated non-neuronal and ES cells. Whether the absence of DNA methylation prevents recruitment of specialized machinery necessary for long-term silencing typical of differentiated non-neuronal cells remains unknown.

How does the inactive yet permissive state escape being converted to an active state? Several diverse enzymatic activities might help to maintain neuronal genes in a state of suspended animation. For example, HDACs, which function to lower levels of acetylated histones; SCPs, which might reduce activity of Pol II; and the histone H3–K4 demethylase LSD1, which is present in CoREST immuno-complexes in ES cells (N Ballas, G Mandel, unpublished data); might all contribute to maintenance of the poised state. Finally, microRNAs have been implicated recently as key players in the self-renewal of stem cells [37]. These small non-coding RNAs might complement the activities of chromatin modifiers that keep transcript levels low, either by blocking translation of neuronal mRNAs or by selective degradation of neuronal transcripts.

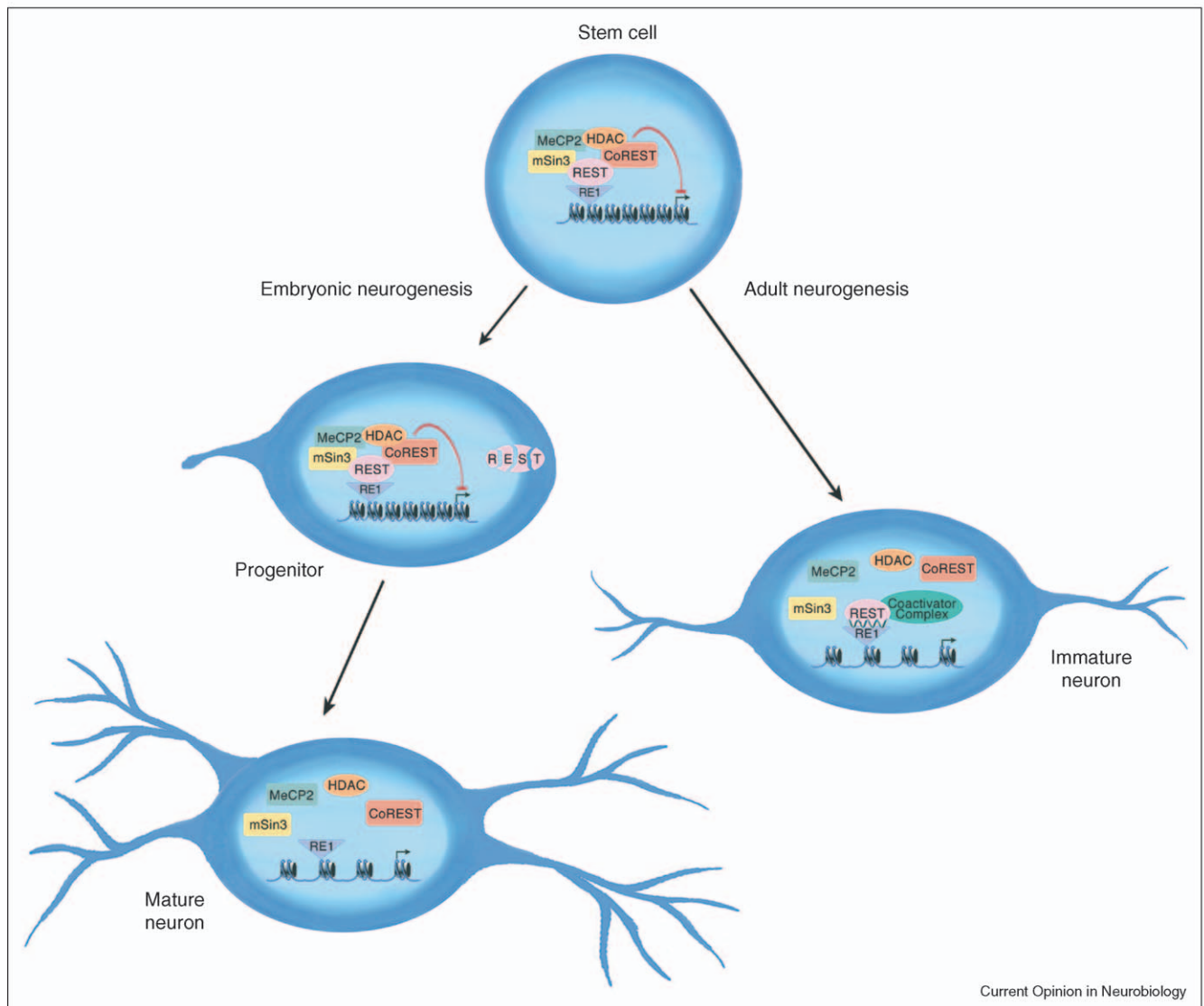
Plasticity versus stability of neuronal gene chromatin, a glance at the outcome

The repression of neuronal gene expression in differentiated non-neuronal and ES cells is associated with two different epigenetic states. Although both states involve HDAC, the consequences for neuronal gene expression are quite different. In particular, whereas there is no basal transcription of several neuronal genes in differentiated non-neuronal cells, these same genes are transcribed at low levels in ES cells [27**]. Furthermore, perturbation of HDAC activity, a modifier associated with active repression, relieves repression of these genes in ES but not in differentiated non-neuronal cells [27**]. It appears that HDAC activity might play a global role in maintaining the plasticity of neuronal gene chromatin because its inhibition also results in neuronal differentiation of multipotent

adult neural stem cells [38[•]]. What might be the natural spring that releases neuronal genes from repression in stem cells? Many genes that are up-regulated by HDAC inhibition, including the neurogenic transcription factor NeuroD that contains an RE1 site [8], are targets of REST, pointing to the disappearance of REST as the key switch for relieving the inactive state of neuronal

gene chromatin. This idea receives support from the recent demonstration that overexpression of a chimera containing the REST DNA binding domain fused to the transcriptional activator domain of VP16, in neural stem cells or in muscle progenitors, induces neuronal differentiation [39,40^{••}]. Interestingly, ES cell chromatin is globally enriched in histone H3 and H4 acetylation in

Figure 2



There are two separate models to explain REST regulation of neuronal genes during embryonic and adult neurogenesis. In both the embryonic and the adult neural stem cell, neuronal genes are actively repressed by a REST repressor complex and chromatin is relatively compact.

(a) During embryonic differentiation, REST is removed at two distinct stages, first at the dividing progenitor stage by proteosomal degradation (broken pink oval), and then at terminal differentiation (mature neuron) by removal from chromatin and transcriptional repression. In the mature neuron, REST corepressors are dissociated from RE1 but still present, chromatin is relaxed and neuronal genes are expressed. **(b)** During differentiation of adult neural stem cells (right), REST remains on neuronal gene chromatin, and a small double stranded non-coding RNA containing RE1 (green wavy line between RE1 and REST), converts REST from a repressor to an activator by dismissal of corepressors and recruitment of coactivators.

addition to di- and tri- methylation of K4 on histone H3, relative to differentiated non-neuronal cells [41], indicating that these modifications might contribute to global plasticity of ES chromatin. Collectively, these findings suggest that the epigenetic modifications that are characteristic of embryonic and neural stem cells are correlated with chromatin plasticity and the ability to differentiate along a neuronal cell lineage.

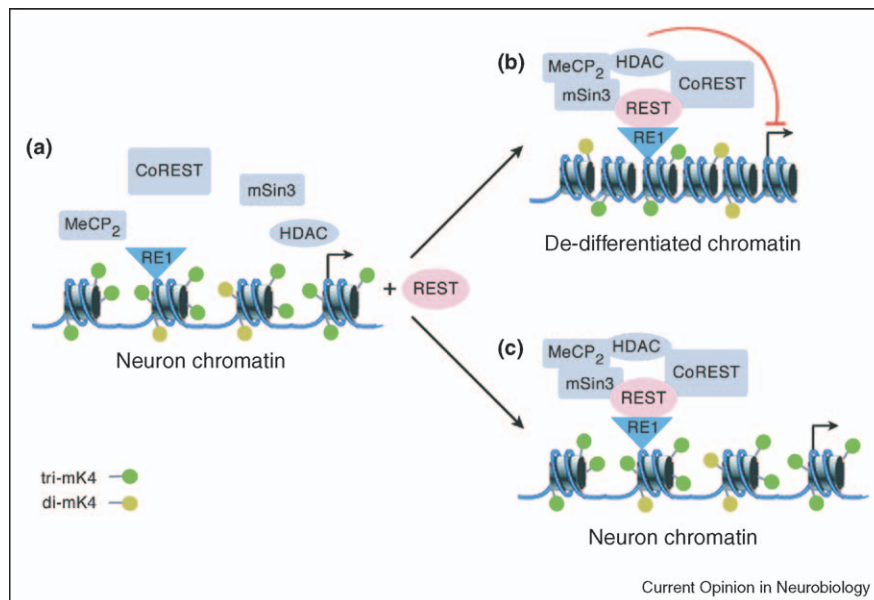
The chromatin state at terminal differentiation: neurons at last

The transition from stem or progenitor cell to a post-mitotic neuron requires disarming REST. During cortical differentiation, post-translational degradation of the REST protein precedes both its dismissal from RE1 sites and transcriptional inactivation of the *REST* gene itself at terminal differentiation [27^{••}] (Figure 2). The identity of transcriptional activators that might function after REST departure is not known, but a novel neuronal protein, termed inhibitor of BRAF35 (iBRAF), is an intriguing candidate. IBRAF expression increases during neuronal differentiation and abrogates REST mediated repression of neuronal target genes (R Shiekhattar, personal communication). In contrast to differentiation during embryogenesis, the differentiation of adult hippocampal stem cells to neurons occurs via a small non-coding double stranded RNA (dsRNA) containing RE1 motif that converts REST from a repressor to an activator of neuronal

genes [42^{••}] (Figure 2). Whether this dsRNA plays a role in differentiation of neural stem and progenitor cells during development has yet to be determined. If so, it must act through a different mechanism that does not depend upon the persistent presence of REST.

At terminal differentiation, the transition to an active chromatin state is accompanied by hyper-methylation of K4 on histone H3, in particular tri-methylation, around the RE1 sites in the proximity of the transcriptional start sites of neuronal genes [27^{••}]. The transcriptional activity of some neuronal genes is adjusted even further by the continued presence of REST corepressors, including HDAC. Perturbation of HDAC activity in neurons results in elevated expression of these genes, suggesting additional plasticity of neuronal gene chromatin in mature neurons [27^{••}]. Indeed, the gene encoding brain derived neurotrophic factor (BDNF), as a representative of this class, can be up regulated either by interference with HDAC activity [27^{••}] or by exposing neurons to 50 mM potassium chloride [27^{••},43,44]. The treatment with potassium chloride correlates with phosphorylation of MeCP2 and its departure [43], along with mSin3A and HDAC [44], from the sites of methylated DNA. It will be important to determine whether MeCP2 is regulated similarly in response to physiological stimuli *in vivo*. Although MeCP2 is a global corepressor, in patients with Rett syndrome (RTT), caused by mutations in MeCP2,

Figure 3



Different possible outcomes of re-expression of REST in mature neurons. **(a)** Chromatin status of mature neurons, in the absence of REST. Active neuronal chromatin is in a relaxed conformation and marked by an increased amount of trimethylated lysine 4 on histone 3 (tri-mK4) compared with the amount of dimethylated lysine 4 on histone 3 (di-mK4). REST reassembles a core corepressor complex. **(b)** Re-expression of REST might result in reprogramming of neuronal genes to a repressed state by reduction of trimethylation of lysine 4 on histone H3 (tri-mK4), and compaction, probably by histone deacetylation, of chromatin. **(c)** Alternatively, REST recruits corepressors, but is unable to reprogram the chromatin to a repressed state (number of trimethyl lysine 4 residues is unchanged from original neuronal chromatin and chromatin stays relaxed) and, therefore, neuronal genes are still transcribed.

the nervous system is selectively affected [45,46]. Furthermore, in mouse models mutations in MeCP2 result in hyper-acetylation of histone H3 in certain areas of the brain [47]. Thus, in neurons, MeCP2 probably plays a predominant role in epigenetic control of chromatin status in the absence of REST.

Conclusions and future directions

Epigenetic regulation of neuronal gene chromatin by REST is fundamental for maintaining stem cells in an undifferentiated pluripotent state and for proper acquisition of neural fate during neurogenesis. The disappearance of REST during cortical neurogenesis appears to be a prerequisite for normal neuronal function in the adult. Are there any situations under which REST is re-expressed in mature neurons and, if so, what are the consequences?

Previous *in situ* hybridization studies of adult rat hippocampal neurons indicated that low steady-state levels of REST transcripts were increased after induction of seizure [48]. No evidence was provided, however, for induction of REST protein. To our knowledge, there is only one example in which both mRNA and REST protein were up-regulated in adult neurons, and that was after a global ischemic insult [49]. Here, REST induction in hippocampal neurons repressed expression of the REST-regulated *GluR2* gene, a subunit of AMPA receptors, and antisense knock-down of REST prevented the suppression [49].

The above findings raise several questions. Are all REST target genes repressed or is the effect gene-specific? Is repression dependent upon neuronal type or physiological stimulus? Does long-term expression of REST promote de-differentiation of mature neurons or are there intrinsic barriers for reversing phenotype? Related to this, is any de-differentiation accompanied by epigenetic reprogramming from an active to an inactive chromatin state (Figure 3)? To date, there is little information on the molecular underpinnings of epigenetic reprogramming. Recent *Xenopus* nuclear transplantation studies have, however, indicated the existence of an epigenetic memory that impedes efficient reprogramming of previously transcribed genes during development [50••]. The ability to reconstitute REST and its corepressor components back onto neuronal gene chromatin in mature neurons (J Chenoweth and G Mandel, unpublished) might provide a new paradigm for investigating the fascinating problem of how to reprogram neuronal gene chromatin of mature neurons either *in situ* or after nuclear transplantation.

Acknowledgements

The authors thank J Chenoweth IV and R Shiekhhattar for permitting us to cite unpublished work, and J Speh for help with the figures. We apologize for omitted citations due to space limitations. G Mandel is an investigator of the Howard Hughes Medical Institute. We acknowledge support from a National Institutes of Health grant to G Mandel.

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