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Review

Chromatin remodeling and neuronal function: exciting links

M. Santos[†,‡], P. A. Coelho[§] and P. Maciel[*,†,‡]

[†]Life and Health Sciences Research Institute, Health Sciences School, University of Minho, Braga, [‡]Institute for Biomedical Sciences Abel Salazar, University of Porto, and [§]Laboratório de Genética Molecular da Mitose, Instituto de Biologia Molecular e Celular, Porto, Portugal
**Corresponding author: P. Maciel, PhD, Life and Health Sciences Research Institute, Health Sciences School, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: pmaciel@ecsaude.uminho.pt*

Regulation of gene expression occurs at different levels, from DNA to protein, and through various mechanisms. One of them is modification of the chromatin structure, which is involved in the definition of transcriptional active and inactive regions of the chromosomes. These phenomena are associated with reversible chemical modifications of the genetic material rather than with variability within the DNA sequences inherited by the individual and are therefore called 'epigenetic' modifications. Ablation of the molecular players responsible for epigenetic modifications often gives rise to neurological and behavioral phenotypes in humans and in mouse models, suggesting a relevant function for chromatin remodeling in central nervous system function, particularly in the adaptive response of the brain to stimuli. We will discuss several human disorders that are due to altered epigenetic mechanisms, with special focus on Rett syndrome.

Keywords: DNA methyltransferase, epigenetics, imprinting, methyl-binding protein, neuronal plasticity, Rett syndrome

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Several articles in this issue address the role of a timely and appropriate regulation of gene expression in the function(s) of the nervous system. Gene expression is particularly varied in neurons, with a large proportion of the coding genome being expressed at any time point (Geschwind 2000; Sandberg *et al*. 2000) often with increased variability of gene products due to alternative splicing of mRNA. Additionally, in the central nervous system (CNS), given the high level of structural and functional specialization, transcripts that are expressed at very low levels may have crucial roles for specific groups of cells and therefore be very important for the function of the whole system.

Regulation of gene expression occurs at different levels, from DNA to protein, and through various mechanisms, some of which are rigidly pre-established and genetically defined, whereas others are necessarily more flexible, as they are required for an adequate response to environmental stimuli. From the point of view of energy cost, it is more useful for the cells to regulate expression at the earliest possible level, i.e. gene transcription, so that mRNAs for unnecessary products are not synthesized. This can be controlled (i) by the availability, in each cell, of transcription factors (in the appropriate activation state and in the presence of the necessary molecular partners) that bind to specific regulating sequences upstream of the genes, allowing their transcription through positioning of the RNA synthesis machinery, but also (ii) by mechanisms of long-range action of other DNA sequences, involving different regulating proteins, with a transcription-enhancing or repressing effect, and/or (iii) through the modification of the chromatin structure, which will define the accessibility of the DNA to these transcription regulators and to the RNA polymerases. The latter mechanisms are involved in the definition of active and inactive regions of the chromosomes, in the dosage-compensation processes such as the inactivation of one of the X-chromosomes in mammalian females, and in the parental imprinting of genes, which makes the expression of a given gene allele dependent on its parental origin. These phenomena are associated with reversible chemical modifications of the genetic material rather than with variability within the DNA sequences inherited by the individual and are therefore called 'epigenetic' modifications.

The link between epigenetic modifications and neuronal function is an exciting new field of investigation in the neurosciences emerging in the post-Human Genome era (Shahbazian & Zoghbi 2002; Tucker 2001). In this review, we will discuss the recent developments in this area of research.

Mechanisms of epigenetic modification

The study of epigenetic instability began more than 75 years ago, when Muller (1940) recovered several examples of flies displaying variegating eye phenotypes after X-irradiation. In

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several human diseases, phenotypic variation has normally been attributed to differences in genetic background and the influences of environment on that genetic background. In disagreement with this idea, experiments in isogenic populations of model organisms such as *Drosophila melanogaster* showed that phenotypic variation does persist and can be transmitted through mitosis and meiosis. These data clearly support the existence of mechanisms providing stable or semi-stable regulation of gene expression apart from nucleotide sequence. This regulation is achieved through the action of epigenetic factors, chromatin-modifying enzymes that can be divided into three distinct categories: (i) histone-modifying enzymes which covalently acetylate, phosphorylate, ubiquitinate, or methylate histones; (ii) DNA-modifying enzymes which methylate CpG-rich sequences; and (iii) ATP-dependent chromatin-remodeling complexes which can disrupt nucleosome structure and increase accessibility to DNA and histones, using the energy from ATP hydrolysis to move histone octamers along DNA molecules (Becker & Horz 2002; Gregory *et al*. 2001; Narlikar *et al*. 2002).

Eukaryotic genome assembles into chromatin; the basic building block of chromatin is the nucleosome, which contains 147 base pairs (bp) of DNA wrapped in a left-handed superhelix 1.7 times around a core histone octamer (two copies each of histones H2A, H2B, H3, and H4). Each core histone contains two separated functional domains: a signature 'histone-fold' motif sufficient for both histone–histone and histone–DNA contacts within the nucleosome, and NH_2-terminal and COOH-terminal 'tail' domains that contain sites for post-translational modifications referred above. Histone covalent modifications can work as recognition signals, directing to chromatin the binding of non-histone proteins that determine its function and subsequently the transcriptional state of the genes. Nearly 40 years of research has resulted in the documentation of a variety of post-translation modification of the histones. The covalent modifications that take place on histones include the acetylation of lysines, the methylation of lysines and arginines, the phosphorylation of serines and threonines, the ubiquitination of lysines, the sumoylation of lysines, and the ADP-ribosylation of glutamic acid residues. All these modifications, except methylation, appear to be reversible. These are the histone modifications that allow the transition between open and condensed states and regulate the accessibility of DNA to several biological processes such as transcription, recombination, replication, and DNA repair. Covalent histone modifications and the histone positioning constitute a potential histone code defining actual or potential transcription sites (Jenuwein & Allis 2001; Richards & Elgin 2002).

The acetylation of lysine residues in histone tails has several roles in the regulation of the nucleosome, such as decreasing the histone–DNA interactions and increasing the accessibility of the DNA for transcription activation. Acetylation can also regulate DNA replication, histone deposition, and DNA repair, by recruiting proteins that have an acetyl-lysine binding module – the bromodomain. The histone acetyltransferase (HAT) is a multisubunit protein responsible for the acetylation of lysines. This acetylation, which promotes transcription, is reversed by histone deacetylases (HDACs) (Marmorstein & Roth 2001). Another histone modification, lysine methylation, has been directly implicated in epigenetic inheritance. Two distinct epigenetic silencing mechanisms are linked to methylation of lysines 9 and 27 on histone H3. Heterochromatic proteins, such as HP1, bind histone H3-containing methyl-lysine 9 and promote gene silencing. The Polycomb protein also binds to histone H3, specifically at methyl-lysine 27, thus promoting gene silencing during development (Sims *et al*. 2003). The histone ubiquitination or sumoylation plays an important role in the regulation of transcription either through proteosome-dependent degradation of transcription factors or through other mechanisms related to the recruitment of modification complexes. Histone ubiquitination is usually involved in positive regulation of transcription, unlike sumoylation of histone H4, which is important for transcriptional repression (Berger 2002; Iizuka & Smith 2003; Zhang 2003). Finally, serine phosphorylation of histone H3 at Ser 10 and Ser 28 has been correlated with mitotic chromosome condensation (Nowak & Corces 2004). Other serine phosphorylation sites have been identified on histone H2A, H2B, and H4. For instance, phosphorylation of histone H2A at Ser 1 is reported to be a hallmark for mitotic chromosome condensation (Barber *et al*. 2004).

In addition to the histone modifications, DNA is also subjected to covalent modifications that are important for gene repression. So far, DNA methylation has been identified in several eukaryotes except in yeast, *Caenorhabditis elegans*, and *D. melanogaster*. In mammals, DNA methylation occurs exclusively at CpG dinucleotides, and different patterns of DNA methylation have been correlated with genome imprinting, inactivation of the X chromosome, and embryonic development. There are essentially two classes of DNA methyltransferases, the *de novo* DNA methyltransferases (DNMT3A and DNMT3B) which define new methylation patterns, and the maintenance DNA methyltransferases. The first identified member of DNA methyltransferases, DNA methyltransferase 1 (DNMT1), is a maintenance DNA methyltransferase. This enzyme uses as substrate hemi-methylated DNA and copies the pattern already established during DNA replication. As a maintenance DNA methyltransferase, one could expect DNMT1 levels in adult brain to be low, as neurons do not undergo mitosis. Instead, not only the level of this protein is quite high but also the level of DNA methylation is higher in adult brain than in other tissues (Brooks *et al*. 1996; Goto *et al*. 1994; Inano *et al*. 2000; Tawa *et al*. 1990). DNA methylation must have a role in the maturation process of the brain as the ablation of DNA methylation maintenance pathway, through a targeted disruption of the *Dnmt1* gene, in mouse CNS precursor cells (but not in postnatal neurons) causes global DNA hypomethylation and neonatal death, due

to defects in neuronal respiratory control of the mutant animals (Fan *et al*. 2001).

DNMT3A and DNMT3B, *de novo* methyltransferases, are essential for mammalian development. Both proteins might be partially redundant, but the critical timing and mutant outcomes of both proteins are different, as shown by the studies of Okano and collaborators (1999) (mutant phenotypes summarized in Table 1). DNMT3A and DNMT3B expression studies performed by Feng and collaborators (2005) also suggest that these proteins have a different functional significance: while DNMT3B is predominant at the beginning of embryonic neurogenesis, DNMT3A appears to play a role at this developmental stage but also later, at postnatal stages, in CNS function. Mutations in the catalytic domain of *DNMT3B* gene have been recognized in a subset of patients with the autosomal recessive human disorder Immunodeficiency, Centromeric instability, Facial anomalies syndrome (ICF, OMIM #242860) characterized by variable immunological defects, centromeric heterochromatin instability, facial anomalies, and mental retardation (Okano *et al*. 1999; Xu *et al*. 1999).

In *Neurospora*, cytosine methylation depends on a conserved DNA methyltransferase, which is directed to chromatin by the histone H3 lysine methyltransferase DIM-5, linking these two types of epigenetic modification.

Imprinting as modification of genetic information affecting behavior

The general idea that genetic information inherited from both parents is equivalent, except for the sex chromosomes, was questioned 20 years ago with experiments that showed that proper development of mice embryos required information from both maternal and paternal genomes (McGrath & Solter 1984; Surani *et al*. 1984). This idea has been consolidated with the identification of several imprinted genes, i.e. genes that display a pattern of expression that is dependent on their parental origin (Smith *et al*. 2004).

The mechanisms underlying the establishment and maintenance of imprinting are not clearly understood, but it is known that the epigenetic mark of the imprinted genes occurs early in the gametogenesis (gonocyte and oocyte development). After the erasing of the inherited methylation pattern, a new one is defined according to the origin of the genetic material (the sex of the parent) (Kafri *et al*. 1992; Monk *et al*. 1987; Sanford *et al*. 1987). For numerous genes, imprinting may not be ubiquitous, but rather tissue-specific, specific to developmental stage or species-specific (Yamasaki *et al*. 2003). Interestingly, there seems to be a differential distribution of the expression of imprinted genes within the brain. This was elegantly demonstrated with studies in mouse chimeras, in which cells that were disomic for maternal genome survived especially in the neocortex, striatum, and hippocampus, while cells disomic for paternal genome were virtually absent in telencephalic structures but present in the hypothalamus, preoptic area, structures important for primary motivated behavior (Allen *et al*. 1995; Keverne *et al*. 1996).

Many explanations for the evolution and origin of genomic imprinting have been proposed, including regulation of gene dosage (Solter 1988) and the conflict over parental investment (Moore 2001). Parental imprinting can be seen as a form of selection of the regions of maternal/paternal genomes contributing for the behavior of the offspring. Maternal investment over its offspring is influenced by the paternal contribution to the offspring genome, and the conflict created might be solved through gene imprinting, each player's (mother, father, and offspring) involvement defending each one's best interest. An example is the involvement of the father in determining the size of the litter and of the mother in provisioning it (Hager & Johnstone 2003).

Clearly, a disturbance in the balance of the two imprinted genomes can result in brain dysfunction, and imprinted genes are recognized to play important roles in a number of different human conditions and in altered social behavior in mammals. Angelman's syndrome (AS, OMIM #105830) is a human disorder presenting severe speech delay, happy affect, epilepsy, and movement disorders (Williams *et al*. 2001). Prader–Willi syndrome (PWS, OMIM #176270) is characterized by diminished fetal activity, obesity, muscular hypotonia, mental retardation, short stature, and small hands and feet. The most common mutations in these two syndromes are deletions of chromosome 15q, and depending on whether the affected allele is the maternal or the paternal one, PWS or AS will develop. Mutations in one particular gene located on chromosome 15q, *UBE3A*, have also been identified in patients with AS without deletions in 15q (Kishino *et al*. 1997; Matsuura *et al*. 1997). *UBE3A* shows an imprinted mode of inheritance, consistent with a gene exclusively or preferentially active on the maternal chromosome. The absence of a functional maternal allele causes AS. The restricted neurobehavioral phenotype of this syndrome might suggest a brain-specific imprinting of *UBE3A*. In fact, Yamasaki and collaborators (2003) showed that *Ube3a*-deficient mice exhibit a neurological phenotype that resembles AS in humans and that *Ube3a* in mice is imprinted specifically in neurons but not in glial cells.

Other very interesting examples of imprinted genes with a role in behavior are the paternally expressed genes (Peg). The *Peg1* gene (also known as *Mest*) is highly expressed in various brain regions of mice and presents an imprinted pattern, with expression of the paternal allele. *Peg1*-deficient mice are viable and fertile; however, the paternal transmission of a mutant allele causes a growth retardation, increased perinatal and postnatal lethality, and abnormal maternal behavior, without placentophagy (Lefebvre *et al*. 1998). *Peg3* also has an imprinted monoallelic paternal expression. *Peg3*-mutant mice have a complete deficit in all aspects of maternal behavior (retrieving, nest building, and crouching). The hypothalamic medial preoptic area (MPOA) is

Table 1: Behavioral phenotypes of mouse mutants for methyl-binding proteins and DNA methyltransferases

Gene	***MBD1***	***MBD2***
Function	Methyl-CpG binding Transcriptional repression	Methyl-CpG binding Transcriptional repression
Animal model phenotype	**Mbd1 $^{-/-}$ (Zhao *et al.* 2003)** Normal development Apparently healthy as adults Reduced neurogenesis Impaired spatial learning ability Marked reduction in DG-specific LTP Histological analysis: no detectable developmental defects	**Mbd2$^{-/-}$ (Hendrich *et al.* 2001)** Possess N-terminal 183 aa of the protein Viable, fertile, and with normal appearance Maternal nurturing defect Reduced litter size and pup weight Failure of the mothers to adequately feed their pups
Observation	Adult neural stem cells (ANCs) from Mbd1 null: Reduced neurogenesis Increased genomic instability	Genomic DNA methylation is not affected Lack intact MeCP1 complex Normal imprinting pattern of various analyzed genes
Gene	***DNMT1***	***DNMT3A and DNMT3B***
Function	Methylation maintenance	*de novo* methylation
Animal model phenotype	**Dnmt1$^{-/-}$ (Fan *et al.* 2001)** Dnmt1 deficiency in embryonic postmitotic neurons: Viability is not affected No obvious demethylation in mutant neurons Dnmt1 deficiency in embryonic CNS precursor cells: Neonatal death (respiratory failure due to abnormal neural control) Substantial demethylation in the brain of mutant embryos No obvious defect in brain structure No increase in embryonic cell death	**Dnmt3a$^{+/-}$ and Dnmt3b$^{+/-}$ mice (Okano *et al.* 1999)** Grossly normal and fertile Dnmt3a $^{-/-}$: Normal at birth Died at about 4 weeks of age Dnmt3b$^{-/-}$: Not viable at birth Growth impairment Rostral neural tube defects
Observation	Dnmt1 is crucial for the function and survival of postnatal CNS neurons	Dnmt3a$^{-/-}$; Dnmt3b$^{-/-}$: Blocks *de novo* methylation

Table 1: Continued

Gene	***MECP2***	
Function	Methyl-CpG-binding domain Transcriptional repression	
Animal model phenotype	**Mecp2 null-mice (Guy *et al.* 2001)** Exons 3 and 4 deleted Born normal Develop neurological symptoms from 3 to 8 weeks of age No signs of cortical lamination or ectopias in the brain Specific loss of MeCP2 in the brain: indistinguishable phenotype Death at around 54 days Mecp2; Mbd2 double mutant: Phenotype was not different from the single mutant	**Mice expressing exogenous MeCP2** **Tau-MeCP2 expression in neurons (Luikenhuis *et al.* 2004)** WT heterozygous for the transgene Healthy and fertile Mutant MeCP2 308 heterozygous for the transgene Healthy and fertile Body and brain weight indistinguishable from wt littermates
	***Mecp2*-deficient mice (Chen *et al.* 2001)** Exon 3 deleted Born healthy Showed abnormal behaviour at around 5 weeks of age Reduced brain size and weight Cell bodies and nuclei of neurons: smaller and more densely packed Mice with specific mutant MeCP2 in postmitotic neurons: Similar but delayed and less severe phenotype	WT and MeCP2 308 mutants homozygous for the transgene Motor dysfunction Mutant pups smaller than wt littermates at weaning No reduction in brain weight Disheveled look Excessive stereotypic scratching
	***Mecp2*-deficient mice (Shahbazian *et al.* 2002a)** Born healthy From 6 weeks of age start to develop neurological symptoms Abnormal motor function and activity Heightened anxiety Abnormal social interactions No abnormalities in the CNS, normal brain weight H3 hyperacetylation in the brain	**MeCP2 transgenic mice (Collins *et al.* 2004)** Mice expressing MeCP2 approximately 2 times endogenous levels Normal at birth Developed severe neurological phenotype at around 10–12 weeks of age Less anxiety-like behavior Enhanced cerebellar motor learning and hippocampal learning Enhanced synaptic plasticity Older animals develop: Clonic and akinetic seizures with abnormal EEG recordings Double mutant MeCP2Tg1; Mecp2 null at 33 weeks remain indistinguishable from wt littermates

known as a regulatory center for maternal behavior, and oxytocin released from the hypothalamic paraventricular and supraoptic nuclei neurons controls milk ejection. The suggestion that *Peg3* could be involved in the modulation of the 'maternal response' is supported by the neural expression pattern of *Peg3* in hypothalamic nuclei, including MPOA, medial amygdala, and hippocampus, and the reduced number of oxytocin-positive neurons in mutant *Peg3* females (Li *et al*. 1999). Interestingly, however, Szeto and collaborators (2004) created a transgenic mouse in a mutant *Peg3* background (Li *et al*. 1999) in which they were not able to see the recovery of the wild-type phenotype; they propose that this result could be due to the low expression level of the transgene during early embryonic development, probably due to the absence of important regulatory elements in the transgene.

Chromatin remodeling and behavior

Chromatin-remodeling complexes were first identified by genetic screens in yeast as targets of mutations that alter the transcription of genes induced in response to extracellular signals (Winston & Carlson 1992). The identified mutant strains were named SWi/SNF (mating type SWItching/Sucrose Non-Fermenting). All different chromatin-remodeling multisubunit complexes contain a core SNF2-related ATPase region. SNF2 family members can be subdivided into several subfamilies according to the presence of protein motifs outside the ATPase region. The SNF2 subfamily includes the human BRG-1, and hBRM subunits of SWI/SNF-related complexes in *Drosophila* and humans. The BRG1- and BRM-associated chromatin-remodeling complexes have been implicated indirectly in the pathology of Williams-Beuren syndrome (WBS, OMIM #194050), an autosomal dominant disorder caused by heterozygosity of a microdeletion at 7q11.2. WBS is characterized by congenital heart disease, infantile hypercalcemia, a characteristic facies (described as elfin facies), and mental retardation. Socially, WBS children present a unique social behavior. Often they take the initiative to approach others, are overly friendly, and are always noted in a group. However, they also present behavioral problems such as attention deficits and anxiety (Morris & Mervis 2000). Interestingly, the Williams syndrome transcription factor (WSTF) encoded by the *WBSCR9/BAZ1B* gene, one of the genes deleted in WBS, is needed to recruit BRG1 and BRM and their associated chromatin-remodeling factors to vitamin D-regulated promoters (Kitagawa *et al*. 2003). Haploinsufficiency of this gene has been implicated as a possible cause of hypocalcemia in WBS patients. WSTF also interacts with ISWI, a SWI/SNF-related ATPase, to form a chromatin-remodeling complex, WHICH, that participates in DNA replication through interaction with PCNA (Bozhenok *et al*. 2002; Poot *et al*. 2004). On the basis of these findings, aberrant chromatin remodeling might play a key role in the pathophysiology of WBS. Another disorder in which chromatin remodeling seems to be affected is Rett syndrome (RTT), which we will explore in greater detail in the next sections, given the abundance of recent data regarding its pathophysiology.

MECP2 and Rett syndrome

The relevance of chromatin modification and remodeling for the function of the mammalian nervous system was first brought to attention when the genetic basis of the pervasive neurodevelopmental disorder known as Rett syndrome was clarified, in 1999 (Amir *et al*. 1999). This syndrome is a major cause of mental retardation in females, affecting 1/10 000–1/22 000 born females; it is characterized by an apparently normal pre- and perinatal development (6–18 months of age), followed by a growth deceleration/arrest and a loss of motor, language, and social acquisitions, leading to lifetime mental retardation, autistic behavior, and motor deterioration (clinical diagnosis criteria reviewed and recently updated by Hagberg and colleagues) (Hagberg *et al*. 1983; Hagberg *et al*. 2002). Stereotypical hand movements (hand washing/wringing, hand clapping/patting or hand mouthing) are often present and constitute a hallmark of the syndrome. Pathologically, a reduction of cortical thickness is observed, in spite of relative preservation of neuronal number, corresponding to a markedly reduced neuronal size and increased cell packing density, with loss of neuronal arborization and decreased synaptic density (Armstrong 2001). The majority of patients with classic RTT are heterozygous for mutations in the *MECP2* gene (Amir *et al*. 1999), which encodes a methyl-CpG-binding protein, MeCP2, known to bind symmetrically methylated CpG dinucleotides and recruit Sin3A and HDACs to repress transcription (Jones *et al*. 1998).

Several animal models for the study of the MeCP2 function *in vivo* have been created in mice (mutants summarized in Table 1), which mimic in many aspects Rett syndrome: a knock-out (*ko*) mouse for the *Mecp2* gene (Guy *et al*. 2001), a mutant that possesses only the C-terminal region of the gene (Chen *et al*. 2001), and a transgenic mouse, MeCP2^{308}, with a hypomorphic allele that truncates the protein at the position 308 (Shahbazian *et al*. 2002a). All these mutants are born normal and symptoms start to develop a few weeks later with progressive motor deterioration, males displaying an earlier onset and being more severely affected than females. As in RTT patients, no gross abnormalities in the brain were detected. The MeCP2^{308} mutant also presented emotional and social behavior abnormalities along with the motor dysfunction.

Expression of MeCP2 in mutant mice that are deficient for the *Mecp2* gene (models by Guy *et al*. 2001 and Chen *et al*. 2001) was shown to rescue the neurological RTT-like phenotype of mutants, the mutant mice expressing the transgene becoming indistinguishable from wild-type (*wt*) littermates.

In the study by Luikenhuis *et al*. (2004), the expression of a mutant *Mecp2* transgene in the postmitotic neurons of mutant mice was sufficient to recover the RTT-like phenotype in these animals. This suggests that the function of MeCP2 must be not in the embryonic development, but at later stages. However, overexpression of MeCP2 had a deleterious effect both on *wt* and on *ko* mice and induced a neurological phenotype that varied in severity according to the protein level (Collins *et al*. 2004; Luikenhuis *et al*. 2004). The MeCP2 protein appears to be highly regulated and its deregulation seems to have severe consequences specifically in the brain. In mice overexpressing MeCP2 (Collins *et al*. 2004), its upregulation affects pathways leading to cerebellar and hippocampal learning and increases synaptic plasticity, in an antagonistic way to the mental retardation presented by RTT patients.

In the embryonic development of humans and mice, MeCP2 expression starts to be detected very early and in the ontogenetically older brain areas (Shahbazian *et al*. 2002b). However, it is only in the mature brain that MeCP2 is expressed at the strongest levels. LaSalle and collaborators (2001) showed that in brain, one can find subpopulations of cells that are MeCP2 'high expression' and MeCP2 'low expression' cells. In RTT pathogenesis, the MeCP2 'high expression' cells seem to be selectively affected. The subpopulation of MeCP2 'high expression' cells was more represented in developed cerebrum than in immature brain (Balmer *et al*. 2002). The results by Mullaney and collaborators (2004) in the rat brain further narrowed the window of MeCP2 critical role to synaptogenesis. The authors showed a higher expression of MeCP2 and higher number of synapses in layer V than in layer VI of the cerebral cortex (first generated), as well as a concordant timing between the expression of MeCP2 and a higher number of synapses in the granule cells of the cerebellum and in the hippocampus, suggesting that MeCP2 might be regulating genes that are important for synapse formation, function, or maintenance rather than previous stages of nervous system development (such as neuronal differentiation or migration).

Neuronal targets of MeCP2

Mutations in the *MECP2* gene are responsible for hyperacetylation of histone H4 in cultured cells from patients with RTT, through impaired formation of the co-repressor complex Sin3A/HDAC, which in turn can affect chromatin architecture (Wan *et al*. 2001). Also, mutant MeCP2^{308} mice display hyperacetylation of H3 in cerebral cortex and cerebellum (Shahbazian *et al*. 2002a). Additionally, MeCP2 has been shown to facilitate lysine 9 methylation in H3 and may serve as a bridge between DNA methylation and histone methylation (Fuks *et al*. 2003; Horike *et al*. 2005). Finally, during postnatal brain development, pairing of homologous 15q11–13 alleles occurs (Thatcher *et al*. 2005) and MeCP2 is involved in this specific pairing that is disrupted in several neurodevelopmental disorders such as RTT. How disruption of these functions leads to the specific developmental dysfunctions that occur in RTT remains unknown. The identification of neuronal targets of MeCP2 is one avenue of research that may provide a clue to RTT pathogenesis, and possibly to an increased understanding of other pervasive developmental disorders such as autism and AS, in which MeCP2 levels appear to be low (Samaco *et al*. 2004).

Most microarray studies have failed to identify any substantial and consistent changes in transcription levels in *Mecp2*-null mice (Tudor *et al*. 2002), clonal cell cultures from individuals with RTT (Traynor *et al*. 2002), or in postmortem RTT brains (Colantuoni *et al*. 2001). These results suggest functional redundancy between the different methyl-binding proteins or a more focused action of MeCP2 as a selective regulator – be it region-specific actions of the protein in the brain, action at a specific developmental stage, involvement of MeCP2 in specific epigenetic events (such as imprinting of certain genes), or in activity-dependent transcription. In any of these scenarios, important differences in the transcription levels of certain genes may exist in the absence of MeCP2, but their detection will only be possible if suitable experimental designs are used.

A recent study by Ballestar and collaborators (2005) combining microarray studies, chromatin immunoprecipitation analysis, bisulfite genomic sequencing, and treatment with demethylating agents, in lymphoblastoid cell lines derived from RTT patients, revealed the deregulated expression of a number of genes, which were shown to have methylated promotors, directly bound by MeCP2. Approximately half of these target genes presented high expression levels in RTT cells when compared with *wt* cells, whereas the remaining half were downregulated, most likely because of an indirect effect of MeCP2 on genes that are in turn regulating these ones. The role of these target genes in the pathogenesis of RTT remains to be clarified.

MeCP2 was shown to be involved in the imprinting control region of the *H19* gene (Drewell *et al*. 2002). *H19* is an example of a gene for which imprinting occurs for the paternal allele. The promoter region of the paternal allele is highly methylated and its silencing was shown to be methylation-dependent and mediated by MeCP2 (Drewell *et al*. 2002). However, the analysis of different imprinted genes, including the *H19* gene, in cultured T-cell clones from blood and in brains from patients with mutations in the *MECP2* gene revealed normal monoallelic expression in all clones and brain samples (Balmer *et al*. 2002), which might suggest an *in vivo* redundancy amongst the methyl-binding domain-containing (MBD) family of proteins.

Horike and collaborators (2005) recently found that *DLX5,* a gene whose product is involved in the synthesis of gamma aminobutyric acid (GABA), is upregulated in RTT. In humans, *DLX5* has an imprinted pattern with expression of the maternal allele, while in mice *Dlx5* is biallelically transcribed, but

preferentially from the maternal allele. The authors found that in the cortex of *Mecp2*-null mice and in human lymphoblastoid cells from individuals with RTT (i) transcription levels were higher than normal and (ii) there was an altered parental imprinting of the gene that was dependent on the type of mutation. Although the target region through which MeCP2 regulates *Dlx5* expression is not known yet, this strengthens the possible link between MeCP2 and imprinting and, for the first time, connects RTT to this epigenetic mechanism. It also provides useful clues to RTT pathogenesis, as affected GABA neurotransmission could explain some of the cognitive symptoms of RTT.

Two other candidate targets of MeCP2 are the *UBE3A* and *GABRB3* genes. These are particularly interesting, as *UBE3A* is linked to AS and *GABRB3* (which encodes the protein GABA receptor β3 subunit), have been consistently implicated in autism, in association studies, and both disorders present some phenotypic overlap with RTT. *UBE3A* and *GABRB3* levels were found to be decreased in RTT, AS, and autism brains. *Mecp2*-deficient mice also display decreased levels of *Ube3a* and *Gabrb3*, in spite of the lack of alterations in the imprinting pattern of the *Ube3a* gene (Samaco *et al*. 2005). A possible mechanism through which MeCP2 regulates the expression of *UBE3A* has recently been proposed: MeCP2 binding to the methylated PWS-imprinting center at the maternal allele where the antisense *UBE3A* gene resides. Mutant MeCP2 would cause an epimutation at this center, affecting the expression of *UBE3A* (Makedonski *et al*. 2005).

Experiments performed in *Xenopus* embryos showed that MeCP2 targets the gene *xHairy2a* during development. In the absence or presence of a mutant form of MeCP2, the expression of the *xHairy2a* gene was misregulated, with consequences in neuronal differentiation. This study showed that MeCP2 interacts with SMRT complex via Sin3A and that mutant MeCP2 had defective binding to SMRT co-repressor complex. It is possible that DNA methylation and MeCP2 binding can modulate the levels of *xHairy2a* expression and have an essential role in early neurogenesis (Stancheva *et al*. 2003).

The most interesting target of MeCP2 identified so far is doubtlessly the gene encoding the brain-derived neurotrophic factor (*BDNF*), one of the genes for which transcription is regulated in a neuronal activity-dependent manner. Data from two different studies showed that MeCP2 is involved in the *Bdnf* gene silencing in the absence of neuronal activation. MeCP2 was shown to bind to the methylated rat *Bdnf* promoter III (equivalent to promoter IV in the mouse) and, upon membrane depolarization of cultured cortical neurons, to dissociate from the promoter and lead to a higher transcription level of the *Bdnf* gene (Chen *et al*. 2003; Martinowich *et al*. 2003). Chen and collaborators (2003) also showed that the release of MeCP2 protein was due to calcium influx that caused a phosphorylation of MeCP2. Given the role of *BDNF* in development and neuronal plasticity (McAllister *et al*. 1999; Binder & Scharfman 2004) and the timing when MeCP2 demand becomes crucial, that coincides with moments of synapse development and maturation, the aforementioned evidence easily fits a model in which MeCP2-regulated chromatin remodeling would underlie neuronal plasticity, which could explain some symptoms of the RTT phenotype, such as reduced dendritic arborization and complexity in some areas of the brain (Armstrong 2001) as well as the clinical finding of mental retardation.

Methyl-DNA-binding proteins and DNA methyltransferases

In addition to MeCP2, four other MBD-containing proteins (MBD1, MBD2, MBD3, and MBD4) exist (Ballestar & Wolffe 2001). Interestingly, null mutations in several of these proteins lead to behavioral phenotypes, as do some mutations in DNA methyltransferases (summarized in Table 1).

MBD1 is expressed in neurons throughout the brain, with highest concentration in the hippocampus (CA1 and DG), and is not expressed in glia. Mice *ko* for the *Mbd1* gene display reduced neurogenesis in the hippocampus, perform worse than *wt* animals when tested in the Morris water maze, and have a reduction in dentate gyrus long-term potentiation (LTP) (Zhao *et al*. 2003).

Mbd2$^{-/-}$-mutant mothers do not present a proper nurturing behavior of their offspring (Hendrich *et al*. 2001). This phenotype resembles the *Peg3*-mutant mothers (discussed above), highlighting a potential connection between *Mbd2* and imprinting. However, altered expression of *Peg3* or other imprinted genes was not detected in *Mbd2*$^{-/-}$ animals. It is possible that if differences exist, the deregulation occurs in a localized and functionally related area of the brain, such as MPOA of the hypothalamus. *Mbd3*$^{-/-}$ animals die before birth, suggesting an essential role of this protein during development (Hendrich *et al*. 2001). The different phenotypes of these two mutants might be explained, in part, by the expression pattern of the corresponding proteins. Expression profiles of MBD2 and MBD3 in the developing brain are not parallel: during development and in adulthood, MBD3 is expressed in ontogenetically younger brain regions, in contrast with MBD2 expression, that is weak in embryonic brain, but pronounced in the adult brain (Jung *et al*. 2003).

In addition to RTT and WBS, there are other human disorders in which mutations affecting chromatin remodeling lead to behavioral phenotypes where, for most of the cases, MR is a cardinal feature. Mutations in the *JARID1C* gene have been recently identified in patients with X-linked mental retardation (XLMR). The protein encoded by this gene belongs to the ARID protein family, which contains several DNA-binding motifs, and is involved in transcriptional regulation and chromatin remodeling (Jensen *et al*. 2005).

All this evidence suggests a role for 'brain chromatin' and its epigenetic modifications in mental retardation. This link

seems to be established early in development and when perturbed has consequences for life.

Chromatin remodeling and interaction with the environment

Some chromatin modification patterns need to be rigidly pre-established and even irreversible, such as the ones involved in developmental determination and differentiation, relevant to the appropriate formation of clearly defined circuits in the nervous system. However, there are many recent pieces of evidence suggesting that in many other cases, a process of dynamic chromatin remodeling is connected to phenomena of cellular and/or system response to extracellular and environmental stimuli.

The first example of this is the response to ischemia. After cerebral ischemia, DNA methylation is known to augment in *wt* mice, rendering the brain more susceptible to damage (Endres *et al*. 2000). The mechanisms through which this happens are not clear, but they might involve altered gene expression, DNA repair mechanisms or changes in mitotic activity. *Ko* animals for the *Dnmt1* gene do not present, after mild brain ischemia, this elevation in the level of DNA methylation, and have a better stroke outcome than *wt* mice, with reduced lesion size and higher number of neurons in the striatum (Endres *et al*. 2000).

Another example is the role of chromatin modifications in rythmicity of expression of the Clock genes. Organisms learn how to properly respond to the environmental changes that occur through the 24-h day or through the different seasons of the year such as temperature and light intensity. The mammalian core timekeeping has been identified as the suprachiasmatic nucleus (SCN) in the hypothalamus (Hastings & Maywood 2000) and allows mammals to adapt behavior and physiological responses to the day: night 24-h cycle (see Oster 2006). The entrainment of the SCN is done by a light pulse which induces a burst of expression of the clock genes (*Per1* and *Per2*) and immediate early genes (*c-Fos*, *Fos-B*, and *Jun-B*) (Albrecht *et al*. 1997; Kornhauser *et al*. 1990; Morris *et al*. 1998). One of the mechanisms involved in transcriptional regulation is chromatin remodeling through histone modification. The data obtained by Crosio *et al*. (2000) support the idea that circadian gene expression might be controlled at the histone level. When a pulse of light was given to mice kept in a 12-h light/12-h dark cycle for 2 weeks, and then for 4 days in constant dark, an increase of the H3 phosphorylation was detected and closely accompanied by the expression of the early gene *c-Fos*. In another study, Etchegaray and collaborators (2003) were able to identify rhythmicity in RNA polymerase II binding and acetylation of H3 in the *Per1* and *Per2* genes and showed that these rhythms were synchronous in the peripheral liver oscillator. It has also been demonstrated that p300, which has intrinsic HAT activity, is part of the CLOCK/BMAL1 complex and that the negative loop of CRY protein in the transcription regulation of *Per* genes is through the p300 protein (Etchegaray *et al*. 2003). Thus, in addition to mutations in circadian genes, loss of function of genes involved in epigenetic modification, namely acetylation and phosphorylation, might be responsible for impairment of rhythmicity.

Activity-dependent gene transcription and chromatin modification: role in synaptic plasticity

Synaptic plasticity underlies the brain's adaptive response to the environment. The mechanisms involved operate through post-translational modifications of proteins at the level of the dendrites (short-term responses) but may also involve the synthesis of new proteins through regulation of gene expression in the nucleus, when long-term responses/long-term memories are concerned (Levenson & Sweatt 2005; West *et al*. 2001). Synaptic activity induced either by external or by endogenous stimuli leads to a calcium influx and depolarization of the membrane. This Ca^{2+} rise is an important element in the activity-dependent gene transcription in the nucleus of neurons. Ca^{2+} influx can be perceived by the cell in different ways (temporal pattern of electrical activity or spatial pattern of Ca^{2+} influx) and by different molecules (second messengers) and the manner in which the signal gets to the nucleus (Ca^{2+} channels, Calmodulin, CREB, and MAP kinase) has a consequence in the interpretation of the different stimuli. This leads to different pathways being activated and consequently different genes activated and proteins expressed (Bradley & Finkbeiner 2002).

In the nucleus, CREB-dependent gene expression plays a crucial role in associating synaptic activity with long-term changes in synaptic circuitry in many kinds of neuronal systems. The phosphorylation of CREB by PKA increases the stability of the complex formed by CREB and CBP, a histone acetyltransferase, and thus regulates CREB-dependent gene expression through chromatin modification (Bito & Takemoto-Kimura 2003). The work by Guan and collaborators (2002) with the early response gene *C/EBP* also showed that the integration of stimuli that were repeatedly presented at independent synapses occurs at the nucleus by changes in chromatin structure that regulate gene expression/protein synthesis.

The data available for MeCP2 (Chen *et al*. 2003; Martinowich *et al*. 2003) provide the first evidence strongly supporting a link between chromatin remodeling and the synaptic or dendritic modifications that underlie the learning process, impaired in RTT and in many other related developmental disorders associated with cognitive deficits which share the clinical outcome of mental retardation.

It can be concluded that epigenetic modifications are essential for proper neuronal development, survival, and function and they may play a role in this system's adaptive response to the environment. We begin to have some evidence for an involvement of chromatin remodeling in plastic CNS

processes, such as the synaptic or dendritic modifications underlying learning. Transcriptional changes and modification of protein expression are known to be crucial for the establishment of many types of long-term memory. Thus, it is conceivable that modification of chromatin could affect these processes, either through an effect on global repression of gene activity or through specific modification of the expression of genes involved in such processes. An increased understanding of the mechanisms of epigenetic modifications and their role in neuronal function should shed light on the basis of many human cognitive and behavioral disorders.

Note added in proof

After this article as been accepted for publication, two independent studies revealed the impairment of synaptic plasticity, LTP and LTD in mouse models of RTT (Asaka Y, 2005; Moretti P, 2006).

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