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Fluoxetine and Cocaine Induce the Epigenetic Factors MeCP2 and MBD1 in Adult Rat Brain

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ABSTRACT

Once bound to methylated CpG sites, methyl-CpG-binding protein 2 (MeCP2) is thought to silence transcription of downstream genes by recruiting a histone deacetylase (HDAC). Mutations within the *MeCP2* gene have been found to cause Rett syndrome, a disorder of arrested neuronal development. Using immunohistochemistry, we found that Mecp2, as well as the methyl-CpG-binding protein MBD1, were significantly induced in normal adult rat brain after repeated injections of fluoxetine or cocaine for 10 days (one injection per day). Mecp2 was not induced by repeated injections of 1-(2-bis(4-fluorphenyl)-methoxy)-ethyl)-4-(3-phenyl-propyl)piperazine (GBR-12909) or nortriptyline. Together, the data indicate that the serotonergic system is predominantly involved. Using real-time reverse transcription-polymerase chain reaction experiments, MBD1 mRNA and both Mecp2_e1 and Mecp2_e2 transcripts were found to be induced by fluoxetine. Induction of the methyl-binding proteins was accompanied with enhanced HDAC2 labeling intensity and mRNA synthesis in response to fluoxetine. In tandem, acetylated forms of histone H3 were found to be decreased. The effect was characterized in three serotonin projection areas, the caudate-putamen, the frontal cortex, and the dentate gyrus subregion of hippocampus. Our data highlight GABAergic neurons as major target cells expressing Mecp2 in response to the serotonin-elevating agents and suggest that serotonin signaling enhances gene silencing in postmitotic neurons.

In vertebrate animals, DNA cytosine methylation is a major epigenetic factor that regulates many cellular events, including developmental gene regulation, X chromosome-inactivation, and genomic imprinting (Fan and Hutnick, 2005). Methyl-CpG-binding protein 2 (MeCP2) is the founding member of a family of proteins that each contains a closely related methyl-CpG binding domain (MBD). Four proteins, named MBD1 to MBD4, have been identified in mammals based on conserved amino acid sequences homologous to the MBD sequence motif of MeCP2 (Hendrich and Bird, 1998; Wade, 2001). Once bound to methylated CpG sites, MeCP2 is believed to silence transcription of downstream genes by virtue of its interaction with a histone deacetylase (HDAC)/Sin3 complex (Nan et al., 1997; Jones et al., 1998; Kriaucionis and Bird, 2003) that favors histone H3 as a substrate (Shahbazian et al., 2002). Recent reports have shown that the X-linked *MeCP2* gene actually has two splice variants, named *MeCP2_e1* and *MeCP2_e2,* that encode two protein isoforms that differ in their N termini, with the previously known *e2* variant encoding the less abundant isoform (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004).

Mutations within the *MeCP2* gene, including large rearrangements, have been shown to be associated with Rett syndrome (RTT) in more than 85% of cases (Amir et al., 1999; Shahbazian and Zoghbi, 2002). RTT, a disorder found almost exclusively in women, is characterized essentially by severe cognitive impairment, autistic behavior, stereotypical hand-wringing movements, and seizures (Hagberg and Witt-Engerstrom, 1987; Naidu, 1997). The involvement of MeCP2 in methylation specific transcriptional repression suggests that symptoms of RTT are the consequence of inappropriate transcription of genes playing an important role for neuronal function.

In a recent ontogenetic study of the expression of Mecp2 protein in normal rat brain (Cassel et al., 2004), we found significant heterogeneity in Mecp2 distribution between various brain areas. In structures including caudate putamen

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ABBREVIATIONS: MeCP2, methyl-CpG-binding protein 2; MBD, methyl-CpG binding domain; HDAC, histone deacetylase; RTT, Rett syndrome; CPu, caudate putamen; GBR-12909, 1-(2-(bis(4-fluorphenyl)-methoxy)-ethyl)-4-(3-phenyl-propyl)piperazine; EST, expressed sequence tag; PCR, polymerase chain reaction; DG, dentate gyrus; 5-HT, serotonin (5-hydroxytryptamine); DA, dopamine; NA, noradrenaline.

(CPu), septum, and hippocampus, very few cells showed detectable Mecp2 protein at birth. The number of immunoreactive cells increased during the first weeks of age, and neosynthesis of Mecp2 protein was still observed in some structures at the age of 2 years. However, the events by which spatiotemporal expression of Mecp2 is regulated remain poorly documented. In the present study, we looked for agents controlling Mecp2 expression in vivo and found that serotonin-elevating agents are able to induce its expression in adult rat brain.

Materials and Methods

Animals. Male Wistar rats (Janvier, France), 11 to 12 weeks old at the end of experiments, were i.p. injected either briefly or repeatedly for 10 days (one injection per day) with either 10 mg/kg fluoxetine hydrochloride (Eli Lilly, Indianapolis, IN), 20 mg/kg cocaine hydrochloride (Sigma, St. Louis, MO), 20 mg/kg GBR-12909 dihydrochloride (Sigma), 15 mg/kg nortriptyline hydrochloride (Sigma), or an equivalent volume of saline (0.9% NaCl). All procedures involving animal care were conducted in compliance with current laws and policies (Council directive 87848, Service Vétérinaire de la Santé et de la Protection animales). Animals were sacrificed 15 h after the last injection, and coronal brain sections were prepared as described previously (Cassel et al., 2004).

Bioinformatics. GenBank contains two identical rat *Mecp2_e2* coding DNA sequences (accession numbers M94064 and NM022673) and four associated expressed sequence tags (ESTs) that include neither the first exon encoding the N-terminal part of Mecp2_e1 nor the 5′-end of the second exon. In contrast, various mouse ESTs corresponding to the 5′-end of *Mecp2_e1* were isolated using the NCBI blast program. Alignment of rat genomic sequence (accession number AC134952) with these mouse ESTs was performed to define the sequence of the rat Mecp2_e1 transcript.

Real-Time Quantitative PCR Analysis. Total RNA was extracted from dissected brain structures and first strand cDNA was generated from 1 μg of total RNA and random primers using Omniscript Reverse Transcriptase (QIAGEN, Valencia, CA) in a total volume of 20 μl in a 1-h reaction at 37°C. The reaction product was used for quantitative real-time PCR assay with a Light Cycler instrument and technology (Roche Applied Science, Indianapolis, IN) with SybR Green I dye for detection and 0.3 μM concentrations of appropriate primers. Rat *Mecp2_e1* specific PCR was carried out using primer I (5′-GGAGAGACTGGAGGAAAAGTCA-3′) and J (5′-CCTTCTTAAACTTCAGGGGTTTC-3′) producing a PCR fragment of 74 bp. Rat *Mecp2_e2* PCR was carried out using primer E (5′-CTGTTTGGGAGAAGCAGAGG-3′) and primer F (5′-TGGTAGCTGGGATGTTAGGG-3′), producing a PCR fragment of 248 bp. *MBD1* gene expression was measured using 5′-CAACCTTCCTGACTTCTTCCA-3′ as a forward primer and 5′-GCCAATCCCTCCTATCTCTTC-3′ as a reverse primer, and *HDAC2* gene expression was measured using 5′-CCCTCAAACATGACAAACCA-3′ as a forward primer and 5′-TGTCAGGGTCTTCTCCATCC-3′ as a reverse primer. PCR comprised an initial denaturation step of 10 min at 95°C, followed by 45 cycles; one cycle consisted of 10 s at 95°C, 5 s at 65°C, and 10 s at 72°C. Clathrin was used as an internal control using 5′-AAGTATCCGTAAGTGGAG-3′ as a forward primer and 5′-GGGGTTAAAGTCACACAG-3′ as a reverse primer. Its amplification was performed in the same conditions as those used for Mecp2 transcripts except for an annealing temperature of 55°C.

Antibodies. The following rabbit polyclonal antibodies were used: anti-Mecp2 antibody (Upstate Biotechnology, Lake Placid, NY) prepared against amino acids 465 to 478 of mouse Mecp2_e2, diluted 1:200 in PBS; anti-MBD1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:200; anti-HDAC1 antibody (Upstate), diluted 1:500; anti-HDAC2 antibody (Upstate), diluted 1:400; and antibody specific for acetylated Lys-9 and Lys-14 on histone H3, diluted 1:250 (Upstate). Antibody binding was detected with secondary biotinylated horse anti-rabbit IgG. For confocal microscopy, mouse monoclonal anti-parvalbumin (Sigma), diluted 1:1000, was also used.

Immunohistochemistry. Immunohistochemistry was carried out as described previously (Cassel et al., 2004). The percentage of immunoreactive cells in each structure was calculated from counts on at least 800 cells by an investigator blinded to the identity of the samples. For each value, 6 to 10 counts were performed on two serial sections from three to five rats. For confocal microscopy, brain sections were incubated overnight at room temperature with rabbit anti-Mecp2 together with monoclonal anti-parvalbumin antibodies. After washing, double staining was performed by incubating slides with Alexa Fluor 488-labeled anti-mouse antibody diluted 1:200 (Invitrogen, Carlsbad, CA) and rhodamine-labeled anti-rabbit antibody diluted 1:200 (Chemicon International, Temecula, CA) for 1 h. Images of labeled cells were acquired using a confocal laser scanning microscope (LSM 410 invert; Carl Zeiss Inc., Thornwood, NY). Nonspecific fluorescence was assessed by incubating cells with secondary antibodies alone and measuring the average intensity for each fluorochrome. This value was then subtracted from individual images.

Results

Figure 1 illustrates Mecp2, MBD1, HDAC1, HDAC2, and acetyl-histone H3 immunohistochemistry in CPu of normal adult Wistar rats after long-term treatment (10 days, one i.p. injection/day) with saline, 10 mg/kg fluoxetine, or 20 mg/kg cocaine. A large majority of cells displayed Mecp2 and MBD1 immunoreactivity exclusively in the nucleus, in agreement

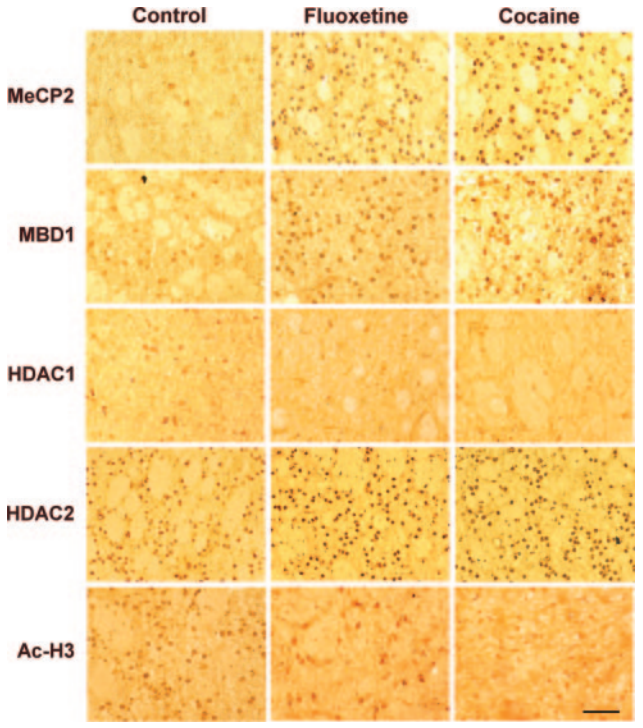

Fig. 1. Photomicrographs showing Mecp2, MBD1, HDAC1, HDAC2 and acetylated histone H3 immunoreactivity in dorsal CPu from rats treated continuously (10 days, one i.p. injection per day) with saline, 10 mg/kg fluoxetine, or 20 mg/kg cocaine. Animals were sacrificed 15 h after the last injection. Coronal tissue sections (15 μm) were incubated with polyclonal antibodies raised against Mecp2, MBD1, HDAC1, HDAC2, or acetylated forms of histone H3. Antibody binding was detected with secondary biotinylated antibody and peroxidase reaction (3,3′-diaminobenzidine tetrahydrochloride and H_2O_2). Scale bar (applicable to all micrographs), 100 μm.

with the proteins binding DNA. The number of immunoreactive cells for both Mecp2 and MBD1 dramatically increased after a series of 10 injections of both pharmacological agents. This was accompanied with substantial enhancement of the labeling intensity. In contrast, no increase in the number of immunoreactive cells for HDAC1 and HDAC2 could be observed in response to the agents. These two enzymes are responsible for the deacetylase activity associated with MeCP2, as shown by cross-immunoprecipitation studies (Nan et al., 1998). However, the intensity of HDAC2 labeling was enhanced in response to long-term fluoxetine or cocaine treatment. We also used an antibody directed against acetylated Lys-9 and Lys-14 of histone H3, two amino acid residues described as being deacetylated by the deacetylase activity associated with MeCP2 (Shahbazian et al., 2002). Using adjacent sections from the same rat brains, we found that the number of cells with detectable acetylated forms of histone H3 was greatly reduced by the repeated treatments, indicating that HDAC activity was probably increased. No difference could be observed in any of the protein expression examined after a single fluoxetine or cocaine injection (data not shown).

Figure 2 shows quantitative analysis of the percentage of Mecp2, MBD1, HDAC1, HDAC2, and acetylhistone H3 immunoreactive cells in the dorsal CPu, the frontal cortex, and the dentate gyrus (DG) subregion of hippocampus, three brain structures receiving significant serotonergic projections. In the CPu, approximately 25% of cells showed Mecp2 and MBD1 immunoreactivity in control rats; the percentage of Mecp2 immunoreactive cells increased dramatically to 85 and 67% in response to fluoxetine and cocaine, respectively, whereas that of MBD1-positive cells rose to 70 and 60% (Fig. 2A). In contrast, no significant change was observed in the number of HDAC-immunoreactive cells remaining at approximately 25% for HDAC1 and 60% for HDAC2, whereas the number of cells displaying acetylated histone H3 decreased from 57 to 28% in the case of fluoxetine treatment and to 25% in the case of cocaine treatment (Fig. 2B). Similar observations were made for these epigenetic regulating factors in two other serotonergic projection areas, the frontal cortex and the DG. Although the Mecp2 and MBD1 induction, as well as the reduction in the acetylated form of histone H3 were less pronounced in these structures, they remained statistically significant. Treatment was not found to induce the expression of the methyl-binding proteins in other hippocampal subregions such as CA1 or CA3 (data not shown).

The data suggest that the serotonergic system was primarily involved because fluoxetine is a selective serotonin (5-HT) uptake inhibitor, whereas cocaine inhibits the 5-HT transporter together with the dopamine (DA) and the noradrena-

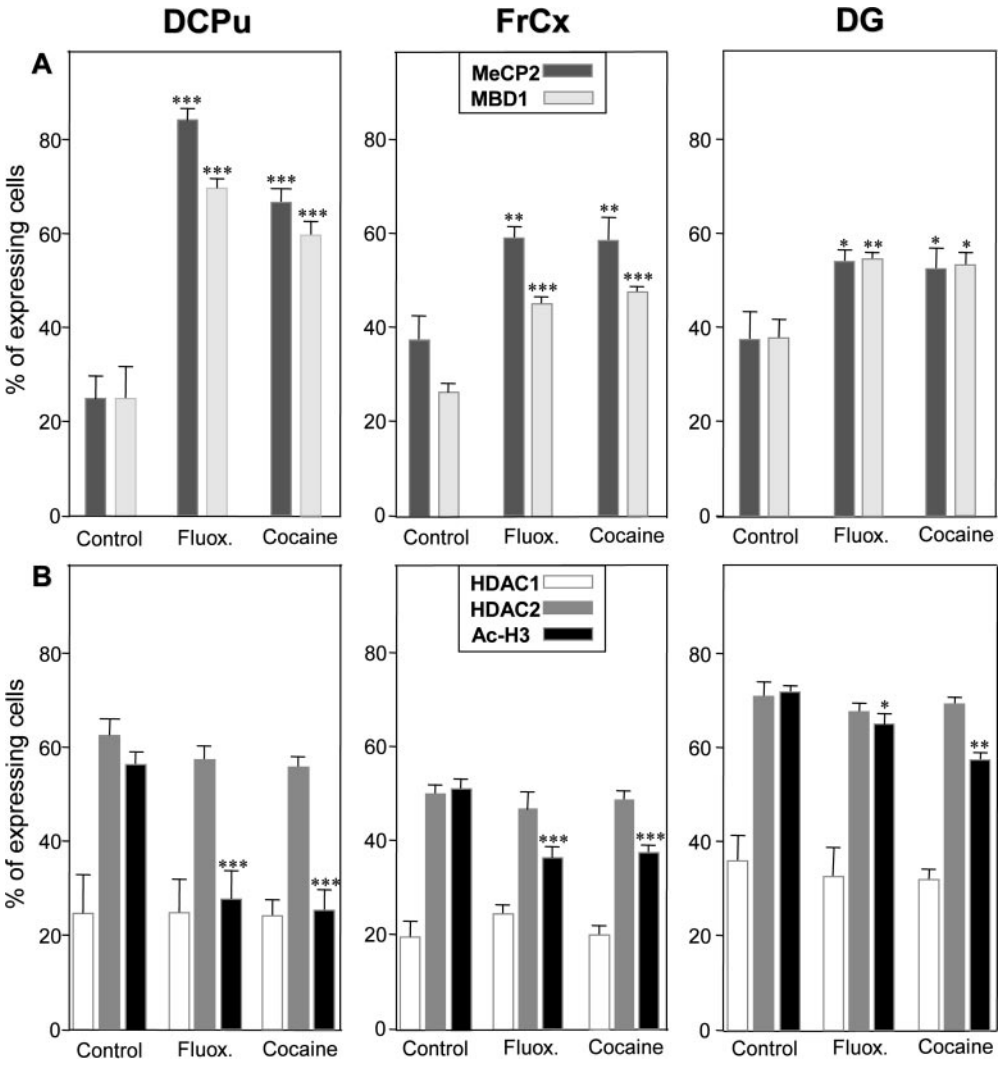

Fig. 2. Quantification of Mecp2 and MBD1 (A), HDAC1, HDAC2, and acetylated forms of histone H3 (B) in dorsal caudate-putamen (DCPu), frontal cortex (FrCx), and dentate gyrus (DG) of hippocampus of rats treated repeatedly with fluoxetine or cocaine. Treatment of rats and immunohistochemistry were carried out as described under *Materials and Methods* and in the legend to Fig. 1. Nuclei were stained with 5 μM bisbenzimide (Hoechst 33258). For each antigen, the percentage of immunoreactive cells was calculated from 6 to 10 counts on two serial sections from three to five rats (at least 800 cells), and expressed as means ± S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t test).

line (NA) transporter (Ritz et al., 1990). To further confirm this, we investigated whether GBR-12909 or nortriptyline (selective inhibitors of the DA and NA transporters, respectively) was able to induce the methyl-binding protein expression. Figure 3 shows quantitative analysis of the percentage of Mecp2 immunoreactive cells observed after treatment with these compounds in the same brain structures as those used to characterize the fluoxetine and cocaine effect. Repeated treatment for 10 days with both reuptake inhibitors was not found to significantly modify Mecp2 expression in the structures examined. Taken together, our data indicate that regulation of methyl-binding protein expression and HDAC activity results from an increase in extracellular 5-HT concentration.

In the DG, we considered cells from the granule cell layer as well as cells from the hilus, where numerous cells displayed enhanced Mecp2 expression in response to fluoxetine (or cocaine). We characterized those cells using double labeling for Mecp2 and parvalbumin, the latter being considered a marker of GABAergic interneurons and, to a lesser extent, of long-axon cells (Celio, 1990). A significant number of GABAergic neurons were also cells in which Mecp2 was induced by fluoxetine (Fig. 4). The hilar cells could be distinguished from cells in the granule layer, which do not contain parvalbumin (Fig. 4, bottom of third row).

To find out whether the increase of methyl-binding proteins resulted from an increase in the amount of mRNA, we measured mRNA expression of both *Mecp2_e1* and *Mecp2_e2* isoforms, as well as that of *MBD1*, by real-time quantitative PCR. Concerning *Mecp2*, position of primers relative to each transcript is illustrated in Fig. 5A. In addition, the N-terminal amino acid sequence of Mecp2_e1 deduced from the rat first exon was found to be highly homologous to that of the human and the mouse protein but revealed an unusual 24 alanine-long expansion encoded by GCC trinucleotide repeats (Fig. 5B). In agreement with what we found for the protein expression, no significant difference in Mecp2 or MBD1 mRNA synthesis could be observed upon a single fluoxetine injection to rats (Table 1). In contrast, the expression of the three transcripts was enhanced in response to repeated fluoxetine administration. Again, the effect was highest in the striatum in which Mecp2_e1, Mecp2_e2, and MBD1 mRNAs were increased by 98, 62, and 76%, respectively. Smaller although statistically significant increases in the amount of the Mecp2 mRNAs were found in frontal cortex and dorsal hippocampus, whereas MBD1 induction in the frontal cortex did not reach statistical significance. Using the same technique, we also found an increase in HDAC2 gene transcription in response to repeated fluoxetine administration; the most pronounced effect took place in the striatum (Table 1), thus confirming the increase in HDAC2 labeling intensity we observed immunohistochemically.

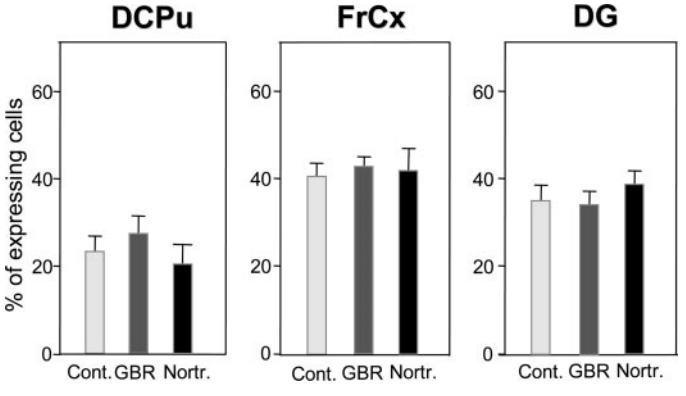

Fig. 3. Quantification of Mecp2 expression in dorsal caudate-putamen (DCPu), frontal cortex (FrCx), and dentate gyrus (DG) of hippocampus of rats treated repeatedly with saline (Cont.), 20 mg/kg GBR-12909, or 15 mg/kg nortriptyline (Nortr.). Treatment of rats and immunohistochemistry were carried out as described under *Materials and Methods* and in the legend to Fig. 2.

Discussion

We report here that expression of the genes encoding the methyl-CpG-binding proteins *Mecp2* and *MBD1* can be stimulated in the adult rat brain by continuously activating the 5-HT neurotransmitter system. The effect could not be repro-

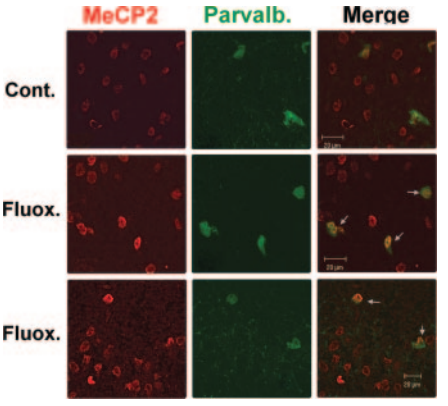

Fig. 4. Double labeling for Mecp2 and parvalbumin by immunofluorescence confocal microscopy in the hilar region of DG. Hippocampal sections were prepared from rats treated for 10 days with saline (Cont.) or fluoxetine (Fluox.). They were incubated with antibodies against Mecp2 and parvalbumin. Secondary labeled antibodies against Mecp2 (Rhodamine, red) and parvalbumin (Alexa Fluor 488, green) were used. Parvalbumin-immunoreactive cells overexpressing Mecp2 in response to fluoxetine in the hippocampus are indicated by white arrows. Scales bars, 20 μm.

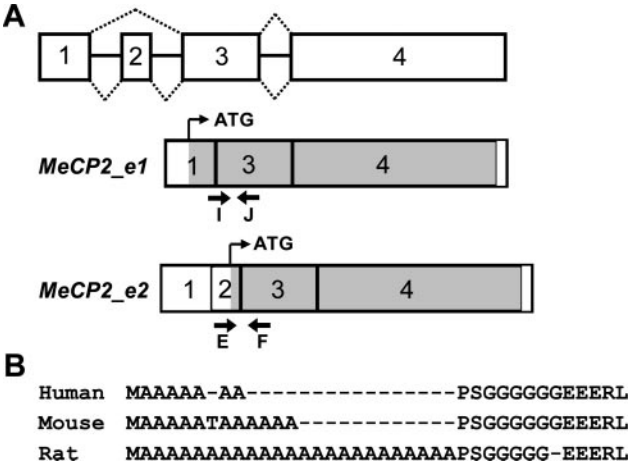

Fig. 5. A, structure of the rat *Mecp2* gene and of the two transcripts generated by alternative splicing. *Mecp2_e1* transcript is generated by splicing exon 1 directly to exon 3, skipping exon 2, whereas *Mecp2_e2* transcript includes all four gene exons. The relative positions of the primers used for RT-PCR experiments are indicated below each transcript. Open boxes represent untranslated regions whereas open reading frames are illustrated by gray boxes. B, N-terminal protein sequence encoded by exon 1 in Mecp2_e1. Human, mouse, and rat sequences are highly homologous but differ in the length of a conserved polyalanine tract.

duced by selective inhibitors of the transporters for DA or NA. The study was conducted at the level of the proteins as well as at the level of the messengers for which we found an increase in MBD1, Mecp2_e1, and Mecp2_e2 mRNAs. The anti-Mecp2 antibody we used does not discriminate between the two Mecp2 isoforms because it is directed against a C-terminal fragment of the protein. The similar induction pattern of Mecp2 and MBD1 proteins observed in response to fluoxetine and cocaine suggests that they share some transcriptional regulatory elements. MBD1 resembles MeCP2 because both are chromosome-bound proteins that repress transcription in a HDAC-dependent manner. In the brain, both methyl-binding proteins are expressed in neurons but not in differentiated glial cells (Akbarian et al., 2001; Zhao et al., 2003). We found a much higher HDAC2 expression in the various brain areas examined compared with HDAC1 expression, suggesting that the former deacetylase enzyme plays a major role in neuronal gene silencing. Moreover, intensity of HDAC2 labeling was found to be enhanced upon repeated treatment by fluoxetine or cocaine. Increase in labeling intensity was corroborated with an enhanced synthesis of HDAC2 mRNA in response to fluoxetine. This observation is further supported by a cDNA microarray analysis in which we found *HDAC2* among the genes modulated by repeatedly administered cocaine, according to the same protocol and dosage as those used in the present study (data not shown).

Fluoxetine was the first molecule of a new generation of antidepressants, the selective serotonin-reuptake inhibitors, and is effective in the treatment of depression from the first week of therapy (Rossi et al., 2004). Our findings, observed after a 10-day period of fluoxetine treatment but not in response to a single injection, therefore suggest that epigenetic regulation may be involved in its mode of action. In effect, genes the transcription of which is repressed by the epigenetic mechanism we describe may contribute to the antidepressant properties of fluoxetine.

5-HT neurons projecting from raphé nuclei ascend to the dopaminergic neuron projection fields in the forebrain, explaining the important effect we observe on methyl-CpG-binding protein synthesis in the striatum in response to enhanced 5-HT neurotransmission. Because 90% of the striatal neuronal population are medium spiny neurons that use GABA as their neurotransmitter (Kita and Kitai, 1988), most of the neurons responding to fluoxetine in this structure by increasing Mecp2 levels are indeed GABAergic neurons. Important 5-HT projections are also taking place in the hippocampus (Parent et al., 1981). In the DG, the raphé serotonergic projection terminates most heavily in the polymorphic layer, on a class of GABA interneurons that influence the firing of dentate granule cells (Halasy et al., 1992). It is noteworthy that we found numerous cells in the hilus of the DG expressing high levels of Mecp2 in response to fluoxetine treatment, a number of them being characterized as GABAergic neurons. This observation suggests that Mecp2 plays an important role for the proper functioning of GABAergic interneurons of the DG. It provides a possible explanation for the occurrence of epileptic seizures commonly observed in RTT (Naidu, 1997) because loss of hilar GABAergic interneurons is the most consistent deficit in patients and in experimental models of temporal lobe epilepsy (Buckmaster et al., 2002).

TABLE 1

Mecp2, MBD1, and HDAC2 mRNA expression measured by real-time quantitative PCR analysis

Values are means ± S.D. (n = 4 to 6) of mRNA expressed in the indicated brain structures of rats treated short- or long-term with saline or fluoxetine. Data represent the amount of each transcript, which was normalized to that of clathrin mRNA in each sample. Values are expressed as percentage of mRNA content in saline-treated animals.

	Short-Term Treatment		Repeated Treatment	
	Control	Fluoxetine	Control	Fluoxetine
Mecp2_e1				
Striatum	100 ± 10	93 ± 16	100 ± 29	198 ± 49**
Fr. cortex	100 ± 19	113 ± 13	100 ± 35	156 ± 21*
Hip	100 ± 17	108 ± 19	100 ± 42	160 ± 17*
Mecp2_e2				
Striatum	100 ± 16	99 ± 22	100 ± 30	162 ± 47*
Fr. cortex	100 ± 20	95 ± 32	100 ± 28	149 ± 39*
Hip	100 ± 20	118 ± 26	100 ± 18	129 ± 20*
MBD1				
Striatum	100 ± 21	100 ± 14	100 ± 18	176 ± 40*
Fr. cortex	100 ± 16	85 ± 15	100 ± 22	126 ± 19
Hip	100 ± 10	83 ± 17	100 ± 7	160 ± 32*
HDAC2				
Striatum	100 ± 34	87 ± 15	100 ± 26	167 ± 33**
Fr. cortex	100 ± 10	97 ± 14	100 ± 12	114 ± 18
Hip	100 ± 11	94 ± 10	100 ± 25	149 ± 30*

* $P < 0.05$.
** $P < 0.01$ (Student's t test).

The epigenetic regulation of gene expression seems to be crucial to the functional integrity of MeCP2-expressing GABAergic neurons. This is further supported by data from the literature, including those reported during the quest for target genes silenced by MeCP2. The maternally expressed *Dlx5* gene showed a loss of imprinting in cells from persons with RTT (Horike et al., 2005). Because Dlx5 regulates GABA synthesis, loss of imprinting of Dlx5 may therefore alter GABAergic neuronal activity in RTT. In addition, the *GABRB3* gene, encoding the β3 subunit of $GABA_A$ receptor, showed reduced expression in multiple RTT and autism brain samples, as well as in *Mecp2*-deficient mice (Samaco et al., 2005).

Accumulation of the methyl-binding proteins and a reduction in histone H3 acetylation suggest that 5-HT signaling is able to remodel chromatin structure, thereby controlling the accessibility of sequence-specific transcription factors to individual genes. Chromatin remodeling in response to cocaine has been shown to constitute an important regulatory mechanism underlying neural plasticity (Kumar et al., 2005; Levine et al., 2005). Hyperacetylation of histone H3 was observed with long-term cocaine treatment at the *brain-derived neurotrophic factor* and *Cdk5* promoters, genes that are induced by long-term cocaine exposure (Kumar et al., 2005). This is in contrast with the fact that MeCP2 has been shown to selectively bind to the *BDNF* promoter and repress expression of the *BDNF* gene (Chen et al., 2003). The fact that several histone modifications can take place in concert at the same histone tail, corresponding to various states of remodeled chromatin (Cheung et al., 2000), may help to explain the apparent discrepancy. Our data highlight the key role of methyl-CpG-binding proteins in this complex regulatory mechanism. Because the mechanism takes place in postmitotic neurons, they also indicate that these neurons are able to reinterpret the DNA methylation code they have acquired during early development.

Acknowledgments

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