

# The loss of methyl-CpG binding protein 1 leads to autism-like behavioral deficits

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Methyl-CpG binding proteins (MBDs) are central components of DNA methylation-mediated epigenetic gene regulation. Alterations of epigenetic pathways are known to be associated with several neurodevelopmental disorders, particularly autism. Our previous studies showed that the loss of *Mbd1* led to reduced hippocampal neurogenesis and impaired learning in mice. However, whether MBD1 regulates the autism-related cognitive functions remains unknown. Here we show that *Mbd1* mutant (*Mbd1*<sup>-/-</sup>) mice exhibit several core deficits frequently associated with autism, including reduced social interaction, learning deficits, anxiety, defective sensory motor gating, depression and abnormal brain serotonin activity. Furthermore, we find that *Mbd1* can directly regulate the expression of *Htr2c*, one of the serotonin receptors, by binding to its promoter, and the loss of *Mbd1* led to elevated expression of *Htr2c*. Our results, therefore, demonstrate the importance of epigenetic regulation in mammalian brain development and cognitive functions. Understanding how the loss of *Mbd1* could lead to autism-like behavioral phenotypes would reveal much-needed information about the molecular pathogenesis of autism.

## INTRODUCTION

Autism and autism spectrum disorders (ASDs) affect approximately 1 in 166 children. These disorders are generally characterized by deficits in social interaction and unusual stereotyped behaviors (1). Individuals with autism and ASDs often suffer multiple mental and emotional problems ranging from obsessive-compulsive and anxiety disorders to learning deficits (1). These chronic coexisting symptoms start early in life and present immense challenges to clinicians and families (1). The cause of autism and its associated symptoms remains unclear, although it is likely that the complex etiology of autism arises from an interplay of genetic predispositions and environmental factors (2). To date, mounting evidence has linked the serotonin system to autism (3); however, the molecular mechanisms that regulate the serotonin system are not fully understood.

Epigenetic modulations, including DNA methylation, small RNA-associated silencing, and histone modifications, could serve as an intermediate process that imprints dynamic environmental experiences on the 'fixed' genome, resulting in stable alterations in phenotypes (4,5). The importance of epigenetic regulation in human diseases has been realized only recently. Disturbance of the epigenetic system can result in an array of multisystem disorders; for instance, epigenetic changes could play a major role in the development of human cancer (6). Mutations in genes that affect global epigenetic profiles can give rise to human diseases, which can be inherited or somatically acquired. For example, mutations in *ATRX* cause hypomethylation of certain repeats and satellite sequences, which lead to mental retardation (7,8). Mutations in *DNMT3B*, a *de novo* methyltransferase, cause ICF syndrome (immunodeficiency, centromeric region instability and facial anomalies syndrome) (9,10). The gross chromosomal

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anomalies observed with these diseases suggest an important role for epigenetics in chromosome architecture. Rett syndrome, the most common form of mental retardation in young girls, is due to germline mutation of *MECP2* (11–14). In addition to Rett Syndrome, altered MeCP2 expression is prevalent in autism and several other neurodevelopmental disorders (15). Also, several inherited syndromes are due to faulty genomic imprinting, including Angelman's syndrome, Prader–Willi syndrome and Beckwith–Wiedemann syndrome (16,17). One striking similarity among these different disorders is that the brain is always affected. Indeed, mice with a conditional DNMT1 knockout in the central nervous system display abnormalities in the brain and have defects in learning and memory (18). Together, these observations strongly imply that epigenetic modulation plays a critical role in the development and normal function of the brain. Altered promoter DNA methylation of serotonin transporter, a gene linked to the incidence of autism, could significantly change its expression level (19,20), suggesting that DNA methylation may play an important modulatory role in the brain's serotonin system. Given that autism is a complex disorder regulated by both genetic and environmental components, DNA methylation could serve as a link between environmental exposure and genetic predisposition.

Methyl-CpG binding proteins (MBDs), including MBD1 and MeCP2, are central components of DNA methylation-mediated epigenetic gene regulation. Extensive *in vitro* analyses have demonstrated a clear role for MBD1 in transcriptional repression, chromatin assembly and heterochromatin structure maintenance (21–24). Mbd1 is highly expressed in both neural progenitors and neurons (25). However, the biological function of MBD1 in the brain is not well established. By generating and analyzing *Mbd1*<sup>-/-</sup> mice, we have demonstrated a novel and clear role for Mbd1 and DNA methylation in adult neurogenesis, synaptic plasticity and learning (25). Recently, a mutation was found in the *MBD1* gene of one autistic patient and his direct relatives (26), suggesting that MBD1 may be important in the modulation of complex brain functions related to autistic phenotypes.

Here we have performed extensive behavioral analyses to assess the roles of Mbd1 in cognitive functions. We found that *Mbd1*<sup>-/-</sup> mice exhibited reduced social interaction, impaired sensorimotor gating, anxiety, depression, learning deficits and abnormal serotonin activity. Furthermore, we found that Mbd1 could directly regulate the expression of one of the serotonin receptors, Htr2c, by binding to its promoter, and the loss of Mbd1 leads to elevated expression of Htr2c. Our results demonstrate the importance of epigenetic regulation in mammalian brain development and cognitive functions.

## RESULTS

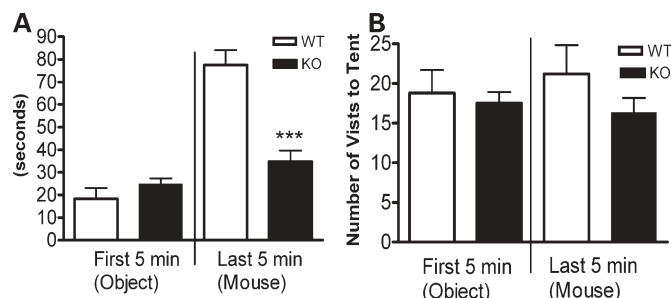
### *Mbd1*<sup>-/-</sup> mice exhibit reduced interest in social interaction

*Mbd1*<sup>-/-</sup> mice exhibit no gross abnormality and have nearly normal lifespan, which allow for detailed behavioral assessment in adult animals. Reduced social interaction is a hallmark of autism (27,28). We, therefore, tested the social interaction of *Mbd1*<sup>-/-</sup> mice using a paradigm modified from Crawley

*et al.* (29). To distinguish between social interaction and exploratory behaviors, we first tested how the mouse responded to a novel toy placed inside a tent the animal could see and smell through, and then we replaced the novel toy with an unfamiliar 'stranger' mouse. Mice with a normal level of social interest would prefer to have more direct contact with a stranger mouse than with a toy (29). As shown in Figure 1, there was a significant difference between *Mbd1*<sup>-/-</sup> (KO) and wild-type (WT) mice in the length of time they spent in direct contact with the tent when it housed a stranger mouse [Fig. 1A, Last 5 min,  $F(1,20) = 26.9$ ,  $P < 0.0001$ ]. Therefore, whereas wild-type mice spent significantly more time in direct contact with the tent when it housed an unfamiliar mouse compared with when it contained a toy, *Mbd1*<sup>-/-</sup> mice did not show more interest in a stranger mouse than a novel toy. Both *Mbd1*<sup>-/-</sup> and wild-type mice exhibited similar levels of exploratory behaviors in response to a novel toy (Fig. 1A, First 5 min). In addition, both *Mbd1*<sup>-/-</sup> and wild-type mice displayed no difference in the frequency of visits to the tent, whether it contained a novel toy (first 5 min) or an unfamiliar mouse (last 5 min) (Fig. 1B). We found neither significant gender differences nor any interaction between gender and genotype. The social interaction deficit of *Mbd1*<sup>-/-</sup> mice was not due to abnormal olfactory function because that *Mbd1*<sup>-/-</sup> exhibited no difference in retrieving the hidden food pellet in a 'buried food test' compared to wild-type mice (KO, 21.2 s  $\pm$  2.5 s; WT 19.9 s  $\pm$  2.0 s;  $P > 0.1$ ,  $n = 4$ ). Overall, these data indicate that *Mbd1*<sup>-/-</sup> mice have normal motor activity as shown previously (25), as well as normal exploratory behavior, but they have significantly reduced interest in social interaction.

### *Mbd1*<sup>-/-</sup> mice have impaired sensorimotor gating

In addition to reduced social interaction, one of the most common clinical features of autism is heightened sensitivity to sensory stimulation, which could be due to reduced sensorimotor gating (30,31). Prepulse inhibition (PPI) is a normal sensorimotor gating process whereby a relatively mild prepulse stimulus, when presented just before the startle stimulus, acts to suppress the response to a strong startle stimulus. A reduction in PPI and deficits in sensorimotor gating have been reported in several cognitive disorders, including schizophrenia, post-traumatic stress disorder, obsessive-compulsive disorder, fragile X syndrome, Asperger's syndrome and autism (30–32). Therefore, we tested PPI in *Mbd1*<sup>-/-</sup> mice between the prepulse stimulus (auditory tone) and the startle stimulus (air puff) using two different interstimulus intervals: 120 and 300 ms. The data were calculated as percent PPI, such that a high percent PPI value reflects strong PPI and hence better sensorimotor gating. As shown in Figure 2, *Mbd1*<sup>-/-</sup> (KO) mice exhibited significantly reduced percent PPI compared with wild-type mice across a range of prepulse intensities at both the 120 ms interval [Fig. 2A, one way ANOVA  $F(1,10) = 196.7$ ,  $P < 0.001$ ] and the 300 ms interval [Fig. 2B,  $F(1,10) = 98.6$ ,  $P < 0.001$ ]. The PPI deficit in *Mbd1*<sup>-/-</sup> mice was not due to deficits in either sensory input or hearing, because the baseline tactile startle responses to either the tone or air puff were no different for wild-type

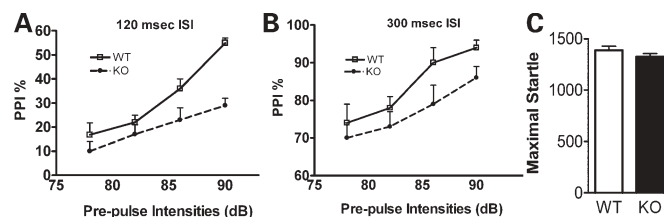


**Figure 1.** *Mbd1*<sup>-/-</sup> mice have impaired social interaction. (A) Both *Mbd1*<sup>-/-</sup> (KO) and wild-type (WT) mice spent similar amounts of time interacting with the tent during the first 5 min. During the last 5 min, when a stranger mouse was in the tent (right panel), WT mice spent significantly more time in contact with the tent, demonstrating a normal level of socialization. KO mice, however, failed to display this interest in social interaction. (B) KO and WT mice exhibited no differences in the number (frequency) of visits to the tent, whether there was a novel object (first 5 min) or a novel mouse (last 5 min) inside the tent. Data are presented as mean ± SEM, *n* = 12; \*\*\**P* < 0.0001.

and *Mbd1*<sup>-/-</sup> mice (Fig. 2C). In addition, stronger prepulse auditory stimuli did lead to greater PPI in *Mbd1*<sup>-/-</sup> mice (Fig. 2A and B), indicating that sensorimotor gating was still present albeit significantly reduced in *Mbd1*<sup>-/-</sup> mice. We found neither significant gender differences nor any interaction between gender and genotype. Therefore, these data indicate that *Mbd1*<sup>-/-</sup> mice have a normal startle response to both auditory and tactile stimuli, but their sensorimotor gating is significantly reduced.

### *Mbd1*<sup>-/-</sup> mice exhibit learning deficits

Although autistic patients exhibit tremendous variation in learning deficits, ranging from severe mental retardation to only mild difficulties in certain areas, learning is affected to some degree in most ASD patients (33). We have previously demonstrated that *Mbd1* deficiency in mice on a 129S4 genetic background leads to reduced adult hippocampal neurogenesis and hippocampus-dependent learning, as shown by the Morris water maze spatial learning tests (25). Here we used two different well-established learning tests, the cued fear conditioning and contextual fear conditioning tests, to further assess the learning ability of *Mbd1*<sup>-/-</sup> mice bred into the C57BL/6 genetic background. These tests make use of mild but unpleasant foot shocks to train mice to associate an auditory tone or a spatial context with the foot shocks; the trained mice are then tested 24 h later using either an auditory tone (cued test) or spatial context (context test), and their learning and memory are assessed by how soon and how long they freeze in fear upon testing. The cued fear conditioning test assesses amygdala-dependent learning ability, whereas the contextual fear conditioning test assesses both hippocampus- and amygdala-dependent learning ability (29,34). We found that *Mbd1*<sup>-/-</sup> mice exhibited deficits in both the cued fear conditioning test (Fig. 3A, Tone test, *P* < 0.05) and the contextual fear conditioning test (Fig. 3B, Context test, *P* < 0.05). Since *Mbd1*<sup>-/-</sup> mice do not have deficits in pain sensitivity for foot shocks (data not shown), these data have confirmed our previous finding (25) and provide evidence that



**Figure 2.** *Mbd1*<sup>-/-</sup> mice have impaired sensorimotor gating as assessed by prepulse inhibition (PPI). (A and B) *Mbd1*<sup>-/-</sup> (KO) mice have reduced PPI compared with WT littermate controls at both 120 ms ISI (A) and 300 ms ISI (B) across a range of prepulse intensities. (C) The tactile startle response was no different for WT and KO mice. Data are shown as mean percent startle at different prepulse amplitudes ± SEM (*n* = 7).

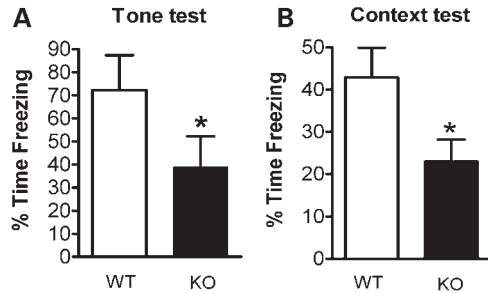
*Mbd1*<sup>-/-</sup> mice have both hippocampus- and amygdala-dependent learning deficits that are present in both 129S4 and C57BL/6 genetic backgrounds.

### *Mbd1*<sup>-/-</sup> mice displayed increased anxiety

Anxiety is frequently comorbid with autism (33). To determine whether *Mbd1*<sup>-/-</sup> mice have anxiety, we assessed their performance in both the light–dark preference test and the elevated plus-maze test (29,35,36). In the light–dark preference test, we found that *Mbd1*<sup>-/-</sup> mice spent significantly less time in a well-lit room than WT mice, suggesting increased anxiety [Fig. 4A, *F*(1,20) = 16.5, *P* < 0.001]. In the elevated plus-maze, we found that *Mbd1*<sup>-/-</sup> mice spent significantly less time in open arms [Fig. 4B and *F*(1,14) = 45.4, *P* < 0.0001] and more time in closed arms [*F*(1,14) = 21.3, *P* < 0.0001], and they exhibited fewer entries into the open arms [Fig. 4C, *F*(1,14) = 24.1, *P* < 0.0001] compared with their wild-type littermates, also suggestive of greater anxiety. The total number of transitions between arms was no different for wild-type and *Mbd1*<sup>-/-</sup> mice (data not shown, *P* = 0.35), indicating that general locomotion was the same between genotypes. We found neither significant gender differences nor any interaction between gender and genotype for either of these tests. The data from these two independent tests indicate that *Mbd1*<sup>-/-</sup> mice have normal levels of motor activity, consistent with our previous findings (25), but exhibit increased levels of anxiety.

### *Mbd1*<sup>-/-</sup> mice exhibited increased susceptibility to depression

Since anxiety and depression are often linked, we next investigated whether *Mbd1*<sup>-/-</sup> mice had increased susceptibility to depression using two well-established behavioral tests, the learned helplessness test and the forced swim test (see Materials and Methods for details). In the learned helplessness test, we used a mild foot shock paradigm, which we knew from our own experience would produce minimal depression in the wild-type C57BL/6 strain of mice (36), allowing for the detection of potential differences in depression between the wild-type and *Mbd1*<sup>-/-</sup> mice. We found that *Mbd1*<sup>-/-</sup> (KO) mice displayed significantly longer latency in escaping the shocks during the test trials [Fig. 5A, *F*(1,64) = 81.5, *P* < 0.0001] compared with their wild-type littermates.



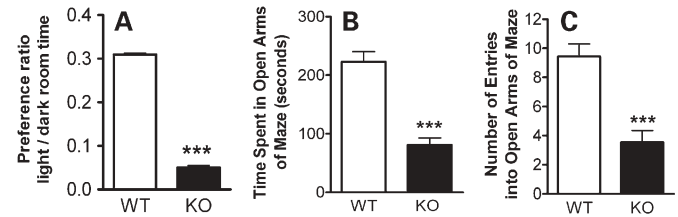
**Figure 3.** *Mbd1*<sup>-/-</sup> mice exhibited deficits in fear-conditioning learning tests. (A) *Mbd1*<sup>-/-</sup> (KO) mice had deficits in the cued fear conditioning test, which assessed amygdala-mediated learning; (B) KO mice had deficits in the contextual fear conditioning test, which assessed both hippocampus- and amygdala-mediated learning. \* $P < 0.05$ ,  $n = 6$ ,  $t$ -test.

In addition, *Mbd1*<sup>-/-</sup> mice had significantly more failed escapes among all test trials [Fig. 5B,  $F(1,14) = 13.01$ ,  $P < 0.003$ ]. Both results indicate that *Mbd1*<sup>-/-</sup> mice have increased depression. To further validate our finding, we performed the forced swim test (37,38). We found that *Mbd1*<sup>-/-</sup> mice displayed fewer escape-directed behaviors [ $F(1,14) = 135.5$ ,  $P < 0.0001$ ] and more non-escape-directed behaviors [ $F(1,14) = 86.7$ ,  $P < 0.0001$ ] (Fig. 5C) compared with WT mice, suggesting increased depression. We found neither significant gender differences nor any interaction between gender and genotype for either of these tests. Since these mice did not have deficits in swimming ability or locomotion (Figs 1–4) (25), these data indicate that *Mbd1*<sup>-/-</sup> mice have an increased susceptibility to depression.

### Mbd1 directly regulates the expression of a serotonin receptor

During behavioral experiments, we observed that both male and female *Mbd1*<sup>-/-</sup> mice exhibited increased ‘wet-dog shake’ behaviors [Fig. 6A,  $F(1,21) = 16.9$ ,  $P < 0.0001$ ]. Wet-dog shakes have been suggested as an indicator of increased serotonin activity through 5-HT<sub>2A</sub>, 2C or 1A receptors in the brain (40,41).

We therefore analyzed the expression levels of several serotonin receptors in the hippocampus and the medial frontal cortex (MFC), two regions associated with anxiety, depression and autistic phenotypes. We found that the mRNA levels of *Htr2c* were significantly higher in *Mbd1*<sup>-/-</sup> MFC (KO) compared with wild-type (WT) littermates (Fig. 6B, WT,  $1.30 \pm 0.11$ , KO,  $2.29 \pm 0.25$ ,  $n = 6$ ,  $P = 0.0035$ ,  $t$ -test). Htr2c is a glycosylated protein with 459 amino acids and a predicted peptide molecular weight of 51.9 kDa. Using Western blot, we detected multiple Htr2c antibody immunoreactive bands (Fig. 6C) that were likely due to glycosylation of this receptor (42). Quantification of either the band corresponding to the predicted 51 kDa or the summation of all the Htr2c antibody immuno-positive bands (45, 51 and 65 kDa) indicated that the expression level of Htr2c protein was higher in *Mbd1*<sup>-/-</sup> compared with wild-type MFC (data not shown). To confirm the protein expression data, we performed a highly specific agonist-mediated serotonin Htr2c receptor binding assay. We found that there was an increase in the

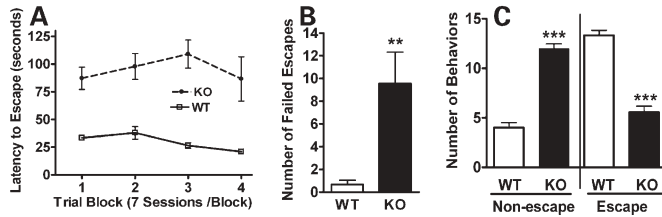


**Figure 4.** *Mbd1*<sup>-/-</sup> mice exhibited increased anxiety. (A) In a light–dark preference test, *Mbd1*<sup>-/-</sup> (KO) mice spent significantly less time in the light room than their wild-type (WT) littermates. (B and C) In an elevated plus-maze test, KO mice spent significantly less time in open arms (B) and had significantly fewer entries into open arms (C) compared with WT littermate controls, while the total numbers of transitions between arms were no different (data not shown). Data presented are mean  $\pm$  SEM,  $n = 9$ –12, \*\*\* indicates significance,  $P < 0.001$ .

binding of serotonin specifically to Htr2c receptor in the *Mbd1*<sup>-/-</sup> brains [ $K_{Dt}(10) 4.45$ ,  $P < 0.002$ ] compared with wild-type brains (Fig. 6D), indicating increased Htr2c protein levels in the mutant brains. Nonetheless, the affinity of Htr2c for serotonin was lower in *Mbd1*<sup>-/-</sup> brains compared with wild-type brains ( $B_{MAX}t(10) 2.9$ ,  $P < 0.019$ ), suggesting that Htr2c receptor, though overexpressed, does not function properly in *Mbd1*<sup>-/-</sup> brains (Fig. 6E). We found no difference between *Mbd1*<sup>-/-</sup> and wild-type mice in the mRNA levels of other serotonin receptors (Htr1a, 2a, 2a, 4) or glucocorticoid receptor in either the hippocampus or MFC. To investigate whether the observed increase in Mbd1 mRNA expression might be due to a lack of an Mbd1-mediated negative regulatory affect in *Mbd1*<sup>-/-</sup> brains, we performed Mbd1-specific chromatin immunoprecipitation and assayed genomic sequences proximal to the *5Htr2c* transcription start site by PCR. Indeed, we found that Mbd1 bound to both the 5'-untranslated region (5'-UTR) and intron1 of the *Htr2c* gene (Fig. 6F). The amount of binding of Mbd1 to *Htr2c* promoter was similar in both male and female wild-type brains but was absent in *Mbd1*<sup>-/-</sup> brains, as expected (Fig. 6G). Furthermore, to confirm that Mbd1 protein was expressed in MFC, we took the advantage of the fact that *Mbd1* heterozygote and mutant mice express  $\beta$ -galactosidase ( $\beta$ -gal) under the endogenous *Mbd1* gene promoter (25). Using an anti- $\beta$ -gal antibody and immunohistochemistry, we confirmed that Mbd1 protein was expressed in the MFC of mouse brains (Fig. 6H and I). In addition, we found no alteration in the level of serotonin in either the forebrains or the blood of *Mbd1*<sup>-/-</sup> mice (data not shown), suggesting that Mbd1 does not regulate serotonin levels. Therefore, these data suggest that Mbd1 directly regulates the expression of Htr2c and that dysregulation of Htr2c in the absence of functional Mbd1 may lead to some of the behavioral abnormalities seen in these mice.

## DISCUSSION

Our data clearly indicate that the epigenetic regulator Mbd1 plays a critical role in the regulation of several autism-related behaviors, including social interaction, anxiety, depression and sensorimotor gating. Since these complex behaviors are regulated by a network of different pathways in mammalian brains, Mbd1 could transcriptionally modulate the key components in



**Figure 5.** *Mbd1*<sup>-/-</sup> mice exhibited enhanced susceptibility to depressive behaviors. (A and B) In a learned helplessness test of depression, *Mbd1*<sup>-/-</sup> (KO) mice exhibited significantly longer latency for escape across all four trial blocks (A) and a significantly greater number of failed escapes (B) than their wild-type (WT) littermates. In a forced swim test (C), KO mice exhibited significantly more non-escape-directed behaviors (floating, twitching and paddling) and significantly fewer escape-directed behaviors (swimming, climbing and thrashing) than WT littermates. Data presented are mean  $\pm$  SEM,  $n = 9$ ; \*\*\* $P < 0.0001$ ; \*\* $P < 0.01$ .

multiple pathways. Based on our behavioral observations and the established link between serotonin and autism, we chose to focus on the brain serotonin system and found evidence that Mbd1 is a key regulator for serotonin 2c receptor.

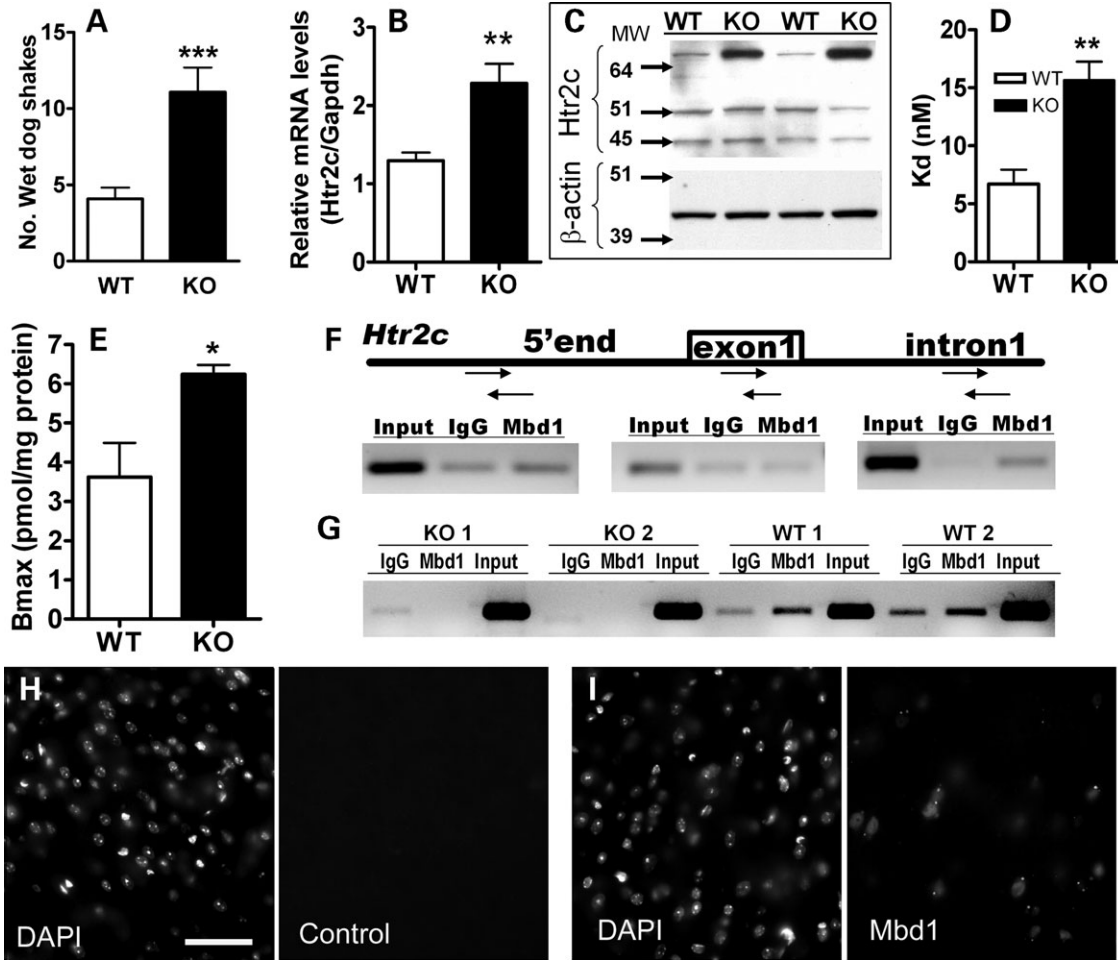
The discovery that MECP2 mutations lead to Rett syndrome and that MECP2 dysregulation is prevalent in autism and other neurodevelopmental disorders revealed the importance of epigenetic regulation in neurodevelopment (11,15). MBD1 and MeCP2 belong to the same family of proteins that interpret CpG methylation status in the genome (43). Although extensive *in vitro* biochemical analyses have demonstrated similar functions for these two proteins, the *in vivo* phenotypes of their respective mouse mutants are distinct. Whereas *Mecp2* null mice exhibit severe neurological phenotypes and motor deficits before reaching adulthood and die before 10 weeks of age, *Mbd1*<sup>-/-</sup> mice are grossly normal, with no motor deficits, and they live nearly normal life spans (11,44,45). The mild phenotypes of *Mbd1*<sup>-/-</sup> mice may explain why mutation of the human MBD1 gene has not been discovered or characterized on a large scale. On the other hand, the majority of people with mild learning and cognitive deficits have not been genetically characterized, either. Mutations in the human MBD1 gene could, in conjunction with other subthreshold gene mutations or polymorphisms, such as the X-linked HTR2C, contribute to the complex genetic background and spectrum of behavioral phenotypes seen in the autistic populations. Nevertheless, our data have demonstrated that *Mbd1*<sup>-/-</sup> mice exhibited several similar behavioral deficits as those seen in *Mecp2* mutant mice. *Mecp2* null mice die soon after reaching adulthood; therefore, most behavioral analyses of the *Mecp2* mutation involve either truncation mutant mice (*Mecp2*<sup>308/y</sup>) (44,46) or brain region-specific knockout mice (47). Both *Mbd1* and *Mecp2*<sup>308/y</sup> mutant mice exhibited reduced social interaction (44,47). Although a strain of *Mecp2* null mice showed signs of reduced anxiety (45), increased anxiety has been clearly demonstrated in *Mecp2*<sup>308/y</sup> mice (46) and null mice (48). In addition, both *Mbd1*<sup>-/-</sup> mice and *Mecp2*<sup>308/y</sup> mutant mice exhibited similar deficits in Morris water maze tests (25,44), contextual fear conditioning (44,45), cued fear conditioning (47) and hippocampal long-term potentiation (25,44). These data suggest that these two MBD proteins may share some functional overlap in the regulation of social interaction, anxiety, depression and learning.

In fact, *Mbd1* and *Mecp2* double mutant and compound heterozygote mice die at postnatal weeks 3–5, much earlier than *Mecp2* single mutant mice (Zhao, unpublished data).

Proper sensorimotor gating enables a reduced response to repeated stimuli. A measure of sensorimotor gating for both human patients and rodent models is the PPI of startle, in which the startle response to a strong stimulus is inhibited when immediately preceded by a weak stimulus (the prepulse). How sensorimotor gating is regulated at a molecular level remains a mystery. Patients with autism or the related Asperger's syndrome, fragile X syndrome and schizophrenia have reduced PPI, which may account for their inability to inhibit repetitive thoughts and actions (30,31,49). The fact that *Mbd1*<sup>-/-</sup> mice have reduced PPI suggests that Mbd1 epigenetic regulation is involved in the regulatory pathways of this process and may provide insight into the molecular mechanism underlying PPI and related behavioral deficits.

The involvement of the serotonin system in autism and ASDs has been well established (3,50–54). Although elevated blood serotonin levels were found in many autistic patients, we did not detect an increased serotonin level in *Mbd1*<sup>-/-</sup> mice, suggesting that Mbd1 does not regulate the levels of serotonin.

The wet-dog shake is a well-appreciated indicator of increased serotonin activity that could be due to either an increased level of serotonin or increased serotonin receptor activity. Early work suggests that the wet-dog shake is related to either Htr2a or Htr2c (40,41). Recently, agonist studies revealed that the 'wet-dog shake' might be due largely to overactivation of Htr2a receptor in the brain (40,41). Since most serotonin receptor agonists and antagonists have overlapping specificities, it is possible that some of the Htr2a agonist-triggered wet-dog shake is partially through Htr2c. Indeed we found that both mRNA and protein expression levels of Htr2c were higher in *Mbd1*<sup>-/-</sup> brains. The serotonin receptor 2c subtype has been implicated in a wide variety of conditions, including obesity, anxiety, depression, obsessive-compulsive disorder, schizophrenia, migraine and erectile dysfunction, and as a consequence has received considerable attention as a target for drug discovery. Consistent with our finding, Htr2c activation has been linked to anxiety behavior, and Htr2c mutant mice show a blunted response to anxiety stimuli (55). In addition, over activation of HTR2C leads to increased anxiety in human patients, and its specific antagonist has been found to have an anxiolytic effect (56,57). Interestingly, although *Htr2c* is localized on the X chromosome and the skewed male-to-female ratio in the incidence of autism is well known, we have not observed any differences between male and female *Mbd1*<sup>-/-</sup> mice in either behavioral analysis or Mbd1 binding to the *Htr2c* promoter. There could still be subtle differences in social interaction deficits between male and female *Mbd1*<sup>-/-</sup> mice that we could not distinguish using our experimental methods. More detailed social interaction analyses will be carried out in the future to further assess gender differences. We also do not rule out the possibility that other serotonin receptors are also regulated by Mbd1 at either the transcriptional or post-transcriptional level. It is possible that we failed to detect any expression changes of other serotonin receptors in subpopulations of neurons due to the limited sensitivity of our



**Figure 6.** Mbd1 directly regulates the expression of serotonin receptor Htr2c. (A) *Mbd1*<sup>-/-</sup> mice (KO) displayed more wet-dog shake behaviors compared with their wild-type (WT) littermates, indicative of abnormal brain serotonin activity; (B) Real-time PCR analyses showing increased *Htr2c* mRNA in the MFC of KO mice; (C) Representative western blot analysis showing increased Htr2c protein in the KO MFC. (D and E) Agonist-mediated serotonin binding assay showing that KO mice exhibited increased serotonin binding to the Htr2c receptor, indicated by the higher  $K_D$  value (D); however, Htr2c receptors in KO brains have lower binding affinity for serotonin, indicated by the higher Bmax value (E). Data are represented as mean  $\pm$  SEM,  $n = 11-12$ . (F) Chromatin immunoprecipitation assay demonstrating that Mbd1 bound to the promoter region of *Htr2c*. (G) Chromatin immunoprecipitation assay demonstrating that Mbd1 bound to the intron 1 of *Htr2c* in WT brains, but not in KO brains. The amount of binding of Mbd1 to *Htr2c* intron 1 was not significantly different between male (KO1 and WT1) and female (KO2 and WT2) brains. (H and I), immunohistochemistry using an anti- $\beta$ -gal antibody showing expression of Mbd1 in the MFC region. Scale bar = 50  $\mu$ m. (H) Negative control using mouse IgG instead of primary antibody. (I) Anti- $\beta$ -gal antibody. \*\*\* $P < 0.0001$ . \*\* $P < 0.01$ ; \* $P < 0.05$ .

assay. Further chromatin immunoprecipitation coupled with high-throughput sequencing will help to elucidate all the brain pathways regulated by Mbd1. Given the recent finding that serotonin transporter expression in humans could be regulated by DNA methylation (19,20), further analysis of Mbd1 regulation of the serotonin system will likely provide fresh insight into the molecular basis of autism-related behavior deficits.

Mbd1 has a clear role in maintaining cellular genomic stability (25) and its mutations have been associated with several types of human cancer (58,59). Interestingly, reduced genomic stability has recently been found in autistic patients (60). The phenotypes of *Mbd1*<sup>-/-</sup> mice suggest that the symptoms resulting from *MBD1* gene mutation in human populations are likely mild, unlike what have been found in Rett Syndrome (*MECP2* mutation). Since most mental disorders are not well categorized, it is possible that the *MBD1* mutation is still

'hidden' in the human population due to its relatively mild effect.

In summary, the behavioral and cognitive phenotypes of *Mbd1* mutant mice suggest that we could use this model to understand the molecular pathways and epigenetic basis of complex cognitive functions, such as social interaction, sensorimotor gating, anxiety, depression and learning, in the context of autism and ASDs.

## MATERIALS AND METHODS

### Mice

All animal procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee. *Mbd1*<sup>-/-</sup> mice under the 129S4 inbred background (25) were crossed into a C57BL/6 background for at least five

generations. F6 heterozygote male and female mice were used in breeding. The postnatal growth of *Mbd1*<sup>-/-</sup> mice is normal with mild reduction of forebrain weight as described (25). *Mbd1*<sup>-/-</sup> mice develop into adulthood with no obvious gross deficit and have a normal life span. All mice used for behavioral analyses were between 2 and 3 months of age. Both male and female mice (age- and sex-matched littermates) were used in the studies, with no significant gender differences found in any of the behavioral analyses.

### Data and statistical analyses

All experiments were recorded using a video camera and scored independently by two experimenters who were blind to genotypes. The results were analyzed by ANOVA using SPSS software (v.14). Because no gender differences were ever detected in any of these tests, the data presented in figures were collapsed across gender.

*Social interaction* was assessed based on a published paradigm with modifications (29,61). We used a total of 24 mice (6/genotype/gender) in these studies. Briefly, an animal was first placed in the test box for 3 min, to allow time for habituation to the environment, followed by the two consecutive 5 min tests. To distinguish between social interaction and novel object exploration, first we tested for 5 min how the mouse responded to a novel toy placed inside a tent that mice could both see and smell through. Then the toy was replaced by an unfamiliar mouse of the same sex and age for 5 min. The number of visits by the animal to the tent (social interacting area) and the length of time the animal spent in direct contact with the tent were recorded during each of the two 5-min periods. The mice were recorded by video camera and independently scored by two experimenters who were blind to the genotypes. The data were analyzed using a multivariate, two-way ANOVA with genotype and gender as factors.

### Buried food test

The purpose of this test was to assess whether the olfaction of the animals are normal as described (62). This test used a standard Teklad mouse chow. *Mbd1* KO and WT ( $n = 4$  each) mice were placed on a food-restricted diet (0.2 g chow per mouse/24 h) starting 2 days prior to testing and during the 4-day experimental period. On each of the four testing days, mice received two trials of tests per day. For each trial a mouse was placed in a clean standard housing cage and permitted to locate, recover and consume a 0.5 g food pellet which was buried approximately 0.5 cm below the surface of a 3 cm deep layer of mouse bedding material (shredded cardboard). Food pellet location was changed for each trial and a clean cage was used for each trial. The latency to find the food pellet was defined as the time between when the mouse was placed in the cage and when the mouse retrieved the food pellet. Mice who did not find the food pellet within 5 min were returned to their home cage and given 0.2 g chow.

*Acoustic prepulse inhibition of tactile startle response (PPI)* was performed using a SR-Lab System (San Diego Instruments) startle apparatus as described previously (35). A total of 14 mice (7/genotype) were used in these studies. Briefly,

the startling stimulus was a 40 ms, 12 psi tactile stimulus (air puff to the back of the animal). The four different intensities of prepulse were 78, 82, 86 and 90 dB tones that lasted 20 ms. Since the background noise level of the chamber was 70 dB, the absolute intensity of tone stimuli were 8, 12, 16 and 20 dB for these tests. Each test began with a 5 min acclimation period, after which each mouse was presented with 60 trials (10 trial types repeated 6 times pseudorandomly) of tests. Trial types were: no startle/no tone control, startling stimulus only, tone prepulse only (four different intensities), tone prepulse trials of 78, 82, 86 and 90 dB (20 ms), followed by the tactile stimulus (120 or 300 ms after onset of the prepulse). The average intertrial interval (ITI) was 15 s and ranged from 10 to 20 s. Maximum startle values for this time period were recorded and analyzed using a macro created in Excel. Percent PPI of the startle response was calculated as  $100 - ([\text{prepulse trial/tactile startling stimulus}] \times 100)$ . A high percent PPI value reflects strong PPI and better sensorimotor gating. A three-way ANOVA (prepulse intensity  $\times$  gender  $\times$  genotype) was used to assess inhibition of the startle response in the presence of a prepulse. A two-way ANOVA (genotype  $\times$  gender) was used to assess startle in the absence of the prepulse.

### Contextual and cued fear conditioning tests

Fear conditioning tests were performed using a Coulbourn Habitest Modular Test System (Coulbourn Instruments, Whitehall, PA, USA) with a stainless-steel grid floor for administration of the foot shock as published [see attached reprint (35,36)]. After 90 s of habituation in the conditioning context, an electric foot shock (0.7 mA) was delivered during the last 2 s of a 30 s clicker (80 dB, 6 clicks/s). The shocks were not painful to the human hand and at this low level felt like a buzzing effect. Mice walk normally and without any alteration in gait after the shock. Extinction testing (24 h later) involved placing the mouse back into the Habitest apparatus and monitoring freezing to the tone (cued test) and environment (contextual test); no foot shock was delivered during extinction testing. Each session was 10 min. The tests were performed by a person who was blind to the genotypes of the animals.

*Light-dark preference test* was performed using a conditioned place preference apparatus modified from published methods (63,64). A total of 24 mice (6/genotype/gender) were used in these studies. The boxes were constructed of Plexiglas with two distinctly different  $20 \times 20 \times 20$  cm chambers connected by a  $9 \times 10 \times 20$  cm anteroom with a removable door. The dark chamber was covered with black and the light chamber with white contact paper. All testing trials were performed under standard illumination in a sound-attenuated room. Mice were placed in the anteroom of the light/dark box and allowed free access to both sides of the box for 10 min. The amount of time spent and number of entries in either the light or dark side of the box were recorded and compared statistically using two-way ANOVA.

*Elevated plus-maze test* was performed using the procedure described (36). A total of 18 mice (5 male and 4 female/genotypes) were used in these studies. Mice were placed in the center square ( $6 \times 6$  cm) of a Plexiglas maze shaped in a

cross that was elevated two feet above the ground and was located in a moderately lit, sound-attenuated room. The supports for the maze were made of clear Plexiglas and were positioned in the middle of the arms, so the mouse was unable to detect them. The maze had two open arms (30 × 6 cm each) that were made of clear Plexiglas floor with no wall. The closed arms were covered with black contact paper and had 6 cm high walls covered with black contact paper. An observer blind to genotype monitored behavior for 10 min. The time spent in the open arms and closed arms and the numbers of entries into each arm were recorded. The data were analyzed using multivariate ANOVA.

*Learned helplessness* was assessed using a Coulbourn Habitest shuttle box (Coulbourn Instruments, Whitehall, PA, USA) with a stainless-steel grid floor. The method used for training and testing learned helplessness was the same as described in our earlier publication (36). During the training period, animals received 20 uncontrollable and unpredictable foot shocks (0.5 mA, 2 s duration). A probability for delivery of the foot shock was assigned at 0.5 every 15 s, and the animal was removed at 30 s after the delivery of the 20th shock and returned to its home cage. After 24 h, the degree of learned helplessness behavior was tested. During the test period, animals were placed into the shuttle box and given 28 shock trials with an ITI of 30 s. An auditory cue (80 dB, 6 Hz clicker) came on at the start of each trial and, after a 1 s delay, the guillotine door was raised. Latency for the mouse to escape through the door was measured. If the animal escaped through the open door, sensors in each compartment of the shuttle box turned off the foot shock and tone and closed the guillotine door. If the animal did not escape within 25 s after the door was open, the shock and tone terminated, and the door closed. A total of 18 mice (5 male and 4 female/genotypes) were used in these studies. The data were analyzed by dividing 28 shock sessions into 4 trial blocks (7 sessions each), and the summed latency of all 7 sessions for each mouse (175 s maximum) were compared. The number of failed escapes (did not escape within the 25 s test period) for each animal was also compared using multivariate ANOVA.

*Forced swim test* was performed as described previously (37,38). A total of 18 mice (5 male and 4 female/genotypes) were used in this study. A mouse was placed in a 30 cm diameter, 46 cm tall cylinder of water (22–25°C, depth 26 cm) and forced to swim for 6 min. Six types of escaping and non-escaping behaviors were scored every 5 s, as described by (39). The floating, kicking and twitching behaviors represent immobility indicative of depression-like behavior. On the other hand, swimming, climbing and thrashing behaviors were considered as escape-directed behaviors. The data were analyzed using a two-way multivariate ANOVA.

*Wet-dog shakes (WDS) behavior* was analyzed as described (65). A total of 23 mice (12 WT, 11 KO, male only) were used in this study. Briefly, mice were videotaped in a standard clean mouse cage with bedding for 10 min and the number of WDS was scored by two independent scorers blind to genotype. Data were compared using a one-way ANOVA.

*Real-time PCR* was performed based on an established method (66). Six *Mbd1*<sup>-/-</sup> and six wild-type mice were sacrificed at postnatal weeks 8–10. The hippocampus and

MFC were dissected out; half the tissue was used for RNA and the other half saved for protein analysis. Total RNAs were extracted using Trizol (Invitrogen Corp, CA, USA). For each sample, four micrograms of total RNA were used in a reverse transcription (RT) reaction (Superscript II, Invitrogen) in a final volume of 20 µl, and 0.05 µl was used in each PCR reaction. For real-time PCR, a 25 µl reaction for each primer set was assembled using 2xSYBR Green PCR Master Mix (Applied Biosystems). A *Gapdh* primer set was used to normalize the results for each sample tested. The following primer pairs were used: Htr1a forward primer, AAT GGG GCG GTG AGA CAG GGT GAG; Htr1a reverse primer, AAG GGC AGC CAG CAG AGG ATG AAG; Htr2a forward primer, GCT GGG CAT CGT GTT CTT; Htr2a reverse primer, AGC GGC TTT CTG TTC TCC T; Htr2c forward primer, TGC CCG TTT TTC ATC ACC AAT; Htr2c reverse primer, CTC CCT CCC AGA CAA AGC AGT; Htr4 forward primer, CAT GGG CTG CTT CTG TTT CTG C; Htr4 reverse primer, GGT CTT TTG TAG CGC TCA TCA TCA. Reactions were run on a 7000 Sequence Detection System (Applied Biosystems).

#### Membrane preparation for western blot and receptor binding assays

MFC and hippocampus were dissected from 8- to 10-week-old mouse brains. Brain tissue was Dounce homogenized in 500 µl homogenization buffer (20 mM Tris, 1 mM EDTA, 320 mM Sucrose, 20 mM β-glycerolphosphate, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, protease cocktail) on ice. Homogenized tissue was centrifuged at 1000 × g at 4°C for 6 min. Supernatants were saved and the pellets resuspended and rehomogenized in homogenization buffer. Following another centrifugation, supernatants were combined. Combined supernatants were centrifuged at 200 K × g for 30 min at 4°C. Supernatants from this centrifugation were discarded and the pellets resuspended in 250 µl extraction buffer (20 mM Tris, 1 mM EDTA, 320 mM Sucrose, 20 mM β-glycerolphosphate, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, 75 mM NaCl, 75 mM KCl, 1% Triton-X 100). Resuspended pellets were Dounce homogenized, incubated on ice for 20 min and centrifuged again at 200 × g for 30 min. The supernatant containing the Triton-soluble membrane fractions were used for western blot analyses. Membranes for the <sup>3</sup>H-serotonin binding study were prepared as described above, with the exception of excluding the second 200 K × g centrifugation. The pellet from the first ultra-centrifugation was resuspended and homogenized in 700 µl homogenization buffer that included 100 nM fluoxetine and 1 nM DPAT. Samples were aliquoted and frozen at –80°C until use.

#### Western blot

Protein concentrations of the Triton-soluble membrane fraction (see above) were determined by Bradford assay (Bio-Rad). Samples (10 µg total protein) were electrophoresed on a 12% SDS-polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Blots were blocked with 0.25% I-Block (Tropix) in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temp-



erature. Blots were probed with a 1:1000 dilution of primary antibody (rabbit anti-mouse Htr2c, ab32172, Abcam) in 0.25% I-Block/TBS-T overnight at 4°C. Blots were washed with TBS-T and incubated in secondary AB (1:20 000 dilution anti-rabbit IgG, Pierce) for 1 h at room temperature. Following another wash, the blot was developed (West Pico, Pierce) and exposed to film. Blots were stripped using Restore Stripping Buffer (Pierce) and reprobed with anti-β-actin IgG (1:2000, Cell Signaling). Blots were washed, incubated with anti-rabbit IgG (1:30 000 dilution, anti-rabbit IgG, Pierce) and developed as above.

### Receptor binding assay

Displacement binding assays were done using <sup>3</sup>H-5HT (75 Ci/mmol; Amersham) in brain membrane homogenates (50 μg protein per tube) with 10 different concentrations of the Htr2c-specific agonist RO 102221 ranging from 0.1 to 500 nM. Incubations were carried out for 30 min at 35°C, followed by rapid filtration on Whatman-D filters using a Brandel Cell Harvester. Non-specific binding was determined in the presence of 1 mM cold 5HT. Assay buffer contained 100 nM fluoxetine, 1 μM MK 212 and 1 nM DPAT to prevent <sup>3</sup>H-5HT binding to 5HT transporter, Htr2a receptor and Htr1a receptor, respectively. The individual displacement binding curves were fit using a one-site hyperbola equation:  $Y = B_{MAX} * X / (K_D + X)$ .  $B_{MAX}$  and  $K_D$  for the KO and wild-type mice were compared using a *t*-test.

### Chromatin immunoprecipitation (ChIP) assays

Antibody to Mbd1 (M-254) was purchased from Santa Cruz Biotechnology and antibody to normal rabbit IgG was purchased from Upstate. ChIP was performed as previously described (68,69), but with the following modifications. Approximately 130 mg of mouse cortex tissue was exposed to formaldehyde at a final concentration of 1% for 15 min and washed twice in cold PBS. Antibodies (5 μg per reaction) were bound overnight to sonicated chromatin at 4°C. Analysis of immunoprecipitated products was done by PCR for the *Htr2c* locus using the following primers: forward 5'-TTCTGCATCATCGATTGTCGT-3' and reverse 5'-GAAAGGTTTGGCTGGAATTG-3' (exon1), forward 5'-AACAGCTGCTGGCTGATTT-3' and reverse 5'-GGAACCAGCAACCTGTTTCAT-3' (intron), forward 5'-GACCCTTGGCCACCTCTTTT-3' and reverse 5'-GCGTGTGCTGTGTTAGCAA-3' (5' end of the gene). In these reactions, PCR was carried out with the following cycling parameters using one-tenth of the immunoprecipitated products: 94°C for 2 min, 38 cycles of 94°C for 45 s, 55°C for 50 s and 72°C for 45 s, followed by 72°C for 7 min. PCR products were separated by gel electrophoresis through 1.5% agarose and bands were detected by ethidium bromide staining.

### Serotonin level measurement

Brain serotonin levels were measured using a chemifluorescence assay as described (67). Briefly, brain issues were first washed with saline, gently blotted dry with Whatman paper, and weighed. Tissues were then homogenized in acidified n-butanol (0.12 N HCl, stock was 12.1 N). The total volume

of homogenized tissue and n-butanol were then brought up to 5 ml in a 15 ml conical tube and centrifuged at 800 × g (1690 rpm) in the R3CB centrifuge with H600A rotor for 15 min at 4°C. The supernatants were then collected and kept at -20°C until use. For the assay, 5 ml n-heptane and 0.6 ml of 0.1 N HCl (containing 0.1% l-cysteine) were added to each 4 ml supernatant sample, followed by vigorous shaking for 10 min, and then centrifuged at 800 × g for 10 min. The organic top layer was removed. The aqueous layer was divided into two 200 μl aliquots as duplicates. A standard curve was generated using serotonin with a serial dilution. Then 1.2 ml of 0.04% o-phthaldehyde in 10 N HCl was added to each 200 μl sample and standard curve. This was mixed well and incubated for 20 min at 100°C. The fluorescence was then read using a spectrofluorometer with 359 nm excitation and 450–500 nm emission with peak emission at 483 nm. The fluorescent readings were then recorded, plotted against serotonin standard and calculated. The results were presented as micro gram serotonin per gram of tissue weight.

### Immunohistochemistry

Heterozygote *Mbd1*<sup>+/-</sup> mouse brain was processed as described (25) and 40 μm sections were stained with a mouse anti-β-gal antibody (1:100, Developmental Hybridoma Bank, Iowa), a goat anti-mouse Cy3 secondary antibody (1:250, JacksonImmuno Research) and DAPI nuclear counter dye. All animal procedures were performed based on a protocol approved by UNM Institutional Animal Care and Use Committee. Immunohistochemistry was performed as described (25). The images were captured using an Olympus BX51 microscope (20×oil) and a Microfire digital camera (Optronics).

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