Defective body weight regulation, motor control and abnormal social interactions in *Mecp2* hypomorphic mice.

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Abstract

MeCP2 is an abundant protein that binds to methylated cytosine residues in DNA and regulates transcription. Mutations in MECP2 cause Rett syndrome, a severe neurological disorder that affects approximately 1:10,000 females. Mice lacking MeCP2 have been generated and constitute important models of Rett syndrome. However, it is yet unclear whether certain physiological events are sensitive to a decrease, rather than a complete lack, of MeCP2. Here we report that a Mecp2 floxed allele (Mecp2\textsuperscript{lox}) that was generated to allow conditional mutagenesis behaves as a hypomorph and the corresponding mutant mice exhibit phenotypical alterations, including body weight gain, motor abnormalities and altered social behavior. Our data reinforce the view that the central nervous system is extremely sensitive to MeCP2 expression levels and suggest that the 3′UTR of Mecp2 might contain important elements that contribute to the regulation of its stability or processing.
Introduction

MeCP2 is a nuclear protein that binds to methylated DNA sequences in the mammalian genome (1). Several studies have shown that MeCP2 associates with transcriptional repression factors and chromatin remodeling activities through a transcriptional repressor domain (2). Other identified domains of MeCP2 include an A/T hook, supposed to participate in the binding to DNA (3,4), and a motif that specifies interaction with WW domain splicing factors located in the C-terminal region (5).

Although MeCP2 is expressed in most cells and tissues, its relevance to neuronal function became apparent with the finding that mutations in the MECP2 gene cause Rett syndrome (RTT, 6). RTT is a childhood disorder characterized by apparently normal early development followed by loss of purposeful use of the hands, stereotypic hand movements, deceleration of brain and head growth, gait abnormalities, seizures and mental retardation (7). RTT was considered for a long time to be of exclusive female occurrence, with a prevalence of approximately 1 in 10,000 births, but an increasing number of male cases with MECP2 mutations are now being reported (reviewed in 8).

The causative role of MECP2 mutations in the etiology of RTT was further confirmed by the generation of mutant mice for Mecp2. Initial attempts to generate Mecp2 null mice resulted in embryonic lethality (9), leading to the creation of conditional Mecp2 alleles using the Cre-loxP recombination system (10,11). Cre-mediated deletion of either exons 3 and 4 (null mutants, 10) or exon 3 (early truncation mutants, 11) demonstrated that MeCP2 is not essential for embryonic development. These Mecp2 mutant mice, as well as a Mecp2 null mutant generated afterwards (12) and a truncated MeCP2 mutant (13),
exhibit RTT-like features: a period of apparently normal development followed by a severe progressive neurological dysfunction that includes abnormal gait and movements, irregular breathing, tremors, decreased mobility and alterations in synaptic plasticity and synapse number (14-16). Null males die at ~8-12 weeks of age (10, 11) while late truncations of MeCP2 allow the mice to survive to adulthood (13, 17, 18).

Engineered alleles carrying intronic insertions sometimes behave as hypomorphs due to disruption of regulatory elements of gene expression or RNA processing (19-21). Since the conditional MeCP2 allele created to delete exons 3 and 4 contains in addition to an intronic insertion, an exonic insertion of a TK-neo cassette, we decided to examine the phenotype of MeCP2^lox/Y males as potential hypomorphs. This is an important issue in the context of human pathology, because situations of decreased function (e.g., through non-coding mutations of MECP2, abnormal protein interactions, etc) are likely to occur in the human population. We found that the MeCP2 floxed allele (MeCP2^lox) behaves as a hypomorphic allele and that the MeCP2^lox/Y male mice exhibit increased body weight and motor abnormalities.

**Results**

**Genomic structure of the MeCP2^lox allele.**

The last exon of MeCP2 contains an unusually long 3’UTR of ~8.6 kb that harbors 4 polyadenylation sites (figure 1A). Expression analysis indicated that the alternative use of the polyadenylation sites produces 4 distinct mRNA transcripts of 1.8, 5.4, 7.2 and
10.2 kb (22). The \textit{Mecp2}^{\text{lox}} alele generated by Guy \textit{et al} carries two insertions: a small insertion that includes a \textit{loxP} site in the distal part of intron 2 and a larger insertion of a human \(\beta\)-globin intron and polyadenylation signal followed by a 1.2 kb TK-neo selection cassette and another \textit{loxP} site (10). Thus, the \textit{Mecp2}^{\text{lox}} allele could potentially only generate the shorter 1.8 kb transcript due to interruption of the 3’UTR sequences by the exogenous insertion (figure 1A). This modification of the repertoire of MeCP2 transcripts could be consequential, since normally the most prevalent MeCP2 transcript in the brain is the 10.2 kb isoform (22, 23), not produced in the \textit{Mecp2}^{\text{lox/y}} mice as demonstrated by the absence of the corresponding amplicon in RT-long range PCR assays (figure 1B). The high degree of sequence conservation of the 3’UTR region (figure 1A) suggests the presence of domains that could be functionally important. In fact, it harbors several highly conserved miRNA recognition elements (24, 25).

**Decreased levels of MeCP2 in neurons of \textit{Mecp2}^{\text{lox/y}} mice.**

To determine if the lack of the major brain specific 10.2 kb MeCP2 mRNA isoform, along with the 5.4 and 7.2 kb isoforms, had any impact on the amount of MeCP2 in cells of the brain, we compared the immunoreactivity for MeCP2 in brain sections of \textit{Mecp2}^{\text{lox/y}} and \textit{Mecp2}^{+/y} littermates. We found that there was a notable decrease in the levels of MeCP2 immunoreactivity in neurons throughout the brain of the \textit{Mecp2}^{\text{lox/y}} mice (figure 2). The specificity of the effect was revealed by staining for calbindin, NeuN and neurophysin. No difference was detected for any of these markers, indicating a specific reduction in MeCP2 expression (figure 2 and data not shown). Remarkably, the decrease in MeCP2 immunoreactivity in the \textit{Mecp2}^{\text{lox/y}} mice was not uniform throughout the brain.
The most dramatic difference in MeCP2 intensity was seen in the hypothalamus (figure 2A and A’) and the mammillary nuclei (supplementary figure 1). The cerebellum was the region that showed the least difference (figure 2E and E’).

Since Mecp2 is located on the X chromosome and subject to X chromosome inactivation, heterozygous females carrying a floxed and a wild type allele simultaneously will be mosaic in terms of the active allele. Thus, for a given neuronal cell type, two subpopulations of cells would co-exist: cells expressing MeCP2 from the floxed allele and cell expressing MeCP2 from the wild type allele. If, as suggested by the previous experiments, the two alleles express different amounts of MeCP2, we should be able to detect such two populations based solely on the determination of MeCP2 expression levels. We therefore immunostained brain sections obtained from Mecp2^{lox/+} females and performed co-staining with neuronal markers for neuronal cell type identification. Due to the difficulty posed by the inherent variability of MeCP2 expression levels present in neurons of wild type brains (26,27), this experiment was feasible only for the Purkinje cells of the cerebellum, identified through immunostaining for calbindin. Even though the cerebellum was the brain region that exhibited the least amount of difference, analysis of the staining in Mecp2^{lox/+} brains showed that we could definitively recognize a population of Purkinje cells expressing low levels of MeCP2 and also Purkinje cells expressing high levels of MeCP2, presumably from the floxed and wild type allele, respectively (figure 3). On the contrary, the staining for MeCP2 was significantly more homogeneous in brains from Mecp2^{+/+} or Mecp2^{lox/lox} females (figure 3). The proportion of calbindin positive cells (Purkinje cells) in the cerebellum that exhibited a level of MeCP2 immunoreactivity below an arbitrarily set threshold value
was 45% for the $\text{Mecp}^2_{\text{lox/}}$ female brain versus only a 5% observed in the $\text{Mecp}^2_{+/}$ brain. These results, besides confirming the observation of decreased MeCP2 immunoreactivity, suggest a cell-autonomous mechanism of regulation of MeCP2 levels. Braunschweig et al compared the level of MeCP2 staining in wild type cells of $\text{Mecp}^2_{+/}$ female brains versus the level of MeCP2 staining in wild type cells of $\text{Mecp}^2_{+/-}$ female brains and found reduced expression of MeCP2 in wild type neurons if they are accompanied by null neurons, suggesting that cells lacking MeCP2 influence negatively the expression of MeCP2 from the neighboring wild type-allele expressing cells (28). Thus, there is a possibility of a non-cell autonomous effect of the floxed allele-expressing cells over the wild type-allele expressing cells that might warrant further investigation.

To determine the degree of MeCP2 deficit in the $\text{Mecp}^2_{\text{lox/Y}}$ brains, we performed quantitative western blot assays on total protein extracts from various brain regions dissected from $\text{Mecp}^2_{\text{lox/Y}}$ and $\text{Mecp}^2_{+/Y}$ mice (figure 4A). The data obtained further confirmed the hypomorph character of the and we calculated that the expression of MeCP2 in $\text{Mecp}^2_{\text{lox/Y}}$ neurons is 90-30% with respect to the wild type expression of MeCP2 (Figure 4B). The biggest decrease in MeCP2 expression was observed for the hypothalamus (30% from $\text{Mecp}^2_{+/Y}$ controls). On the other hand, non-brain tissues such as the liver of the $\text{Mecp}^2_{\text{lox/Y}}$ mice expressed similar amounts of MeCP2 to their wild type counterparts (figure 4B).
Mecp2\textsuperscript{lox/Y} mice have reduced MeCP2 mRNA steady-state levels.

In order to establish whether the decrease in MeCP2 was due to a reduced neuronal translatability of the 1.8 kb transcript versus the other isoforms, or to a decreased stability or expression of the mRNA, we determined the amount of MeCP2 transcripts in total RNA obtained from Mecp2\textsuperscript{lox/Y} and Mecp2\textsuperscript{+/Y} brain samples. We compared the steady state levels of MeCP2 mRNA in total RNA obtained from whole brains, excluding the cerebellum, by quantitative real time RT-PCR with primers directed to exons 2 and 3 of MeCP2 (fig 4C). Since the reduction in MeCP2 immunoreactivity in the cerebella of Mecp2\textsuperscript{lox/Y} mice was minimal, as compared to wild type mice, we decided to exclude it in order to eliminate any possible hindering effect. The level of MeCP2 mRNA was reduced to 55\% in brains of Mecp2\textsuperscript{lox/Y}, as compared to the amount of MeCP2 mRNA present in brains from Mecp2\textsuperscript{+/Y} mice (figure 4C and data not shown). These data suggest that sequences present in the 3’UTR region might participate in the regulation of MeCP2 mRNA expression, stability or processing and further confirm the hypomorph character of the Mecp2\textsuperscript{lox} allele.

Phenotypic characterization of the Mecp2\textsuperscript{lox/Y} mice.

Mecp2\textsuperscript{lox/Y} mice are born at an expected Mendelian ratio and show no overt phenotype for 2 months. However, we noticed that with age most Mecp2\textsuperscript{lox/Y} mice became considerably heavier than their wild type littermates and so we measured their body weight from 4 to 20 weeks of age (figure 5A). Significant weight differences began to be observed at 9 weeks of age and became most marked between 14 and 17 weeks of age.
The average weight of 17 weeks old $\text{Mecp2}^{\text{lox/Y}}$ mice was 39.7 ± 3.7 g, compared with 32.1 ± 1.2 g for wild type littermates. The weight increase was mostly caused by fat accumulation (figure 5B), which was particularly obvious in the inguinal fat deposit. Individual fat pads of 14-17 weeks old $\text{Mecp2}^{\text{lox/Y}}$ mice weighed two to three times more (up to 6 times more in extreme cases) than those of their wild type counterparts. We observed no substantial difference in the amount of food consumed between $\text{Mecp2}^{\text{lox/Y}}$ and $\text{Mecp2}^{+/Y}$ mice, suggesting that the obesity observed is not due to changes in food intake (data not shown).

We also evaluated $\text{Mecp2}^{\text{lox/Y}}$ male mice for general health, including the appearance of fur and whiskers and testing normal reflexes. We did not find consistent differences between $\text{Mecp2}^{\text{lox/Y}}$ and wild type littermates in coat condition, or piloerection, features present in mice lacking functional MeCP2 (10,11,14). Hindpaw clasping and forepaw stereotypies are manifested by null and truncating mutants of $\text{Mecp2}$ (10-13). We did not detect the presence of stereotypies in the $\text{Mecp2}^{\text{lox/Y}}$ mice. However, a subtle but consistent clasping was observed in these hypomorphic mice starting at 6 weeks of age. The response to the visual placing reflex (forepaw extension when lowered toward a visible surface) and the reactions to a gentle touch from a cotton swab to the whiskers on each side of the face were both in the normal range (data not shown). No differences in olfaction or motivation to eat food were detected between wild type and $\text{Mecp2}^{\text{lox/Y}}$ mice. Fasted mice from both phenotypes were equally successful in locating and retrieving hidden (buried) food. Similar latency ($P=0.44$) to find the food was measured for $\text{Mecp2}^{\text{lox/Y}}$ animals (49.3± 13.2 sec) and for their wild type littermates (62.5 ± 10.6 sec). Also, both groups of mice ate the uncovered food at the end of the test.
We then compared the spontaneous exploration of the elevated plus maze of the hypomorphic mutants and their wild type littermates, as a sensitive measure of anxiety. It has been shown that MeCP2 mouse mutants display signs of altered anxiety levels, consistent with Rett syndrome symptoms (12, 29, 30). Analysis of the behavior of \textit{Mecp2}^{lox/Y} mice in the elevated plus maze indicates that they perform similarly to their wild type littermate controls, spending 65.5 ± 7.3 % of the time in the closed arms (versus 68.2 ±3.0 % of wild types P>0.05, figure 6A). This result indicates that a significant decrease in MeCP2 levels does not induce anxious responses to this particular test. To confirm this result we subjected the mice to the open field paradigm, another test commonly used to measure anxiety levels in mice. Again, using this test we did not find evidence of elevated anxiety in \textit{Mecp2}^{lox/Y} mice compared to their wild type littermates (table I, supplementary). Also, we did not observe any noticeable indication of stress when handling the animals, supporting a lack of anxiety-related behaviors in these mice.

The open field test also serves to measure novelty-induced locomotor activity. Examination of the data indicated that there were no significant differences in average speed (45.05 ± 1.7 cm/min for \textit{Mecp2}^{+Y} versus 46.12 ± 1.6 cm/min for \textit{Mecp2}^{lox/Y}) or total traveled distance (1942.8 ± 291.3 cm versus 2640.8 ± 299.9 for \textit{Mecp2}^{+Y} and \textit{Mecp2}^{lox/Y}, respectively). However, when we challenged the mice to walk in a thin wooden dowel connecting two elevated platforms, we observed that the wild type mice clearly outperformed their \textit{Mecp2}^{lox/Y} littermates (figure 6B). When placed in the middle of the suspended dowel, the \textit{Mecp2}^{lox/Y} mice took significantly more time to reach the platform than the \textit{Mecp2}^{+Y} mice. In addition, the total number of platform reachings was fewer for the \textit{Mecp2} hypomorphs. The number of falls (0.5 ± 0.3 for \textit{Mecp2}^{+Y} versus 0.2
± 0.2 falls for $Mecp2^{lox/Y}$ mice), as well as the latency to start moving towards the platform, however, was not different between the genotypes (figure 6B). This task obviously requires more precision in movement control than the open field, suggesting a deficit in fine motor control in the $Mecp2$ hypomorph mice. The hanging wire test, which measures the ability to grip a horizontal wire with the forepaws and to remain suspended for a maximum time of 1 min, is another test that we used to measure motor control in these mice. $Mecp2^{lox/Y}$ mice displayed a normal ability to grasp the wire and to suspend themselves. The number of falls did not differ significantly ($P>0.05$) between $Mecp2^{lox/Y}$ and $Mecp2^{+/Y}$ mice (2.4 ±0.44 versus 2.0 ± 0.44 falls, respectively).

An important feature of RTT patients that was also recapitulated in mouse models of the disease is the presence of abnormal social interactions. We tested the sociability of $Mecp2^{lox/Y}$ mice, as well as their preference for social novelty by the use of the three chamber test (31). This test is based on the fact that mice are highly social individuals and display a natural tendency to approach and engage in social interactions with an unfamiliar mouse. After letting the subject mice to freely explore the test chamber for 10 min, their tendency to socially interact with another mouse was measured by giving the subject mouse the choice to be with an unfamiliar mouse (stranger1, confined in a wired cage) versus an empty wired cage during 10 min. Afterwards, a second unfamiliar mouse (stranger 2) was introduced into the empty wired cage to allow the subject mouse to choose which mouse to interact with, the now familiar stranger 1 or the unfamiliar stranger 2, for an extra 10 min period to measure social novelty preferences (32). A two way ANOVA with repeated measures determined that no preference for any place was evident for either $Mecp2^{lox/Y}$ or $Mecp2^{+/Y}$ mice during the habituation period ($P>0.05$).
Also, mice from both genotypes showed the same response to the addition of an unfamiliar mouse. \( \text{Mecp}^2\text{lox/Y} \) or \( \text{Mecp}^2\text{+/Y} \) mice spent more time in the chamber side that contained the stranger (stranger 1) versus the side with the empty cage (object), suggesting normal sociability (figure 6C, left). However, a statistically significant difference was observed when their behavior related to social novelty was tested. Mice of both genotypes spent significantly more time interacting with the unfamiliar mouse than with the one that they previously interacted with, but interestingly, \( \text{Mecp}^2\text{lox/Y} \) spent comparatively less time in the compartment containing the familiar mouse and more in the one that contained the unfamiliar than the \( \text{Mecp}^2\text{+/Y} \) mice (figure 6C, right). The quality of the interactions in which the animals engaged seemed to be similar for the hypomorphs and their wild type littermates (data not shown). Thus, these behavioral measures suggest that a reduction in MeCP2 levels alters the social behavior of \( \text{Mecp}^2\text{lox/Y} \) mice.

**Discussion**

The recent advent of mouse molecular genetics has dramatically increased our understanding of gene function and the pathological consequences of their malfunction. In particular, the use of knockout mice has led to many insights into the physiological function of many genes (33). However, for some cases, loss of function analysis requires the complementation of more sophisticated alleles to decipher gene function. For instance, a mutation which induces embryonic lethality precludes the study of gene
function in later stages of development. This seemed to be the case for null germ line mutations of MeCP2 (9) and prompted the generation of conditional alleles of \textit{Mecp2} through the use of Cre-loxP system. One such conditional allele, the \textit{Mecp2}^{lox} allele (or \textit{Mecp2}^{tm1Bird/J} as it is now distributed by the Jackson laboratories) was generated by Guy et al by inserting ~470 bp upstream of exon 3 a \textit{loxP} site and ~800 bp downstream of the stop codon in exon 4 a human β-globin intron and polyadenylation signal followed by a \textit{loxP}-flanked TK-neo cassette (10). \textit{Mecp2}^{lox} mice were bred to mice with ubiquitous expression of Cre recombinase and the resulting offspring, carrying a deletion of exons 3-4, constitute an important mouse model of Rett syndrome (10, 27, 34, 35).

Since the insertions in the \textit{Mecp2}^{lox} allele lie in non coding sequences, it could be assumed that mice carrying this allele would be phenotypically indistinguishable from wild type mice. In fact, \textit{Mecp2}^{lox/Y} mice look overtly healthy and are fertile. However, the insertion in exon 4 results in the elimination of 3 out of the 4 main mRNA isoforms derived from alternative use of polyadenylation sites, including the most prevalent brain isoform, a large transcript of 10.2 kb, potentially generating a hypomorphic allele. Although it has been reported that the half lives of the 1.8 kb and the 10.2 kb transcripts do not differ significantly in Raji cells (36), the stability and/or translatability of these transcripts could differ in brain cells. Indeed, it has been recently reported that MeCP2 protein levels are regulated by the brain-enriched microRNA miR132 (25). Therefore, we set out to determine if the lack of the major brain MeCP2 mRNA isoform derived from alternative use of polyadenylation sites was of any phenotypic consequence.

We found that the immunoreactivity for MeCP2 was decreased to 90-30% of wild type levels in neurons of \textit{Mecp2}^{lox/Y} mice. This reduction in MeCP2 was widespread but
with some regional differences, the biggest difference presented by hypothalamic neurons and the smallest difference was observed for the cerebellum. A decrease in MeCP2 levels was also observed in $\textit{Mecp2}^{\text{lox/lox}}$ female mice in a pure 129 genetic background, indicating that this is not a sex or genetic background-specific effect (data not shown). The 3’UTR of $\textit{Mecp2}$ contains some elements involved in the regulation of mRNA stability and translation (24, 25, 36, 37), although their relevance for the expression of MeCP2 remained fully speculative. Recently, Klein et al (25) found that MeCP2 translation is repressed by a microRNA (miR132). Interestingly, the $\textit{Mecp2}^{\text{lox}}$ mRNA lacks the recognition element for miR132 and therefore one might anticipate an increase in MeCP2 levels in $\textit{Mecp2}^{\text{lox}}$ mice. In fact, treatment of cultured cortical neurons with an oligonucleotide designed to block the interaction of miR132 with the corresponding MeCP2 microRNA recognition element increased MeCP2 protein levels (25). Our finding that MeCP2 levels are actually reduced in $\textit{Mecp2}^{\text{lox}}$ mice indicates that there is more to the regulation of MeCP2 protein levels in neurons than the miR132 microRNA recognition element. Indeed, our data suggest that in the array of 3’UTR elements that orchestrate the neuronal-specific regulation of MeCP2 protein levels, the repressive elements might be less important than the positive regulators.

One could propose several different mechanisms to explain the reduction in MeCP2 protein levels in brains of $\textit{Mecp2}^{\text{lox/Y}}$ mice. However, a definite answer to this interesting question will necessitate knowledge not yet available. For instance, we do not yet know if a particular cell produces only one or a combination of MeCP2 mRNA isoforms, if the stability of the isoforms varies, if it differs in different cells or how the selective use of alternative polyadenylation site is regulated. Nevertheless, the step-wise
process of eukaryotic mRNA polyadenylation: sequence recognition, cleavage and addition of the poly(A) tail (reviewed in 38 and 39) suggest that the presence of only one of the endogenous Mecp2 polyadenylation sites should not impact on the amount of mRNA transcribed from the floxed allele, unless the displacement of the distal 3’UTR sequences modified the activity of an unrecognized transcriptional enhancer. Our finding that the amount of total MeCP2 mRNA in brains of \(\text{Mecp2}^{\text{lox/Y}}\) was approximately 50% of that of \(\text{Mecp2}^{+/Y}\) mice, can not rule out the presence of an interrupted transcriptional enhancer. Further, since the floxed allele contains exogenous intronic and polyadenylation sequences and a TK-neo cassette, these elements could possibly affect the stability or rate of transcription of the modified allele.

We studied the \(\text{Mecp2}\) hypomorphic effects on mice of a 129S1/SvImJ; B6/CBA hybrid background. Post-weaned \(\text{Mecp2}^{\text{lox/Y}}\) mice exhibited increased body weight, diminished ability to perform coordinated movement and balance and abnormal social interactions compared to their wild type controls.

The striking decrease in MeCP2 levels in the hypothalamus, area specialized in the integration of the control of energy homeostasis, suggest that the origin of the overweight phenotype could stem from misexpression of MeCP2 hypothalamic targets. Notably, abnormalities in body weight were observed in mice lacking functional \(\text{Mecp2}\) (10-13), supporting the involvement of MeCP2 in regulation of energy balance. However, although some patients carrying \(\text{MECP2}\) mutations develop obesity (40-43), most RTT girls are underweight (44). This seemingly discrepant data could be explained by the presence of different genetic modifiers (supported by the observation that the effect of MeCP2 mutation on body weight was dependent on genetic background [10]), the fact
that the mice have *ad libitum*, self-provided food whilst RTT girls are usually fed by others at regular intervals, or by species-specific differences between mice and humans in the molecular determinants of energy homeostasis.

*Mecp2* hypomorphic mice do not display gross movement abnormalities in their home cage or in a new environment such as the open field and they could easily hang themselves from a suspended wire with their forepaws. However, they exhibit a subtle clamping of their rear paws indicating some neurological deficit. Furthermore, when forced to perform a task that requires simultaneous coordination of fore and hind paws motion in conjunction with balance control, *Mecp2*\(^{lox/Y}\) mice are less able than their wild type littermates. These data imply that MeCP2 expression levels are relevant for the normal function of central structures controlling movement coordination. Alternatively, this latter phenotype could be the result of differences in motivation to reach the three-walled platforms or even in the perception of safeness of the platform versus the suspended rod. The idea of abnormal perception is not supported by the normal behavior displayed by the *Mecp2*\(^{lox/Y}\) mice on the elevated plus maze, in which they spent more time in the safety confines of the closed arms. We also disfavor the idea of a lack of motivation, since the *Mecp2*\(^{lox/Y}\) mice moved towards the platforms, although at a slower pace. Thus, we believe that our data reinforces the importance of MeCP2 expression levels in motor control.

Reduced MeCP2 expression has been observed in autism frontal cortex samples (45, 46) and in peripheral blood cells from autistic patients (37) suggesting a causative link. Interestingly, the patients with lower MeCP2 expression reported by Coutinho et al had alterations in the 3′UTR. In addition to these antecedents, we detected alterations in
the social behavior of the \( \textit{Mecp2} \) hypomorphic mutant. Although \( \textit{Mecp2}^{\text{lox/Y}} \) mice were able to distinguish between another mouse and an object or even a familiar mouse from an strange one, the hypomorphic mice exhibited a different response in terms of the time allocated to the interaction with the familiar (\( \sim 70\% \) of wild type) and unfamiliar mice (\( \sim 120\% \) of wild type). Thus, the normal reduction in olfactory investigation after an initial exposure, or habituation, seems to be exaggerated in the \( \textit{Mecp2} \) hypomorphic mice and could be interpreted as enhanced social discrimination. A more detailed study of the social behavior of \( \textit{Mecp2}^{\text{lox/Y}} \) mice, as well as a determination of the status of the pathways involved in processing the olfactory and visual cues that constitute the basis of social recognition will shed light into this phenotype. Nevertheless, these results suggest that a decrease in MeCP2 levels might not be enough by itself to produce autistic features, but could act as a predisposing factor for the development of multifactorial disorders of social behavior. The \( \textit{Mecp2}^{\text{lox/Y}} \) mice, with decreased levels of MeCP2 in the brain, will be a useful resource to test this hypothesis by mating them to mice carrying other potentials susceptibility-increasing mutations.

In summary, our study establishes that the \( \textit{Mecp2}^{\text{lox}} \) allele is hypomorphic and therefore interested researchers in generating tissue-specific deletions of Mecp2 through cre-mediated recombination should be cautious; that the 3′UTR of \( \textit{Mecp2} \) might contain important regulatory regions whose mutation could contribute to pathological conditions including anomalies of body weight and motor control; and finally that the phenotype caused by a significant reduction in MeCP2 levels in mice is much less severe than the phenotype caused by the lack of functional MeCP2 or a more modest increase in MeCP2 levels. This last conclusion, together with the demonstration that post-symptomatic
activation of MeCP2 expression leads to phenotypic reversal in a mouse model of RTT (47) brings hope for future therapeutic approaches aimed at restoring MeCP2 expression.

Materials and methods

Animals

$\text{Mecp2}^{\text{lox}}$ mice, generated by homologous recombination-replacement of intron 2 to exon 4 of $\text{Mecp2}$ with the same region flanked by loxP sites and a selectable cassette, were a generous gift from A. Bird (10).

Experiments were performed with mixed background F1 male mice derived from breeding of $\text{Mecp2}^{\text{lox}}$ females backcrossed for 6 generations on a 129S1/SvImJ genetic background with B6/CBA males. Animals were kept in an animal room under SPF conditions at a room temperature of 20 ± 2°C, in a 12/12 h light/dark cycle with free access to food and water. All experiments were approved by the Centro de Estudios Científicos Animal Care and Use Committee.

Fluorescent Immunohistochemistry

Mice were anaesthetized with an intraperitoneal injection of 2% avertine (2,2,2-tribromoethanol, 0.02 ml/g body weight, Sigma) and transcardially perfused (chilled saline for 1 min followed by [pH 7.4] containing 4% paraformaldehyde [Sigma] for 10 minutes). Brains were quickly dissected, post-fixed overnight in 4% formaldehyde at 4°C, cryoprotected for 24-36 h in 30% sucrose, embedded in O.C.T. (Tissue Tek) and frozen
on dry ice. 50 µM serial sagittal sections were collected through the whole brain. Floating sections were blocked for 1 h in 0.1 M PBS containing 2% normal goat serum and 0.3% Triton X-100. Sections were then incubated for 24 hrs at 4°C in blocking solution with primary antibodies as follows: anti-MeCP2 C-terminal 1:100 (Upstate); anti-MeCP2 N-terminal 1:500 (Sigma); anti-Neurophysin 1:500; anti-Calbindin D28K 1:600 (Sigma); or anti-NeuN 1:600 (Chemicon). Sections were washed five times with 0.3% Triton X-100 in 0.1 M PBS and incubated for 24 hrs at 4°C with secondary antibodies conjugated with Alexa Fluor 488 (Molecular Probes) or Cy3 (Jackson Immunoresearch) at a 1:800 dilution. Nuclei were stained with a solution containing 300 nM of 4’,6’-diamino-2-phenylindole (DAPI) for 15 minutes at room temperature. The expression levels of MeCP2 in Mecp2^lox/Y and Mecp2^+/Y mice were compared by normalizing immunolabeling with the use of the NeuN and calbindin antibodies. The same neuronal groups were identified and the intensity of NeuN or calbindin immunolabeling was compared to the intensity of MeCP2 in brains from both genotypes (3 mice of each genotype). Images were captured using a Zeiss Axiovert 100M confocal microscope or an Olympus CX31 epifluorescence microscope equipped with a QImaging 3.3 RTV cooled CCD camera. Images were processed with LSM Image Browser software (Zeiss) and Adobe Photoshop 7.0 (Adobe). To set up a threshold value to differentiate high and low MeCP2 expressing neurons in the cerebella of Mecp2^+/+ and Mecp2^lox/+ females, we obtained signal intensities with the LSM Image Examiner Software by placing the cursor through MeCP2 & Calbindin double-positive Purkinje cells (Z-stack projection of 12 sections; merged images), this way obtaining individual profiles and histograms for the marked cells. The threshold value was set at intensity ratio of green channel / red channel of 0.6.
**Western Blot Analysis**

Brain regions were dissected from 1 mm coronal sections and homogenized in lysis buffer containing 125 mM Tris (pH 6.8) and 1% SDS supplemented with 1X protease inhibitor cocktail (Sigma, P8340). 25 µg of protein were electrophoresed on 4-8% SDS polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad) and blocked for 1 h at room temperature with freshly prepared TBS-T buffer containing 5% nonfat dry milk. Membranes were incubated for 2 h at room temperature with rabbit polyclonal antibodies against MeCP2 1:2,500 (Upstate) or β-Tubulin 1:1,000 (Santa Cruz Biotechnology), washed and incubated with anti-rabbit HRP-conjugated IgG 1:40,000 (Pierce) for 2 h at room temperature. Bands were visualized with SuperSignal West Femto chemiluminescent substrate according to the manufacturer’s instructions. Densitometry of immunoreactive bands was quantitated with Quantity One software (Bio-Rad).

**Real-time RT-PCR**

Total RNA from brain (without cerebellum) was extracted with Trizol (Invitrogen, CA) according to the method provided by the manufacturer. RNA was quantitated by measuring absorbance at 260 nm and stored at –80 °C until used. RNA was treated with DNase I (Epicenter, WI) for 30 min at 37°C and reverse transcribed at 45°C for 90 min by using random primers and AffinityScript™ MultiTemperature (Stratagene, CA) to synthesize single stranded cDNA. PCR mixtures were prepared with
Quantimix Easy Syg Kit for Real Time DNA amplification and quantification (Biotools, Spain). All amplification reactions were performed in triplicate from 25 ng cDNA using the Rotor-Gene 6200 (Corbett, Australia) in a total volume of 10 µl, each reaction containing 1 µl of diluted cDNA. The Real Time program used consisted of an initial period of 10 min at 95°C, followed by 40 cycles at 95°C for 25 s, 55°C for 25 s and 72°C for 30 s. The Results were analyzed with the Rotor-Gene 6000 Series Software 1.7 (Corbett) and all values were normalized to the levels of the cyclophilin 1 mRNA. Primers used were the following: forward 5-CTCCATAAAAATACAGACTCACCAGT-3 and reverse 5-CTTAAACTTCAGTGGCTTGTCT-3 for Mecp2 (NM_010788) and forward 5-GGCAAATGCTGGACCAAACACAA-3 and reverse 5-GTAAAATGCCCAGCAAGTCAAAG-3 for cyclophilin-1 (NM_008907).

**Phenotypic Testing and Behavioral Overview**

All behavioral testing was done on *Mecp2<sup>lox/Y</sup>* and *Mecp2<sup>+/Y</sup>* male littermates. Since 4 weeks of age routine observation for Rett-like symptoms such as clasping and stereotypic hand movement and body weight determination were performed once a week. At 12 weeks of age, each mouse was subjected to a battery of behavioral tests performed always in the same order: elevated plus maze, open field, dowel test, hanging wire test, social behavior and olfactory test. For all experiments the data were presented as mean ± SE of 9 *Mecp2<sup>lox/y</sup>* and 11 *Mecp2<sup>+/y</sup>* mice. Statistical significance was set at a minimum of p< 0.05.
**Elevated Plus Maze**

Mice were placed in the center of a cross-shaped maze elevated 45 cm from the floor with two open and two closed arms. The behavior of the mice was observed and the time spent in either the closed or open arm or in the center of the maze was recorded. The data were analyzed using the Student’s test.

**Open Field**

Mice were tested for their motor, rearing and exploratory activity in a photobeam system open field (Med associated Inc.). The mice were placed at the center of the field and the activity of the mice was recorded during 30 min, in 10 min intervals. Data were analyzed by two-way ANOVA.

**Motor Abilities and Reflexes**

Motor abilities were tested by assaying dowel and hanging wire tests and sensory reflexes by visual placing, whisker touch and righting positioning. The dowel apparatus consists of two elevated platforms that are enclosed by walls in every side except in the side that connects them through a 70 cm long dowel of 0.7 cm radius. Before testing, mice were placed on one of the platforms and allowed to habituate for 1 min, then moved to the opposite platform for another min. Thereafter, mice received a short training, placing them on the dowel 10 cm away from one of the platforms. All mice reached the platform in the first 90 sec, thus were further assessed. Next, mice were placed in the middle of the dowel and the time of first arrival, the total number of arrivals and the number of falls were recorded. The hanging wire test was performed by hanging the mice
by its forepaws from a suspended wire and recording the number of falls in 1 min. Data were analyzed by Student’s t test.

**Social Behavior and Interaction**

We used a social interaction paradigm that assesses social interest and social recognition. Mice were placed into a three-chamber divided cage (60 x 40 x 22 cm) and allowed to habituate and explore the new environment for 10 min. Thereafter, the social interest was assessed by placing an adult mouse into a wire mesh enclosure located in one cage extreme and an empty wire mesh enclosure in the other extreme and allowing the subject mouse to explore for 10 min. After that time, social recognition was assessed by placing a new adult mouse into the empty wire mesh enclosure and allowing 10 more min. of exploration. After finishing the test, all animals were returned to their home cages. Data were analyzed by Student’s t test and two-way ANOVA.

**Olfactory test**

Olfaction was tested as in Moy *et al.*, 2007 (48). Mice were fasted for 20 h and introduced in a 28x28x11 cm cage containing a layer of 3 cm of bedding material and allowed to explore for 5 min. The subject mouse was then removed and a food pellet (of approximately 2 cm long) was buried into the bedding material. The mouse was reintroduced in the cage and allowed to find the hidden food for 15 min. The latency to find the food pellet was recorded. The data were analyzed by independent-samples t-test.
Acknowledgements

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Conflict of Interest Statement

The authors declare no conflict of interest.

REFERENCES


Legends to figures

Figure 1. Genetic modifications of the MeCP2 allele result in the absence of normally produced transcripts. (A) The conserved 3’UTR of MeCP2 is interrupted in the MeCP2\textsuperscript{lox} allele (modified from the UCSC Genome Browser, http://genome.ucsc.edu). The genomic structure of MeCP2 is on top with its last 2 exons depicted by boxes. Tall boxes are coding and half size boxes are UTR sequences. Arrowheads mark the binding sites for microRNAs reported to affect the expression of MeCP2 (see also supplementary figure 2), black arrows indicate the polyadenylation sites and white arrows below MeCP2 indicate the sites of insertion of exogenous sequences in the MeCP2\textsuperscript{lox} allele. Marked by a horizontal bracket is the region of the 3’UTR displaced by the insertion of the selection cassette and therefore absent in the MeCP2\textsuperscript{lox} mRNA. Note the high degree of conservation in the 3’UTR region. (B) Absence of longer alternative polyadenylated transcripts in MeCP2\textsuperscript{lox/Y} mice. Long range-PCR of either genomic DNA (gDNA) or cDNA obtained from MeCP2\textsuperscript{+/Y} and MeCP2\textsuperscript{lox/Y} brains using primers whose binding sites are located upstream and downstream of the cassette insertion site in exon 4 of MeCP2 (horizontal arrows in A) for the detection of MeCP2 transcripts surpassing the first polyadenylation site. In spite of robust amplification of a band of the expected size from the gDNA, we did not detect amplification products from the MeCP2\textsuperscript{lox/Y} derived cDNA.

Figure 2. Diminished MeCP2 expression in brains of MeCP2\textsuperscript{lox/Y} mice. Confocal images of double immunofluorescence for the detection of MeCP2 (green) and NeuN (red), in midsagittal brain sections from MeCP2\textsuperscript{+/Y} (A-H) and MeCP2\textsuperscript{lox/Y} (A’-H’) mice. A-E’ are
merged images of the green and red channels, as well as the left panel of F-H’. The middle and right panels of F-H’ are the individual green (MeCP2) and red (NeuN) channels, respectively. The intensity of the NeuN staining was similar for both phenotypes, whilst the MeCP2 signal was fainter in the MeCP2<sup>lox/Y</sup> neurons.

Magnification bars: 100 µm (A-E’) and 20µm (F-H’). Ht: hypothalamus, Cx: cerebral cortex, DG: dentate gyrus, Hpc: hippocampus, Cbl: cerebellum, CPu: caudate-putamen.

**Figure 3.** Occurrence of two distinct subpopulations of Purkinje cells in brains of heterozygous MeCP2<sup>lox/+</sup> females. Confocal images (Z-stack projection of 12 sections covering completely the shown Purkinje cells) of co-immunostaining for MeCP2 (green) and calbindin (red) in the cerebella of MeCP2<sup>+/+</sup> (A-C) and MeCP2<sup>lox/+</sup> (D-F) female mice. A and D are merged images of both channels. Insets: We obtained signal intensities of high and low MeCP2 expressing neurons with the LSM Image Examiner Software by dragging the cursor through MeCP2 and calbindin double-positive Purkinje cells (see white lines), obtaining individual profiles and histograms for the selected cells. Y-axis represents signal intensity and X-axis represents distance covered (in µm). Green traces correspond to MeCP2 staining and red traces to calbindin staining. B, C, E and F are images solely of the green channel. In C and F, the output intensity was modified using the “cont” command on the Zeiss LSM Image Browser software for easier discrimination of two subpopulations of Purkinje cells; high and low (arrows) MeCP2 expressors.

**Figure 4.** The reduction in levels of MeCP2 in MeCP2<sup>lox/Y</sup> mice was accompanied by differences in the steady-state level of mRNA. A. Protein extracts prepared from 14-16
weeks of age $Mecp2^{+/Y}$ (left lane of individual panels) and $Mecp2^{lox/Y}$ (right lane of individual panels) male mice were subjected to western blot for the immunodetection of MeCP2 (~72 Kb, top) and $\beta$-tubulin (~50KD, bottom). B. Normalized quantification of band intensities, from at least 3 independent determinations shows a reduction of MeCP2 protein levels (P<0.05 by Student’s t test) in all brain regions but not in the liver. C: Expression of Mecp2 was analyzed in mice from both genotypes by real time quantitative RT-PCR from total RNA from whole brain minus cerebellum obtained at 14-16 weeks of age. Cyclophilin was used as a normalizing control. The primers used to amplify MeCP2 anneal at exons 2 and 3 and therefore recognize all alternatively polyadenylated mRNA isoforms. **: P<0.01 by Student’s t test, n=8.

**Figure 5.** Weight and fat mass of $Mecp2^{+/Y}$ and $Mecp2^{lox/Y}$ mice. A, Growth curves of sibling male mice (P < 0.005 for genotype, by repeated measure ANOVA, *: P<0.05 and **: P<0.005 by post-hoc Student’s t test). B, Weights of inguinal/gonadal (IG), retroperitoneal/perirenal (PR) and intrascapular brown (BF) fat pads and testis (Ts), kidney (Kd), heart (Ht) and liver (Lv) in male $Mecp2^{+/Y}$ and $Mecp2^{lox/Y}$ mice (n = 6; *, P < 0.01, by Student’s t test).

**Figure 6.** Motor deficits and altered social behavior in $Mecp2^{lox/y}$ mice. A. 12 week-old $Mecp2^{lox/y}$ mice did not display increased anxiety-like behaviors as assessed by the elevated plus maze test. B, $Mecp2^{lox/y}$ displayed impaired motor coordination compared with wild type mice on the dowel test. No differences were observed in the latency to initiate movement when placed on the dowel (Latency Time), but $Mecp2^{lox/y}$ mice took
more time to reach the platform (*, P < 0.05, **, P < 0.01, by t test). Depicted are the time it took for the mice to arrive for the first time to the platform (First Arrival) and also the average of all the reaching times in the 2 min interval (Average Time). C, Test for sociability and social novelty preference in \textit{Mecp2}^{loxy} mice. The time spent (\% of total time) in the chamber side with stranger 1 (stranger 1) or with the empty cage (Object) during the sociability part of the test is shown in the left half of the picture. Depicted on the right half of the picture is the percentage of time spent in the chamber side with stranger 1 (Familiar) or with stranger 2 (Unfamiliar) during the preference for social novelty test. Grey bars: \textit{Mecp2}^{+/Y} (n=9), white bars: \textit{Mecp2}^{loxy/Y} (n=11). Mean +/- S.E.M. values are presented. *: P<0.05 by Student’s t test.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A

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B

Relative immunodetection (%)

0 50 100 150

Cx Ht CPu Hpc BS Lv

C

MeCP2 expression levels (as % of control)

0 20 40 60 80 100

+Y lox/Y

Genotype

**
Figure 5.

A

Body Weight (gr)

Weeks of Age

B

Tissue Weight, % of Control

IG  PR  BF  Ts  Kd  Ht  Lv

Mecp2<sup>+/y</sup>  Mecp2<sup>lowy</sup>
Figure 6.

A

% of Time

Open Center Closed

B

Time (s)

Latency Time First Arrival Average Time

C

% of Time

Stranger 1 Object Familiar Unfamiliar