Neurobiology of Disease 59 (2013) 257–266

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Loss of MeCP2 function is associated with distinct gene expression changes in the striatum

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ARTICLE INFO

Article history:
Received 1 May 2013
Revised 5 July 2013
Accepted 2 August 2013
Available online 13 August 2013

Keywords:
Methyl-CpG binding protein 2
MeCP2
Rett syndrome
RTT
Gene expression
Striatum

ABSTRACT

Rett syndrome (RTT) is a neurodevelopmental disorder characterized by developmental regression beginning 6–18 months after birth, followed by a lifetime of intellectual disability, stereotyped behaviors, and motor deficits. RTT is caused by mutations in the gene encoding MeCP2, a methyl-CpG binding protein believed to modulate gene transcription. Gene expression studies of individual brain regions have reported that *Mecp2* loss-of-function leads to both activation and repression of its gene targets in mice. Conditional deletion of MeCP2 from different brain regions has revealed unique insights into the role of these structures in mediating particular RTT-like phenotypes. However, the function of MeCP2 in the striatum, a major brain region involved in motor control and executive cognitive functions, has yet to be studied. Here, we characterized the gene expression changes in the striatum of *Mecp2* mutant mice. We found a number of differentially expressed genes in the striatum of both constitutive *Mecp2*-null mice and mice lacking MeCP2 only from forebrain GABAergic neurons. These changes only occurred when MeCP2 expression levels had reached mature levels and RTT-like symptoms were manifest, supporting a role for MeCP2 in maintaining proper brain function. Many of the gene expression changes identified in the striatum have not previously been shown to change in the hypothalamus or cerebellum. Bioinformatic analysis of differentially expressed genes in striatum as well as hypothalamus and cerebellum revealed that loss of MeCP2 does not affect the global landscape of gene expression. Additionally, we uncovered a number of differentially expressed genes in the liver of *Mecp2*-null mice suggesting an important role for MeCP2 in non-neuronal tissues. Collectively, our data suggest that the differential expression of genes following loss of MeCP2 occurs in a tissue- or cell-type specific manner and thus MeCP2 function should be understood in a cellular context.

Introduction

RTT is a severe X-linked neurological disorder that affects 1 in 10,000–15,000 females. Patients with RTT experience a relatively normal first 6–18 months of life after which they enter a period of developmental stagnation or regression. Subsequently, patients lose learned motor and communication skills, and develop hand stereotypies and gait apraxia (Katz et al., 2012; Neul et al., 2010). Approximately 95% of RTT cases are associated with mutations in the gene encoding methyl-CpG binding protein 2 (MeCP2).

Genetic studies using *Mecp2* knockout mice, or mice carrying RTT-associated mutations, have established a causal role of MeCP2 in the etiology of RTT (Chen et al., 2001; Goffin et al., 2012; Guy et al., 2001; M. Shahbazian et al., 2002; Pelka, 2006). Conditional deletion of *Mecp2* from the mouse brain recapitulates the phenotypes observed in *Mecp2*-null mice such as reduced brain and body weight, decreased locomotor activity, motor incoordination, gait and breathing abnormalities (Chen et al., 2001; Guy et al., 2001). These studies demonstrate that RTT is primarily a neurological disorder and that MeCP2 is indispensable for proper brain function. Furthermore, conditional deletion of *Mecp2* from specific brain regions or specific population of brain cells have demonstrated that different symptoms of RTT may manifest through loss of MeCP2 function from particular brain regions or cell types (Adachi et al., 2009; Chao et al., 2010; Fyffe et al., 2008; Lioy et al., 2011; Samaco et al., 2009; Ward et al., 2011). However, while specific neurotransmitter systems and neuronal populations play important roles in the etiology of individual RTT-like phenotypes, the large-scale disruption of MeCP2 function is required for the appearance of phenotypes that mimic constitutive *Mecp2*-null mice (see Goffin and Zhou, 2012 for review).

MeCP2 is a highly abundant nuclear protein that binds methylated CpGs (5mC) *in vitro* and *in vivo* (Skene et al., 2010). Through its interaction with co-repressor complexes, such as Sin3A and histone deacetylases 2 (HDAC2), MeCP2 was originally postulated to repress gene transcription (Nan et al., 1997); however initial gene expression studies did not find significant changes in gene expression upon loss of MeCP2 (Kriaucionis et al., 2006; Tudor et al., 2002). To reduce the potentially

Abbreviations: RTT, Rett syndrome; MeCP2, methyl-CpG binding protein 2; WGCNA, weighted gene co-expression network analysis.

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Available online on ScienceDirect (www.sciencedirect.com).

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http://dx.doi.org/10.1016/j.nbd.2013.08.001

confounding effects of cellular heterogeneity in the brain, other studies examined gene expression changes using individual brain regions; these studies identified hundreds to thousands of genes whose expression is affected by MeCP2 dysfunction in the hypothalamus and cerebellum (Ben-Shachar et al., 2009; Chahrour et al., 2008). Notably, most of these differentially expressed genes were down-regulated in *Mecp2*-null mice and up-regulated in *Mecp2*-overexpressing mice, arguing against a primary role for MeCP2 in gene repression. This additional role for MeCP2 in gene activation may result from the recruitment of CREB by MeCP2 (Chahrour et al., 2008) or the binding of MeCP2 to 5-hydroxymethylcytosine (5hmC), which is enriched over active genes (Mellén et al., 2012; Szulwach et al., 2011). Since the pattern of 5mC and 5hmC localization across the genome is cell-type dependent (Mellén et al., 2012), genes that have been previously identified as differentially expressed in the hypothalamus and cerebellum may reflect the composite changes across multiple cell types.

One brain region that has received little attention in the RTT field is the striatum. This brain region serves as the input center for the basal ganglia, a subcortical region of the brain that processes and integrates information associated with motor control and various executive cognitive functions. Indeed, hand stereotypies, impaired motor coordination and disrupted cognitive functions are common for all RTT patients. In addition, RTT patients often display a volumetric decrease in the size of the striatum (Reiss et al., 1993; Subramaniam et al., 1997). Deletion of MeCP2 from forebrain GABAergic neurons leads to the expression of several RTT-like phenotypes (Chao et al., 2010). However, the role the striatum plays in mediating these phenotypes is not known. Additionally, the genes regulated by MeCP2 in the striatum have yet to be defined. Given that 97–98% of striatal neurons are GABAergic medium spiny neurons (MSNs), this cell homogeneity affords this region a unique advantage for the study of MeCP2-regulated gene expression.

In this study, we profiled gene expression changes in the striatum of mice following the conditional deletion of MeCP2 from the striatum during early development and at an adult stage. We identified 21 differentially expressed genes only in adult mice and not during development, highlighting the importance of MeCP2 in maintenance of proper brain function. We also examined gene expression changes in the striatum of constitutive *Mecp2*-null mice and found that 59 genes are significantly down-regulated and 68 genes are significantly up-regulated in the absence of MeCP2. We found that the majority of differentially expressed genes in the striatum are distinct from those identified in the hypothalamus and cerebellum. Weighted gene co-expression network analysis revealed that the overall transcriptome differs largely between brain regions rather than between WT and *Mecp2*-null genotypes, indicating that the gene expression landscape is not globally affected by loss of MeCP2. Together, our data suggests that MeCP2 regulates gene expression in a region-specific manner, most likely driven by cell type, and that the aggregated differences in gene expression within individual cells and regions lead to the pathogenesis of RTT. Given that MeCP2 is a 5mC and 5hmC binding protein and these methylation patterns likely differ between different cell types, we propose that the role of MeCP2 in regulating target gene expression should be studied with individual cell types in consideration.

Results

Differentially expressed genes in the striatum of Mecp2 conditional knockout mice

The striatum serves as the major site of input and integration for cortical, thalamic, and midbrain afferents in the basal ganglia, a brain region essential for maintaining proper sensorimotor and executive cognitive functions. The manifestation of motor and cognitive disturbances in RTT, together with the observed decreased striatal volume in RTT patients (Reiss et al., 1993; Subramaniam et al., 1997), point to a possible role for the striatum in the etiology of RTT. A causal role for the striatum in the pathogenesis of RTT, or a biochemical maker of striatal dysfunction, however, has yet to be examined.

Given the potential role for MeCP2 in the repression and activation of gene transcription, we examined whether loss of MeCP2 alters gene expression within the striatum. The striatum affords a unique advantage for gene expression studies because it is composed primarily of GABAergic MSNs. This homogeneity in cell type reduces the confounding effects that multiple unique cell types with highly divergent gene transcriptional programs can have on gene expression analyses (Heiman et al., 2008). Since altered neuronal activity caused by the loss of MeCP2 from afferent inputs to the striatum may indirectly affect striatal gene expression, we conditionally deleted MeCP2 from forebrain GABAergic neurons, including striatal MSNs, while preserving expression in those neurons that provide inputs to the striatum. We achieved this by breeding floxed *Mecp2* mice (*Mecp2*$^{2lox/+}$) (Chen et al., 2001) to mice expressing Cre-recombinase localized to striatal MSNs and forebrain GABAergic interneurons (Dlx5/6-cre) (Monory et al., 2006). The behavioral consequences of deletion of MeCP2 from forebrain GABAergic neurons using *Dlx5/6-cre* have previously been determined (Chao et al., 2010). These mice do not show any of the overt RTT-like phenotypes – such as hypoactivity, gait, breathing difficulties or premature lethality – that are readily apparent in constitutive *Mecp2*-null mice. However, behavioral characterization of these mice revealed phenotypic alterations involving social interaction preference, motor coordination and repetitive behaviors (Chao et al., 2010). To determine the efficiency of cre recombinase in the striatum of these mice, we performed immunohistochemistry using an antibody directed against MeCP2 in the male progeny of mice with the *Mecp2*$^{2lox/y}$; *Dlx5/6-cre* genotype (referred hereafter as cKO) and their *Mecp2*$^{2lox/y}$ (WT) littermates. Indeed, we demonstrate a significant loss of MeCP2 immunoreactivity from the striatum of cKO mice but not their WT littermates (Fig. 1A). In addition, close examination of MeCP2 expression revealed the existence of <1% of MeCP2-positive cells in the striatum (Supplementary Fig. S1).

RTT-like symptoms in mice manifest in an age-dependent manner. Mice show relatively normal behavior during the early stages of postnatal development followed by the gradual appearance and increased severity of RTT-like phenotypes. We therefore examined striatal gene expression levels at two developmental time points: the first at P7 during early postnatal development when MeCP2 protein levels are still increasing and the second following development at P90 when MeCP2 protein levels have plateaued (Skene et al., 2010). In addition, behavioral testing has previously shown that cKO mice exhibit motor deficits at this time point (Chao et al., 2010). We therefore dissected the striatum from male cKO and WT littermates at P7 and P90 and obtained genome-wide whole transcript coverage expression data using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays (Gene Arrays). We first examined whether striatal gene expression changed over the course of development from P7 to P90. Using the criteria of a fold-change of ≥ 1.2 with FDR ≤ 0.05 we identified over 7000 genes that were differentially expressed between P7 and P90 in both the WT and cKO samples (Figs. 1B and C). These data demonstrate that there is a large-scale reorganization of the striatal transcriptome across development and that this occurs to a similar extent in both WT mice and mice lacking MeCP2 from striatal neurons.

We also compared gene expression changes between cKO mice and their WT littermates at P7 and P90. During early postnatal development at P7 we did not detect any significant differential gene expression between genotypes using the aforementioned criteria (Fig. 1D). In contrast, in adult mice at P90 we identified the differential expression of 21 genes in the cKO mice relative to the WT littermates (Fig. 1E, Supplementary Table 1). These results demonstrate that the conditional loss of MeCP2 from striatal neurons leads to changes in the expression of a small number of genes only following postnatal development. Therefore, MeCP2 may not be required for striatal gene regulation during the early stages of development, but rather plays a critical role

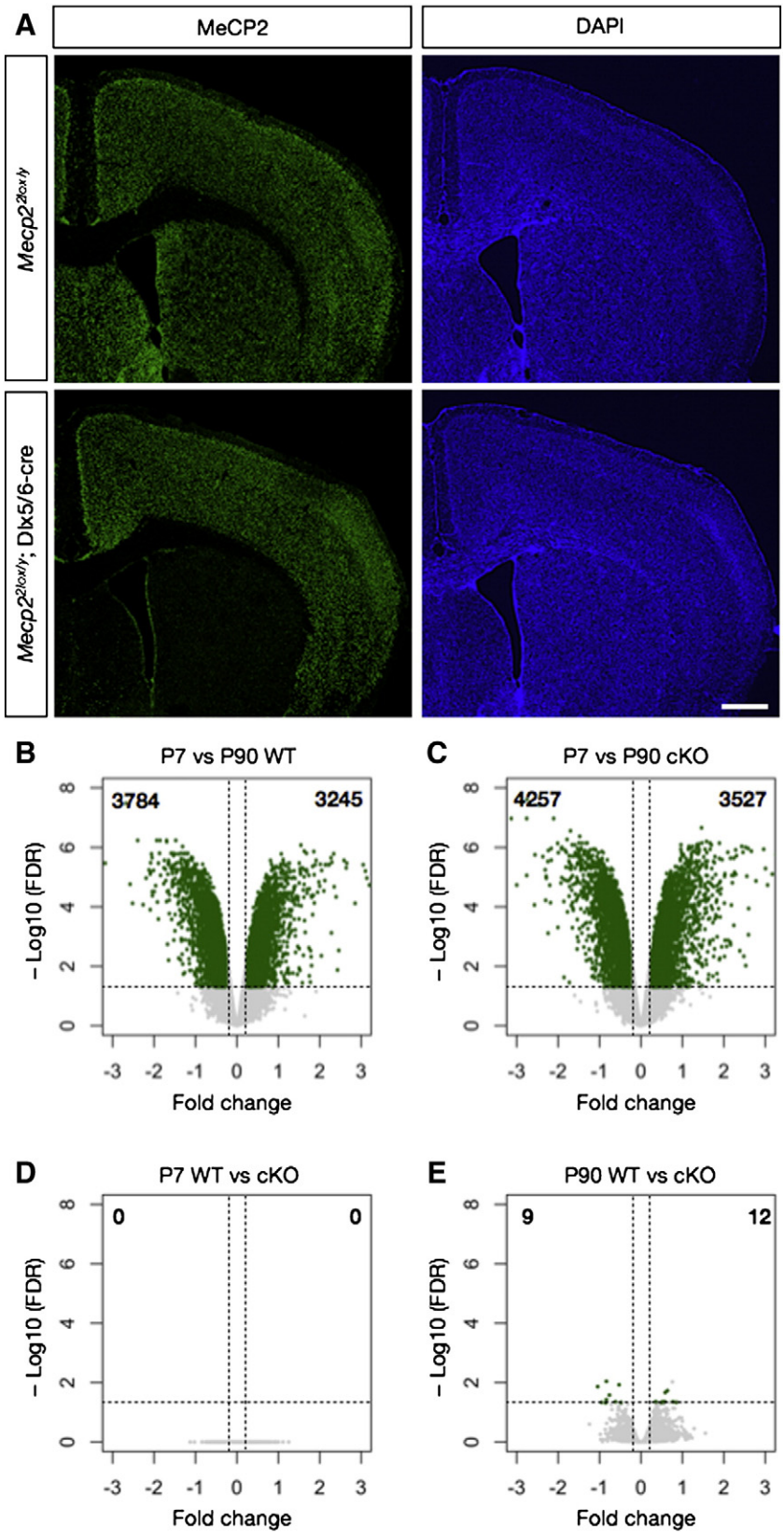

Fig. 1. Gene expression changes in the striatum of *Mecp2* conditional knockout mice. (A) Immunohistochemistry for MeCP2 expression in $Mecp2^{2lox/y}$ and $Mecp2^{2lox/y}$; *Dlx5/6-cre* mice at P90. The presence of *Dlx5/6* leads to a substantial loss of MeCP2 from striatum. Scale bar corresponds to 500 μm. (B-C) Volcano plots depicting fold-change and FDR-correct *p*-values for striatal gene expression between P7 *versus* P90 in WT and $Mecp2^{2lox/y}$; *Dlx5/6-cre* (cKO) mice. (D-E) Volcano plots for gene expression changes between WT and cKO mice at P7 and P90. Green dots represent genes showing a fold change of ≥ 1.2 with FDR ≤ 0.05 that were considered differentially regulated.

in maintaining proper brain function. In support of this hypothesis, conditional deletion of *Mecp2* from adult mice is sufficient to recapitulate numerous RTT-like symptoms (Cheval et al., 2012; McGraw et al., 2011; Nguyen et al., 2012) and restoration of MeCP2 in adult mice can reverse RTT-like neurological phenotypes (Guy et al., 2007).

Differentially expressed genes in the striatum of Mecp2 constitutive knockout mice

The limited number of differentially expressed genes in the striatum of cKO mice is in contrast to previous studies examining gene expression changes in the hypothalamus or cerebellum of constitutive *Mecp2*-null mice where hundreds to thousands of genes are differentially expressed (Ben-Shachar et al., 2009; Chahrour et al., 2008). This difference may result from the differences in cellular composition between brain regions, the identity of the mouse model used, the array platform used or the cell autonomous and non-cell autonomous effects of MeCP2. To address the possibility that the identity of the brain region mediates these differences, we examined gene expression changes in the striatum of the same constitutive *Mecp2*-null mice using the same Affymetrix GeneChip Mouse Exon 1.0 ST Arrays (Exon Array) used in previous studies (Ben-Shachar et al., 2009; Chahrour et al., 2008). Striata were dissected from symptomatic P60 *Mecp2*-null mice (Guy et al., 2001) and their WT littermates, extracted total RNA, converted to cDNA and hybridized to individual exon arrays. Since exon arrays have 1.2 million probe sets with approximately 40 probes per gene, the calculation of gene expression levels is a greater challenge than that required for gene arrays: that is, multiple probes will be mapped to individual genes with varying intensities, leading to difficulty in calculating the level of gene expression. Multiple distinct, but related, methods have been developed to analyze exon array data with respect to the calculation of gene expression levels. To analyze differentially expressed genes in a comprehensive manner we applied four different methods to measure gene expression levels: assessing expression levels using the mean values from individual probes for a given gene, the median values from individual probes for a given gene, the Affymetrix Power Tools (APT) software package and the JETTA software package (Seok et al., 2012).

Using these different methods, we have compiled four lists of genes that are differentially expressed in the striatum of WT and *Mecp2*-null mice. These four lists of genes overlap with one another to a large degree, but unique genes are also identified by each method. Using criteria of fold change of ≥ 1.2 with FDR ≤ 0.05 we identified a total of 127 genes that are differentially expressed in the striatum of *Mecp2*-null mice in at least one of the four approaches (68 up-regulated genes, 59 down-regulated genes) (Supplementary Dataset S1). Of these, 67 were identified using at least two of these analytical methods (Supplementary Dataset S1) and 57% of those genes dysregulated in P90 cKO mice were also differentially expressed in *Mecp2*-null mice (Supplementary Table 1). Importantly, we find that *Mecp2* is significantly down-regulated, whereas interleukin-1 receptor-associated kinase 1 (*Irak1*) is significantly up-regulated in *Mecp2*-null mice. Since the *Irak1* gene is located only 3 kb distal to *Mecp2*, this alteration is likely due to the altered genomic environment caused by deletion of *Mecp2* exons 3 and 4 (Urdinguio et al., 2008).

To verify the exon array analysis, we randomly selected six genes from our list of differentially expressed genes (*Exph5*, *Robo3*, *Drd3*, *Satb1*, *Dsg1c*, and *Dlk1*) and analyzed their mRNA levels in WT and *Mecp2*-null striatal tissues using quantitative real-time PCR (qRT-PCR). We also included *Mecp2* and *Irak1* as positive controls. We found that all eight of these selected genes show expression changes that are consistent with those seen in our exon array data (Fig. 2A), validating our approach in using exon array analysis to identify differentially expressed genes in the striatum of *Mecp2*-null mice.

To characterize the major biological themes present in differentially expressed striatal genes, we assigned gene ontology (GO) annotations to the genes and tested for significant enrichment of functionally related terms. As expected, most of the enriched terms involve neural morphology or function. We found that the 59 down-regulated genes are significantly enriched with functions associated with ion and chemical homeostasis (Fig. 2B), whereas the 68 genes up-regulated in *Mecp2*-null mice are highly enriched for functions associated with transcriptional regulation, development and differentiation (Fig. 2C). Therefore, the differentially expressed genes identified from the striatum are likely functionally related to neuronal function.

Differentially expressed genes in the striatum are distinct from other brain regions

To determine whether gene expression changes upon loss of MeCP2 are consistent across the brain regions, we compared the identified 127 differentially expressed striatal genes with genes previously shown to be significantly altered by loss of MeCP2 (defined as those genes showing fold change of ≥ 1.2 with FDR ≤ 0.05) in the hypothalamus (Chahrour et al., 2008) or cerebellum (Ben-Shachar et al., 2009) between WT and *Mecp2*-null mice. Unsupervised hierarchical clustering of differentially expressed genes between *Mecp2*-null and WT littermates in these three brain regions revealed largely distinct classes of genes affected by the loss of MeCP2 (Fig. 3A). Comparison of these differentially expressed genes revealed that only a small number of genes are altered in all three brain regions (Fig. 3 and Supplementary Dataset S2). Indeed, only 2 genes (including *Irak1*) are commonly up-regulated between brain regions (Fig. 3B), whereas 3 genes (including *Mecp2*) are commonly down-regulated (Fig. 3C). In addition, comparison between differentially expressed genes in the cerebellum of *Mecp2*-null mice and their WT littermates identified using RNA-seq (Mellén et al., 2012) revealed that only three genes were commonly down-regulated (including *Mecp2*) in the striatum. Furthermore, analysis of the GO terms for these differentially expressed genes yields a transcriptional signature for the striatum that is different to that observed in the hypothalamus or cerebellum (Fig. 2B and C) (Ben-Shachar et al., 2009; Chahrour et al., 2008). These data therefore support a model in which MeCP2 regulates gene expression in a region-specific manner.

Similar gene co-expression networks in WT and Mecp2-null mice

Given the observations that MeCP2 binds to 5mC and 5hmC across the genome (Mellén et al., 2012; Skene et al., 2010), it has been suggested that MeCP2 may affect gene functions at a global scale in addition to specific genes. To assess the global effect of MeCP2 on gene transcription, we analyzed gene expression level data from striatum, hypothalamus and cerebellum of *Mecp2*-null mice and their WT littermates using hierarchical clustering algorithms. We found that the overall gene expression profiles are clustered based on brain regions but not genotypes (Fig. 4A). These data demonstrate that the overall biochemical signature of an individual brain region is maintained in the absence of MeCP2.

To gain higher-order and system level insight into how loss of MeCP2 affects the expression of functionally related genes, we next applied weighted-gene co-expression network analysis (WGCNA) to gene expression data from the striatum, cerebellum and hypothalamus. WGCNA examines groups of genes whose expression profiles are highly correlated across samples and give rise to gene modules containing subsets of genes with similar biochemical and functional properties as well as anatomical localization (Johnson et al., 2009; Oldham et al., 2008; Zhang and Horvath, 2005). This technique has recently been used to identify modules of dysregulated genes in autism patients, providing evidence for convergent molecular pathologies in ASD (Voineagu et al., 2011). We therefore constructed a co-expression network using the entire data set, composed of both *Mecp2*-null mice and their WT littermate samples from the striatum, hypothalamus and cerebellum. Gene expression profiles were classified into nine modules (ME1–9) with the expression levels of each module summarized by the first principle component (the module eigengene).

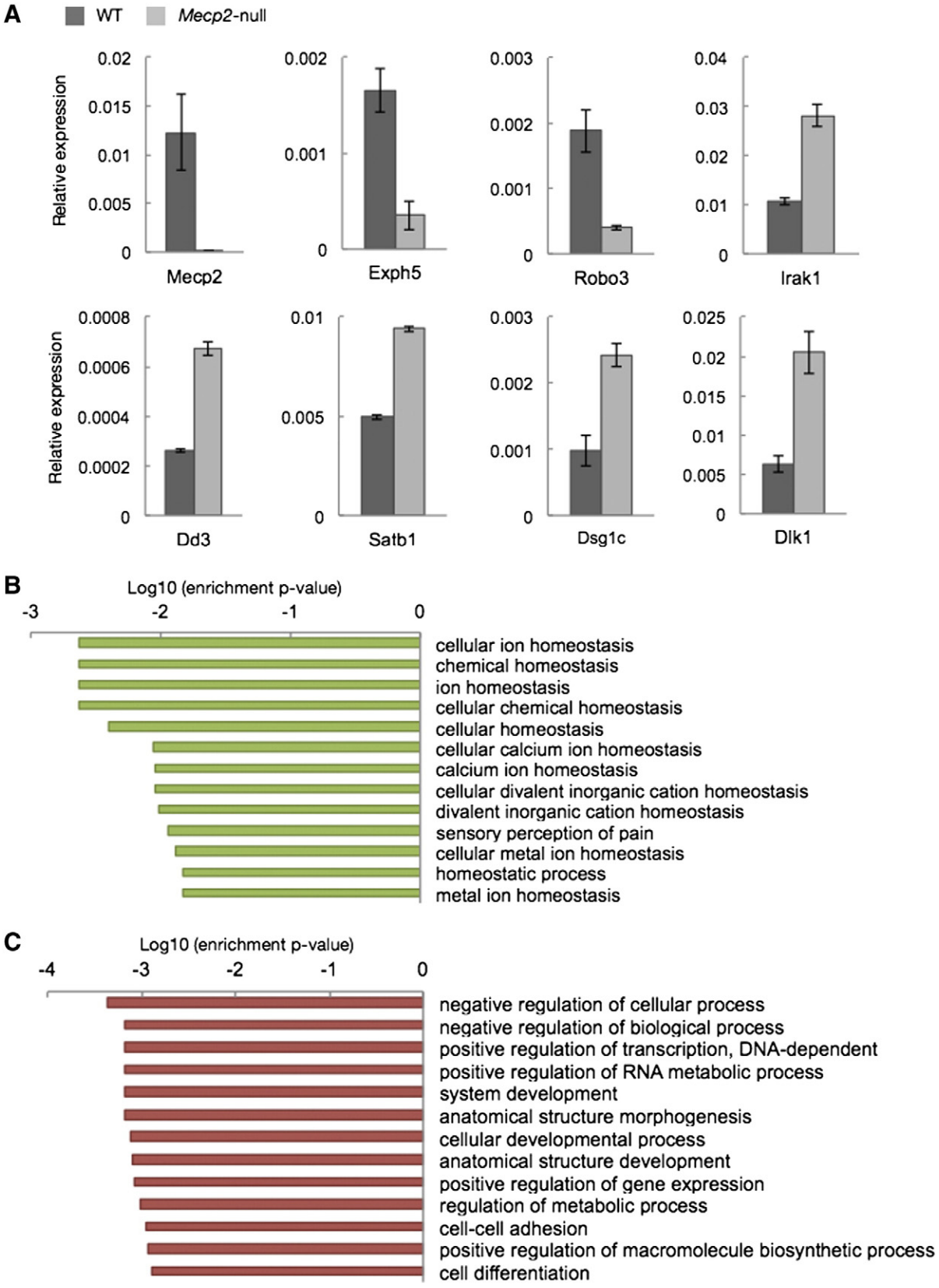

Fig. 2. Gene expression changes in the striatum of *Mecp2* constitutive knockout mice. (A) Validation of differentially expressed genes in the striatum of WT and constitutive knockout (*Mecp2*-null) mice by quantitative real-time RT-PCR. (B) Gene ontology (GO) analysis for genes down-regulated in the striatum of *Mecp2*-null mice compared to their WT littermates. (C) GO analysis for genes up-regulated in the striatum of *Mecp2*-null mice compared to their WT littermates. The enrichment *p*-values were calculated by hypergeometric test and adjusted by the Yekutieli method using GOEAST software.

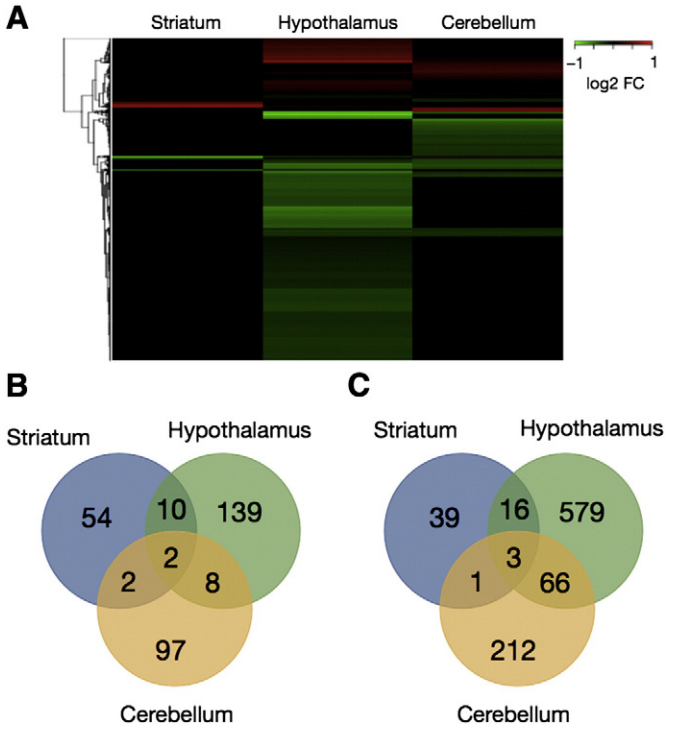

Fig. 3. Differential gene expression in different brain regions of *Mecp2*-null mice. (A) Heat map of the differentially expressed genes in the striatum, hypothalamus and cerebellum of *Mecp2*-null mice. Expression values are color-coded according to the legend to the right. The dendrogram depicts unsupervised hierarchical clustering based on fold-changes. (B) Venn diagram depicting the overlap between genes up-regulated in striatum, hypothalamus and cerebellum. (C) Venn diagram depicting the overlap between genes down-regulated in the striatum, hypothalamus and cerebellum.

We first asked whether global differences in the organization of the brain transcriptome exist between different brain regions. Indeed, eigengene values across all modules were markedly different between the striatum, hypothalamus and cerebellum (Fig. 4B and Supplementary Fig. S2), consistent with the existence of robust modules of co-expressed genes that are related to specific cell types and reflect anatomical localization and functional divergence (Oldham et al., 2008). We then sought to identify discrete groups of co-expressed genes showing transcriptional differences between *Mecp2*-null mice and their WT littermates. We found no significant differences in module eigengenes between WT and *Mecp2*-null mice across the striatum, hypothalamus or cerebellum (Fig. 4B and Supplementary Fig. S2). Therefore, the loss of MeCP2 function does not alter the general organization and molecular distinctions of transcriptomes in different brain regions. Finally, we wanted to determine whether those genes differentially expressed in the striatum were distributed across modules or clustered together. We found that 70% of differentially expressed genes in the striatum are clustered within three modules: 33% of genes within module 1, 25% in module 3 and 12% in module 4 (Fig. 4C). These data suggest therefore that loss of MeCP2 from the striatum leads to alterations in genes that are functionally related to one another. The overall transcriptome differs largely between brain regions rather than between WT and *Mecp2*-null genotypes.

Evaluation of gene expression variation in Mecp2-null mice

MeCP2 has been suggested to organize global chromatin structure and tighten transcriptional regulation by dampening transcriptional noise (Muotri et al., 2010; Skene et al., 2010). This raises the possibility that loss of MeCP2 function may lead to the stochastic dysregulation of genes within a given brain region across individual animals: that is, altered gene expression profiles within different brain regions of *Mecp2*-null mice may reflect an increase in inter-subject variance in

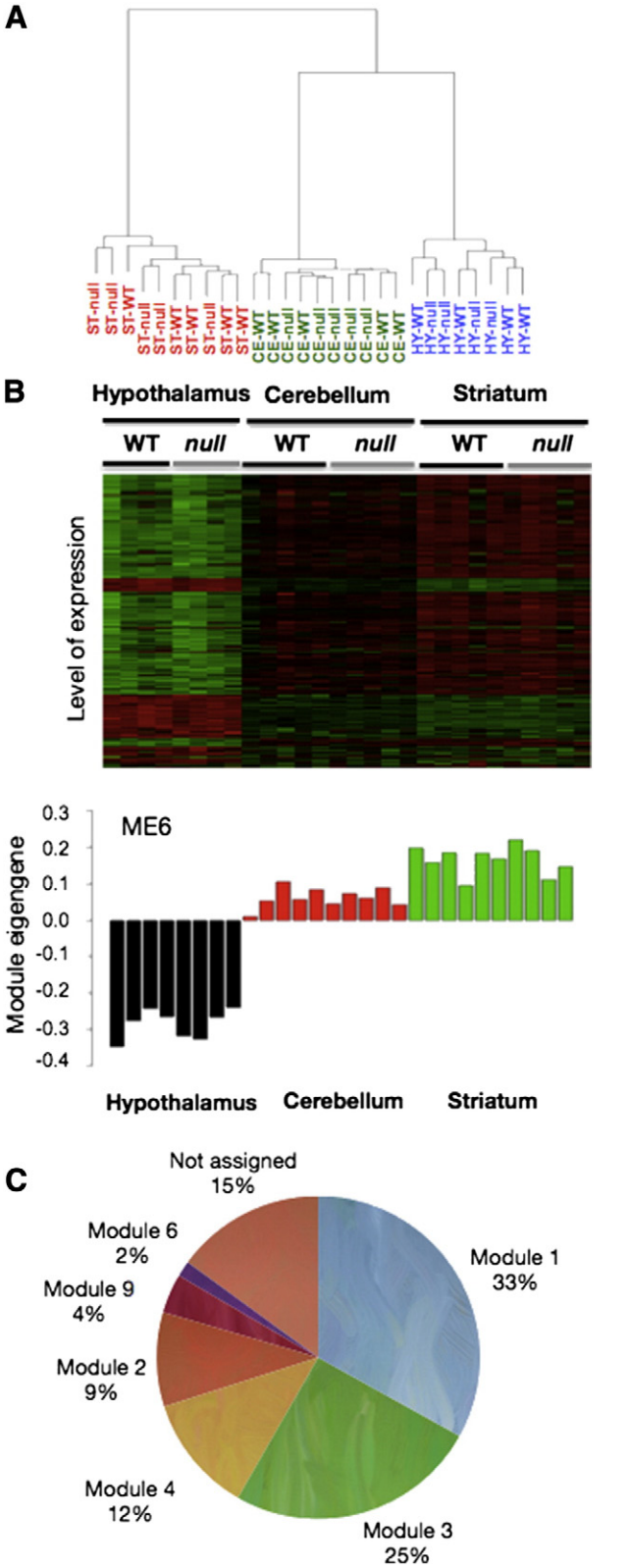

Fig. 4. Gene expression network analysis in different brain regions of WT and *Mecp2*-null mice. (A) Unsupervised hierarchical clustering of the gene expression profiles of the striatum (ST), hypothalamus (HY), and cerebellum (CE) in *Mecp2*-null (*null*) and WT mice. (B, top) Heat map of the gene expression profiles in *Mecp2*-null and WT data sets across three brain regions. (B, bottom) A representative module (ME6) of co-regulated genes generated based on topological overlap of gene expression data from WT and KO mice. A clear separation of eigengene values can be seen based on brain region but not genotype. (C) Localization of differentially expressed genes within modules assigned using WGCNA in the striatum.

gene expression. To examine this possibility we calculated the variance for individual gene expression levels across animals in the striatum, hypothalamus and cerebellum of *Mecp2*-null mice and their WT littermates. However, we found no significant differences in gene expression variance in any of these three brain regions (Bartlett's test, Supplementary Fig. S3). To investigate whether the abundance of gene expression may override individual variation across the transcriptome, we further classified all expressed genes into three groups based on their expression levels (low, medium or high), and calculated expression variance within these individual groups. Again, we found no significant differences in gene expression variance between genotypes in any of these groups (Supplementary Fig. S4). These data indicate that increased variance in gene expression levels is not the major contributor to the differential expression of genes between *Mecp2*-null mice and their WT littermates. Therefore, loss of MeCP2 function is unlikely to modulate gene expression levels through a stochastic disruption of gene regulation processes; rather MeCP2 appears to modulate gene expression through a coordinated, cell-type specific mechanism. Consistent with this, we found that MeCP2 also affects neuronal morphology in a brain region- and cell type-specific manner (Wang et al., 2013).

Loss of MeCP2 function affects both neuronal and non-neuronal genes

Distinct gene expression changes occurring in different brain regions would be expected if those genes were particularly enriched in a single brain region. To investigate this possibility, we isolated liver tissue from *Mecp2*-null mice and normalized gene expression data from the striatum, hypothalamus and cerebellum to that of liver in order to identify region-enriched genes. We next generated a classification scheme by a direct comparison of WT gene expression profiles between our liver dataset and the datasets from the striatum, hypothalamus, and cerebellum (Fig. 5A). Genes exhibiting higher fold expression (fold change ≥ 2 with FDR ≤ 0.05) in the three brain regions relative to liver were considered brain-enriched genes. Among this class of genes, those that only appeared in a single brain region relative to the remaining two were considered specific brain region-enriched genes. Using these criteria, we identified striatum- (622 genes), hypothalamus- (1109 genes) and cerebellum-enriched genes (714 genes) (Fig. 5A and Supplementary Dataset S3). To verify the fidelity of this classification scheme, we obtained the top 50 highly expressed genes in the striatum from the Allen Mouse Brain Atlas and confirmed that 98% of the genes from the atlas are present in our list of striatum-enriched genes (Supplementary Dataset S4). A similar analysis with hypothalamus- and cerebellum-enriched gene lists revealed 83% and 89% overlaps, respectively.

We next examined whether genes differentially expressed between WT and *Mecp2*-null mice were classified as brain region-enriched genes or demonstrated similar expression levels in different brain regions. We found that the majority of genes deregulated in *Mecp2*-null datasets tended to be those expressed across different brain regions or showed similar expression levels in the liver. Indeed, only a small portion, 3 to 10%, of all differentially expressed genes, are enriched in a single brain region (Fig. 5A). Thus, brain region-specific differential gene expression in *Mecp2*-null mice is unlikely to be a consequence of the enrichment of those genes in that particular brain region.

MeCP2 is ubiquitously expressed throughout the body although its function in non-neuronal tissues is not well understood (Shahbazian et al., 2002). To examine whether loss of *Mecp2* function affects gene expression in non-neuronal tissues, we profiled gene expression levels from the liver of *Mecp2*-null mice and their WT littermates. We found that 161 genes are differentially expressed in the livers of *Mecp2*-null compared to their WT littermates (Fig. 5B and Supplementary Dataset S5). Of these genes, 73 are up-regulated and were enriched for lipid, fatty acid and small molecule metabolism as determined by GO analysis (Fig. 5C). Since MeCP2 expression is relatively low in the liver (Shahbazian et al., 2002), these expression changes may be indirectly associated with MeCP2 due to general alterations in metabolism not yet characterized in *Mecp2*-null mice. Nevertheless, these data suggest that *Mecp2*-null mice may have altered liver metabolism consistent with a recent finding that mice lacking MeCP2 show numerous alterations in liver metabolism (Pitcher et al., 2013). It is possible therefore that these altered metabolic processes may also contribute to the pathology of RTT.

Discussion

RTT is a devastating neurological disorder that leads to the manifestation of a number of diverse symptoms including gait apraxia, loss of purposeful hand movements, respiratory abnormalities and loss of communication. The particular symptoms observed in RTT suggest that striatal dysfunction may play an important role in its etiology. Indeed, volumetric analyses of RTT individuals have consistently identified a decrease in the size of the neostriatum, even when taking into account the smaller brain size of these patients (Reiss et al., 1993; Subramaniam et al., 1997). The striatum serves as the input center for the basal ganglia, a subcortical region of the brain which processes and integrates information associated with learning and memory, motor control and various executive cognitive functions. The conditional deletion of MeCP2 from forebrain GABAergic neurons, including MSNs of the striatum, leads to the development of a number of symptoms in mice including impaired motor coordination, ataxia and social deficits (Chao et al., 2010). The role that MeCP2 loss-of-function in the striatum plays in the development of these symptoms, however, is unclear. Similarly, the identity of genes affected by loss of MeCP2 in this region has not been investigated.

In order to examine changes in gene expression within the striatum we utilized two mouse models: mice lacking MeCP2 only from forebrain GABAergic neurons including MSNs of the striatum, and constitutive *Mecp2*-null mice. Using the first model, we profiled gene expression changes in the striatum across development from P7 to P90. We found considerable changes in the gene expression profiles of the striatum during these time points, with over 7000 genes being affected developmentally in both cKO mice and their WT littermates. In contrast, we observed only a small number of differentially expressed genes between cKO mice and their WT littermates. These alterations only occurred at P90 when MeCP2 levels are high and not during early postnatal development. These data suggest therefore that MeCP2 is not required for striatal gene regulation during early development; rather, MeCP2 appears to be essential for maintaining proper brain function at later postnatal stages. Indeed, conditional deletion of *Mecp2* from adult mice is sufficient to recapitulate many RTT-like symptoms and altered hypothalamic gene expression (Cheval et al., 2012; McGraw et al., 2011; Nguyen et al., 2012).

Notably, the number of differentially expressed genes in the striatum is markedly different in the cKO striatum compared to those previously observed in the hypothalamus and cerebellum of constitutive *Mecp2*-null mice (Ben-Shachar et al., 2009; Chahrour et al., 2008). To address the possibility that these differences were the result of different mouse models we repeated our striatal gene expression studies using symptomatic *Mecp2*-null mice and exon arrays similar to previous studies. Indeed, up to 127 genes were differentially expressed in symptomatic *Mecp2*-null mice at P60 compared to WT littermates. The greater number of genes identified in the striatum of *Mecp2*-null mice relative to cKO mice may result from differences in cell autonomous and non-cell autonomous effects of MeCP2 loss-of-function. Most of the genes identified in cKO mice were also identified in *Mecp2*-null mice suggesting that they are indeed regulated by MeCP2.

Among the small number of differentially expressed genes found in the striatum, the majority of the affected genes were not identified in analyses of the hypothalamus or cerebellum. These data suggest that MeCP2 regulates gene expression in a region- or cell-type-specific manner. Furthermore, the majority of these region-specific changes in

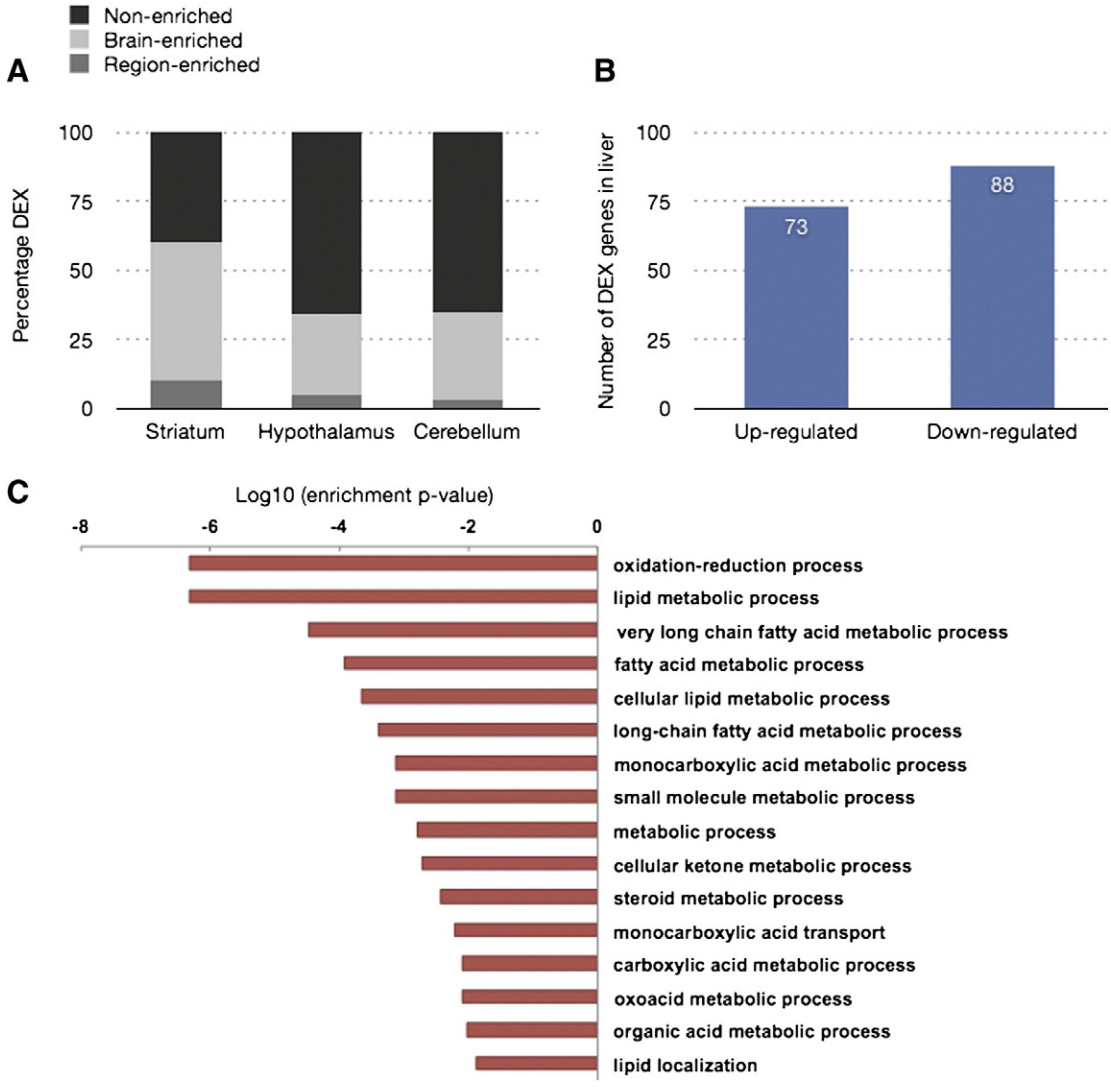

Fig. 5. Distribution of differentially expressed genes (DEX) in WT and *Mecp2*-null mice. (A) Genes differentially expressed in WT and *Mecp2*-null tissues were separated into those that show enrichment within their representative brain region, those enriched in brain *versus* liver, and those that are not enriched. (B) The number of genes up-regulated and down-regulated in the liver of *Mecp2*-null mice compared with WT littermates. (C) Gene ontology (GO) analysis showing biological functions of the genes up-regulated in the liver of *Mecp2*-null mice compared to their WT littermates. The enrichment *p*-values are calculated by a hypergeometric test and adjusted by the Yekutieli method using GOEAST software.

gene expression involve genes that are typically expressed in multiple brain regions and non-neuronal cell types. Distinct gene expression profiles in different brain regions are likely governed by local alterations to chromatin structure in addition to cell-type specific 5mC and 5hmC localization (Mellén et al., 2012; Szulwach et al., 2011). Thus region-specific alterations in gene expression may reflect differential binding of MeCP2 to 5mC and 5hmC, respectively.

Our analysis of the gene expression changes in the striatum revealed considerably fewer genes differentially expressed by loss of MeCP2 relative to the hypothalamus and cerebellum that together exhibited >2000 genes differentially expressed (Ben-Shachar et al., 2009; Chahrour et al., 2008). The larger number of genes altered in these two regions may reflect the greater heterogeneity in cell types within these regions in comparison to the striatum that is primarily composed of MSNs. It would be interesting to determine whether individual cell types, such as granule cells, Purkinje cells and Bergmann glia from the cerebellum, demonstrate a more restricted alteration in gene transcription in *Mecp2*-null mice. Moreover, analysis of gene expression at a systems level using WGCNA revealed modules of genes functionally and anatomically classified based on brain region and are unaltered by loss of MeCP2. However, those genes that are differentially expressed in the striatum cluster within discrete sets of modules arguing that these genes are functionally related to one another. These findings suggest that MeCP2 does not alter the gene expression landscape globally, but rather shows a brain region- or cell-type-specific effect. Thus, while individual gene expression changes in individual cell types may be small, the composite effects, however, lead to global disruption in brain function and the pathogenesis of RTT.

Most of the work regarding MeCP2 has centered around its function in the brain since brain-specific deletion of MeCP2 is sufficient to recapitulate most RTT-like symptoms observed in constitutive *Mecp2*-null mice (Chen et al., 2001; Guy et al., 2001). Although MeCP2 is ubiquitously expressed, its function in other tissues remains unknown. In this study, we also examined gene expression changes in the liver of symptomatic *Mecp2*-null mice and identified 161 genes that were differentially expressed, with 73 upregulated genes being enriched for lipid, fatty acid and small molecule metabolism. These data may indicate that *Mecp2*-null mice may have altered liver metabolic processes. Indeed, it has been suggested that *Mecp2*-null mice have a metabolic disorder (Pitcher et al., 2013). *Mecp2*-null mice were found to have marked increases in sera levels of triglycerides and cholesterol and decreased sera levels of insulin-like growth factor binding-protein 2. These alterations were associated with an increase in fat, but not lean mass and insulin resistance. Since MeCP2 expression remains low in

the liver (M.D. Shahbazian et al., 2002), it is not known whether these alterations in the expression of genes related to metabolism in the liver are directly caused by loss of MeCP2 function in the liver, or are secondary to occur indirectly through altered metabolic pathways in *Mecp2*-null mice. Further study into the function of MeCP2 in the liver and the greater characterization of metabolic processes affected by loss of MeCP2 will provide greater insight into the role of metabolism in the pathogenesis of RTT.

In summary, we identified a number of differentially expressed genes in the striatum of *Mecp2*-null mice. These differentially expressed genes in the striatum were largely unique to those previously identified in the cerebellum or hypothalamus. We have therefore uncovered a previously under-appreciated feature of MeCP2 in regulating gene expression in a region-specific manner within the brain. We demonstrate that differential gene expression in different regions of the *Mecp2*-null brain does not occur due to region-specific expression or increased gene expression variance within a particular functional biochemical network. Differential gene expression is likely caused by other region-specific mechanisms such as local differences in 5mC and 5hmC patterns. Our findings add new possibilities to the molecular function of MeCP2 and identify genes that may be novel therapeutic targets for the treatment of RTT

Materials and methods

Mouse strains, sample collection, and RNA extraction

Experiments were conducted in accordance with the ethical guidelines of the National Institutes of Health and with the approval of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Conditional deletion of *Mecp2* from forebrain GABAergic neurons was achieved by breeding $Mecp2^{flox/+}$ females (Chen et al., 2001) to transgenic Dlx5/6-cre males. Constitutive *Mecp2*-null mice (Guy et al., 2001) were generated by breeding $Mecp2^{-/+}$ females to WT males. All mice were maintained on the C57BL/6 background. Striata were dissected from cKO mice at P7 and P90 and immediately frozen in liquid nitrogen. Striatal and liver tissue were dissected from constitutive Mecp2-null mice at P60 and flash frozen in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA), DNase I treated, and purified using the miRNAeasy mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA).

Microarray target preparation and hybridization

Microarray services were provided by the Penn Microarray Facility, including quality control tests of the total RNA samples by an Agilent Bioanalyzer and Nanodrop spectrophotometry. All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, 100 ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by poly(T) and random oligomers that incorporated the T7 promoter sequence. Second-strand cDNA synthesis was followed by *in vitro* transcription with T7 RNA polymerase for linear amplification of each transcript, and the resulting cRNA was converted to cDNA, fragmented, assessed by the Bioanalyzer, and biotinylated by terminal transferase end labeling. cRNA yields and 5 μg of labeled cDNA was added to Affymetrix hybridization cocktails, heated at 99 °C for 5 min and hybridized to Affymetrix Mouse Exon 1.0 ST GeneChips (for constitutive *Mecp2*-null mice and their WT littermates) or Affymetrix GeneChip Mouse Gene 1.0 ST Arrays (for cKO mice and their WT littermates) according to the manufacturer's instructions.

Microarray data analysis

The bioinformatics analysis of all the microarray datasets used in this manuscript was processed in the MAC terminal window and R environment. The raw CEL files of hypothalamus (accession number GSE11150) and cerebellum (accession number GSE15574) datasets were downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database.

Four methods were used to process the raw exon array data and to calculate gene-level expression values. The Affymetrix power tools (APT) command line program was used to process raw data using the robust multichip average method and generate gene-level expression values based on the core probesets using median polish. The JETTA bioconductor package was used to normalize raw data using the quantile method and to calculate gene-level expression values based on the core probesets using a median polish. The last two methods utilized the affy and xmapcore (exonmap) bioconductor packages. Raw data was processed using the robust multichip average method. Probesets that mapped uniquely to gene exons using the exmapcore package were used for subsequent analysis. Gene-level expression values were calculated using either the median or mean value of all uniquely-mapped exon probsets. Differentially expressed genes were identified using the limma bioconductor package. A linear model was fitted for every gene, and the p-values of differentially expressed genes were calculated using the empirical Bayes moderated *t*-test. Next, multiple comparisons were processed to adjust the raw p-value of *t*-test using Benjamini and Hochberg's method and to generate the false discovery rate (FDR). The significance threshold was set at a fold change ≥ 1.2 with a FDR ≤ 0.05 for identifying differentially expressed genes between *Mecp2*-null mice and their WT littermates. The significance threshold was set at a fold change ≥ 2 with a FDR ≤ 0.05 for tissue and region-enriched genes between the striatum, hypothalamus, cerebellum, and liver datasets. Genes that were identified by at least one of the four methods described were considered to be differentially expressed genes.

The GOEAST online software using default settings was used for Gene Ontology (GO) enrichment analysis. Gene ID conversion was performed using the DAVID Bioinformatics Resources.

Weighted gene co-expression network analysis (WGCNA)

The weighted gene co-expression network was built using the WGCNA package in the R environment. The JETTA package was used to generate the gene expression profiles of WT and constitutive *Mecp2*-null mice, and a network was constructed using all striatum, cerebellum, and hypothalamus datasets. Network constructions were performed using the blockwise modules method in the WGCNA package. Using the scale-free topology criterion, the power of six was chosen to construct the total network. Dynamic hybrid tree cutting was used to cut the hierarchical clustering trees and to identify individual modules. The minimum module size was set to 30 genes and the minimum height for merging modules was set to 0.1. Default parameters were used for other settings.

Quantitative real-time PCR

For each sample, 1 μg of total RNA was digested with DNAseI (Invitrogen, Carlsbad, CA) and reverse-transcribed by oligodT-priming using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA). The amount of each transcript of interest present in the sample was measured by quantitative real-time PCR on 1/10th of the resulting cDNA using SYBR Green detection (Applied Biosystems) on an ABI 7900HT instrument. Each sample was measured in triplicate using exon-spanning qRT-PCR primers. GAPDH amplification served as an internal RNA control for relative quantification. The following mouse primers were used for qRT-PCR analysis: *Gapdh* (F: 5′-AATGGTGAA GGTCGGTGTG-3′; R: 5′-GTGGAGTCATACTGGAACATG-3′), *Mecp2* (F: 5′-CATACATAGGTCCCCGGTCA-3′; R: 5′-CAGGCAAAGCAGAAACATCA-3′), *Irak1* (F: 5′-GTCTTGGATAGCCTGCAACTG-3′; R: 5′-TGAGGGATTTGT CAGAGTGAAC-3′), *Drd3* (F: 5′-TTATCCACATCCCTGAAGCTG-3′; R: 5′-

ACAGTGGGTATTAAGAACGTGAG-3′), *Exph5* (F: 5′-TCGGTCATCCTTTGCT TCAC-3′; R: 5′-AAGGTCTTACACGCTCATCG-3′), *Satb1* (F: 5′-TCTAGGA AGAGGAAGGCTTGG-3′; R: 5′-CGCAGAAAACTGGTAACATGG-3′), *Dsg1c* (F: 5′-GGAGCTTAGAGTTAGAGTGATGG-3′; R: 5′-CAAGTTATTTGGCTCA TCGGC-3′), *Dlk1* (F: 5′-TGTCAATGGAGTCTGCAAGG-3′; R: 5′-ATTCG TACTGGCCTTTCTCC-3′), *Robo3* (F: 5′-TTGTGACTAAGCCCCAGAAC-3′; R: 5′-CTGACTAGGGAAAAGCAGGAC-3′).

Data deposition

The datasets that were generated and reported in this paper have been deposited into the National Center for Biotechnology Information GEO database through accession number GSE42895.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.08.001.

Acknowledgments

We thank members of the Zhou laboratory for critical readings of the manuscript. This work was supported by NIH grants NS058391 and NH091850 (ZZ), International Rett Syndrome Foundation (ZZ and DG), and a Molecular Biology Training Grant (T32-GM072290 to BJ). Z.Z. is a Pew Scholar in Biomedical Sciences.

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