Characterization of MeCP2e1 Transgenic Mice

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Rett Syndrome (RTT), a neurodevelopmental disorder characterized by loss of speech and purposeful limb movement, seizures, breathing abnormalities, and often autistic features, is caused by mutations in methyl CpG binding protein 2 (MeCP2). In 2004, a second isoform of MeCP2, called MeCP2e1, was discovered. While MeCP2e1 appears to be the predominant isoform in the brains of both humans and mice, little is known about its biological function and importance.

To study the role of MeCP2e1 in neurodevelopment, transgenic mice with a mutant *Mecp2* exon 1 translational start site were generated, creating the first known mouse model with targeted deletion of Mecp2e1. Mice were phenotypically characterized using a panel of behavioral and motor function tests, as well as characterization by immuno-staining of Mecp2e1 deficient and hemizygous control cerebral cortices. Results indicate that *Mecp2e1* knockout mice have a distinct phenotype which includes decreased anxiety and decreased sociability. Immuno-staining results appear to confirm the loss of Mecp2e1 in the cortical brain tissue of Mecp2e1 deficient mice. Study results show notable overlap with, as well as significant differences from, previous *Mecp2* mouse models, providing support that *Mecp2e1* transgenic mice may supply a useful and unique model for exploring RTT therapy.

INTRODUCTION

Mutations in *MECP2* leading to defects in methyl CpG binding protein 2 (MeCP2) account for the majority of Rett Syndrome cases (*1*). Rett Syndrome (RTT) is a both mice and humans (*9*). Like the previously studied MeCP2e2 isoform, MeCP2e1 possesses both a methyl DNA neurodevelopmental disorder characterized by loss of speech and purposeful limb movement, seizures, breathing abnormalities, and often autistic features (*2*). An X-linked disorder, RTT primarily affects females, at an incidence of about 1 in 10,000-15,000 births. Patients develop normally for the first 6-18 months of life and then display regression of physical and cognitive development, including deceleration of head growth, loss of verbalization, and loss of purposeful hand movements followed by a period of stabilization (*3*). Since the protein MeCP2 is known to be involved in gene transcription and expression, abnormal epigenetic regulation is thought to be the underlying cause of RTT pathogenesis (*1*).

MeCP2 is a DNA-binding protein that preferentially binds methylated CpG dinucleotide regions of DNA (*4, 5*). *In vitro* studies have lent substantial support to the model of MeCP2 as a transcriptional repressor of methylated promoter regions (*5, 6*). However, continuing studies seem to suggest that the role of MeCP2 extends far beyond transcriptional repression (*7, 8*). After large-scale mapping of neuronal MeCP2 binding sites, it was found that more than half of promoters bound by MeCP2 are actively expressed (*8*). In another study, MeCP2 appeared to activate the majority of gene targets as well as associate with transcriptional activators (*7*).

To further complicate the study of MeCP2 function, a second isoform of the protein generated by alternative splicing was discovered in 2004 (*9, 10*). The new isoform, designated MeCP2e1, appears to be the predominant form in brain tissues of binding domain (MBD) and a transcriptional repression domain (TRD) (*11*). These features point to the role of MeCP2 as a transcriptional

repressor of gene expression. However, MeCP2e1 possesses a unique amino terminus distinct from the MeCP2e2 form due to alternative splicing (*9*). The MeCP2e1 amino terminus may confer unique biological properties to its isoform. Discerning these distinctive properties which may eventually reveal the biological function of each isoform in brain and other tissues (*9, 12*). Differences in MeCP2 isoform function may also explain variation in neurodevelopmental phenotypes associated with mutations in MeCP2e1 or MeCP2e2 (*13, 14*). Variants in exon 1 of *MECP2*, which is present only in MeCP2e1, have been linked to mental retardation and developmental delay (*13*). *MECP2* Exon 1 mutations also seem to be associated with a more severe RTT phenotype (*14*).

Given its predominance in the brain, identifying the biologic function of MeCP2e1 should be relevant to RTT and other disorders. Notably, compared to MeCP2e2, MeCP2e1 is more similar to analogous forms of the protein in lower organisms, including zebrafish and frog (*9*), suggesting that MeCP2e1 is the ancestral form of the protein. As the older protein form, MeCP2e1 may function in a more fundamental and dramatic role than MeCP2e2. Additionally, mice deficient in both isoforms of MeCP2 were only partially cured of neurological symptoms when MeCP2e2 expression was re-introduced (*15*), further suggesting a vital role for MeCP2e1 in the brain. Taking these findings into consideration, it is hypothesized that MeCP2e1 is relevant to RTT pathogenesis and normal brain development.

To study the role of MeCP2e1 in neurodevelopment, transgenic mice with a mutant *Mecp2* exon 1 translational start site were generated. It is predicted that these mice will have a number of physical and neurological features in common with existing *Mecp2* deletion mouse models of RTT. Mice will be subject to behavioral and motor function tests, as well as characterization for the loss of Mecp2e1 by immuno-fluorescent staining. It is expected that the transgenic mice will lack expression of Mecp2e1, while expression levels of Mecp2e2 will be comparable to wild-type mice littermates.

If a mouse model lacking Mecp2e1 is successfully generated, there will be significant implications for future MeCP2 research. The role of Mecp2e1 in normal development may shed light on the isoform's contribution to RTT and other autism-spectrum disorders. This information may someday be used to develop therapies for patients with RTT and similar neurological conditions.

METHODS

Mouse Phenotyping

Mice were scored according to a "Sickness" scoring system with one point assigned for each trait observed in the mouse, including hind limb clasping, forelimb washing, matted fur, skin lesions, absence of curling, body deformity, and reduced ambulation. Hind limb clasping, the retraction and curling of hind paws, and forelimb washing, a repetitive circular motion of the forelimbs, are behaviors only seen in *Mecp2* knockout mice. Matted fur was defined as tufting of the fur that occurs when mice stop grooming. Skin lesions were raw areas of skin resulting from over-grooming. Matted fur and skin lesions could occur on the same mouse. Absence of curling was tested by picking the mouse up by the tail and noting whether the mouse curled its body upward and side to side in escape efforts. Body deformity was defined as a shortening or fattening of the mouse body compared to normal mice. Reduced ambulation was characterized by an observable lack of movement around the cage by the mouse. Scores were averaged and compared weekly for Mecp2e1 deficient males (hemizygous), *Mecp2* females carrying one mutated copy of *Mecp2* (heterozygous) and wild-type females. The Elevated Plus

Maze and Social Novelty Test were performed as previously described (*16*, *17*). Ataxia was assayed by Treadscan (CleverSys Inc, Reston, VA), according to manufacturer's instructions.

Histology

Intact brains were removed from *Mecp2e1* transgenic mice and wild-type littermates at 6-10 weeks, fixed for 72 hours in 4% formaldehyde, washed with 1XPBS and dehydrated in 70% ethanol prior to paraffin embedding and sectioning by microtome as previously described (*18*).

Immuno-fluorescent (IF) Staining

5.0 micron tissue sections were processed and stained with the Mecp2e1 custom antibodies JLN (Aves Inc, Tigard OR), CSN (non-commercial source), and MEN8 (Sigma-Aldrich, St Louis, MO) diluted at 1:100 to 1:200 in immuno-fluorescent (IF) stain buffer [phosphate buffered saline (PBS), 1% fetal calf serum (FCS), and 0.5% Tween-20] as described previously (*19*). Primary antibodies were incubated on slides for 18 hours at 37°C, and then washed five times in PBS/0.5% Tween-20 for five minutes with shaking. Secondary antibodies, including Alexa 488-goat anti-chicken antibody and Alexa 594-goat anti-mouse antibody (Molecular Probes), were diluted 1:100 in IF staining buffer and incubated on slides for 2 hours at 37°C. Five washes with shaking in PBS/0.5% Tween-20 for five minutes followed. Slides were mounted in Vectashield (Vector Laboratories) containing 5µg/ml DAPI, coverslipped, and sealed with nail polish.

Fluorescent Microscopy and Imaging

Slides were viewed with a 100x oil immersion objective on a Zeiss Axioplan 2 (Carl Zeiss, Thornwood, NY) fluorescence microscope equipped with a Retiga EXi high-speed uncooled digital camera (QImaging, Surrey, BC, Canada), fluorescent filter sets, and automated xyz stage controls. A Macintosh computer running iVision (Scanalytics, Vienna, VA, USA) software with Multiprobe, Zeissmover and 3D extensions was used for acquiring and processing slide images. Blue, green, and red filters were used to visualize fluorescence. Images were merged using the same iVision software to create a representative image of total fluorescence. All images were taken in real-time preview mode using a 100x oil objective with a 1x zoom. Each image set used the same exposure times, microscope settings and image processing settings for optimal visual clarity. Slides were not blinded prior to image analysis.

RESULTS

Mecp2e1 knockout mice have a distinct phenotype.

To evaluate the phenotype of the *Mecp2e1* transgenic mice, a panel of behavioral and motor function tests was used. The Elevated Plus Maze, Ataxia (Treadscan) Screening, and Social Novelty Test were chosen based on their appropriateness in previous studies with transgenic mice (*20*). These tests were also chosen because they each assay for characteristics of the Rett Syndrome phenotype in humans (*16*, *20*). In addition to these tests, a "Sickness" scoring system was used to score overall fitness and health in Mecp2e1 deficient and hemizygous males along with heterozygous and wild-type females.

The Elevated Plus Maze (EPM) measures anxiety in mice using a maze assay. In the maze, which is elevated 40-70 cm above a surface, anxiety is measured by comparing the amount of time spent in the open arms of the maze with time spent in arms covered by sidewalls (*17*). Mice with higher levels of anxiety are believed to spend more time in the arms with sidewalls, while less anxious mice generally spend more time in the open arms of the maze (*17*). To control for activity, the number of entries into the closed and open arms is scored simultaneously. In this test,

Mecp2e1 deficient males spent more time in the open maze arms, suggesting that they are less anxious than wild-type littermates. This did not appear to be the result of overall hyperactivity or faster movements by Mecp2e1 deficient mice based on measurements of total distance traveled by mice. Additionally, no significant difference between Mecp2e1 deficient and hemizygous male mice was observed in Ataxia Screening, which measures abnormal locomotion. Similar Ataxia Screening results between Mecp2e1 deficient and hemizygous male littermates further suggest that the results of the EPM are due to differences in anxiety between littermates, not differences in motor function. Taken together, these results indicate lower levels of anxiety in Mecp2e1 deficient mice, contrary to previous findings with total *Mecp2* exon 3 and 4 knockout mice (*20*).

Measurements of sociability were assayed by the social novelty test. In this paradigm, the test mouse is placed into the center of an enclosure with 3 compartments. After an acclimation period, an object mouse is placed in a neighboring transparent compartment. An additional mouse is later placed in another transparent compartment. A video tracking system records the time that the test mouse spends in each compartment socializing with the object mice (*16*). When compared to hemizygous control littermates, Mecp2e1 deficient mice spent more time in an empty compartment than with stranger mice, indicating lower sociability. These results were unexpected given the previous studies of social behavior in *Mecp2* exon 3 and 4 knockout mice (*20*).

A "Sickness" scoring system was developed to account for abnormal phenotypic features, including hind limb clasping, forelimb washing, and reduced curling in response to tail suspension along with the presence of matted fur, skin lesions, gross body deformity, and reduced ambulation. For each abnormal characteristic that a mouse exhibited, one point was assigned. In this assay Mecp2e1 deficient and hemizygous control littermates were observed and scored weekly. By 9 weeks postnatal, Mecp2e1 deficient mice had an average score of 2.875, which was significantly higher ($p>0.0011$) on the "Sickness" scoring system than hemizygous male littermates, which had an average score of zero. Mecp2e1 deficient mice also had significantly higher ($p>0.0465$) "Sickness" scores than heterozygous females, which averaged 0.143. These results suggest that a total loss of MeCP2e1 expression in males is more detrimental to health than the loss of a single Mecp2e1 allele in females that have two alleles.

Mecp2e1 expression appears to be absent in Mecp2e1 knockout mice.

Immuno-fluorescent staining and microscopic analyses were used to qualitatively compare Mecp2e1 and Mecp2e2 expression in cortices of *Mecp2e1* knockout and hemizygous control cerebral cortices. 5.0 micron coronal cross-sections from 11 week old mice were immunostained with CSN and JLN antibodies for Mecp2e1 detection and with MEN8 antibodies for combined Mecp2e1 and Mecp2e2 detection. Because MEN8 staining of neurons is specific for both Mecp2 isoforms, expression of Mecp2e2 was used to compare relative levels of JLN and CSN staining for Mecp2e1. Nuclei and heterochromatic chromocenters were visualized by DAPI staining.

As no commercial antibodies exist for MeCP2e1 detection, both CSN and JLN antibodies were custom made in chicken for the purpose of MeCP2e1 detection. However, designing MeCP2e1 specific antibodies is difficult due to the repetitive amino acid sequence encoded by *MeCP2* exon 1. Nonetheless, the CSN antibody was designed to target amino acids encoded by exon 1 of *MECP2* with some amino acids derived from exon 3 to provide diversity. Similarly, JLN

was designed to target amino acids from exons 1 and 3, with the majority of amino acids from exon 1. Recognition of *Mecp2* exon 1 amino acids is expected to make CSN and JLN antibodies specific for Mecp2e1; however, because each antibody targets slightly different amino acid sequences, it is possible that different immuno-staining patterns would be observed for each antibody. The MEN8 antibody is a commercial product that targets both Mecp2 isoforms as it targets common amino acids from *Mecp2* exon 3.

As expected, immuno-staining with CSN and MEN8 together in control hemizygous mice revealed CSN fluorescence in the nucleoplasm of cortical neurons. Neuronal chromocenters also showed CSN fluorescence in control mice; however, these signals appeared predominantly in the nucleoplasm, similar to previous findings (*19*). This staining pattern suggests that expression of Mecp2e1 is found primarily in neuronal nucleoplasm as well as in neuronal chromocenters. Also consistent with previous findings, MEN8 staining in hemizygous control mice showed strong fluorescent signals in the heterochromatic chromocenters and nucleoplasm of cortical neurons. Together, these results reaffirm the previous hypotheses that total Mecp2 expression is localized to chromocenters and nucleoplasm, while Mecp2e1 is mainly expressed in the nucleoplasm (*19*).

Immuno-staining of MeCP2e1 knockout mice with both CSN and MEN8 antibodies revealed an overall reduction in fluorescent signal in the nucleoplasm and chromocenters of cortical neurons. This effect was most pronounced in the apparent absence of CSN signal in the nuclei of cortical neurons, indicating a lack of Mecp2e1 expression in knockout mice. MEN8 signal was still detectable in the neuronal chromocenters of Mecp2e1 knockout mice, but was much weaker than the signal seen in hemizygous control littermates. This reduction in signal is consistent with the expectation that total Mecp2 fluorescent signal will be diminished in the absence of Mecp2e1. Lack of CSN fluorescence and reduced MEN8 fluorescence suggests that expression of Mecp2e1 is absent in knockout mice.

Although CSN and JLN are designed to preferentially recognize the Mecp2e1 isoform, immunostaining of Mecp2e1 deficient adult cortex suggests that JLN may recognize nuclear proteins other than Mecp2. Overall, combined JLN and MEN8 staining of adult cortex from hemizygous control mice was very similar to the staining patterns seen in CSN/MEN8 combination staining. Consistent with expectations of Mecp2e1 expression, fluorescent signals for JLN were seen mostly in the nucleoplasm, but also in the chromocenters of cortical neurons. As expected in the hemizygous control mice, MEN8 fluorescence was most apparent in neuronal chromocenters, but was also visible in nucleoplasm.

However, immunostaining results from JLN and MEN8 staining of knockout mice deviated from expectations in some key aspects. Unexpectedly, cortical neurons from Mecp2e1 deficient mice exhibited greater JLN fluorescence in the nucleoplasm than hemizygous littermates. This marked difference from CSN staining patterns is likely due to the difference in amino acid sequences used to make each antibody. MEN8 signals seen in combined JLN/MEN8 staining suggest that JLN may be binding to unknown proteins. Similar to the results seen in CSN/MEN8 staining, MEN8 fluorescence was reduced in knockout mice. This result indicates a reduction in total Mecp2 expression, consistent with a loss of Mecp2e1 expression. Results of MEN8 staining indicate a loss of expression of Mecp2e1 in knockout mice, with increased amounts of nonspecific binding by the JLN antibody.

DISCUSSION

Genetically engineered mice were developed to study the biological function of the protein isoform Mecp2e1. Behavioral studies, overall health and fitness assays, and immuno-fluorescent staining of brain cortex were performed on Mecp2e1 deficient mice. Behavioral measures of anxiety revealed lower levels of anxiety in Mecp2e1 knockouts, while sociability measures suggested decreased sociability. Phenotypic abnormalities were measured by a "Sickness" scoring system that assayed for abnormal traits including hind limb clasping, forelimb washing, reduced curling in response to suspension, along with matted fur, skin lesions, gross body deformity, and reduced ambulation. Results indicate that MeCP2e1 deficient males suffered the greatest impact to overall health, while heterozygous females also showed higher "Sickness" levels. *MeCP2e1* wildtype females and hemizygous males did not have significant sickness scores.

Some phenotypic overlap exists between the *Mecp2e1* transgenic mice in this study with other transgenic mice lacking *Mecp2* expression, including mouse models of RTT (*20*-*22*). Unlike exon 3 and 4 knockout mice, Mecp2e1 deficient mice showed anxiety levels lower than controls (*20*). This finding is disconcordant with several other studies, which show negligible differences in anxiety levels (*21*, *23*). However, other mice models of Rett with a truncated form of Mecp2 demonstrate increased levels of anxiety (*22*). These diverse findings indicate the complexity involved in expression of *Mecp2*, depending on the type of mutation involved. Also, the mice in this study are the first known targeted deletion of Mecp2e1. Thus, it is expected that there would be differences in the phenotype of novel Mecp2e1 deficient mice compared to previous total Mecp2 knockouts.

Delay before symptom onset was another feature found in both Mecp2e1 deficient and other *Mecp2* mouse models. Severe neurological symptoms were observed in *Mecp2* exon 3 and 4 knockout mice at 6 weeks postnatal (*20*). Similarly, the "Sickness" score, which may indicate neurological defects, became markedly increased in knockout mice compared to wild-type littermates and heterozygous females at 6 weeks postnatal. Like the total Mecp2deficient mice, heterozygous females also exhibited symptoms of neurological defect. However, the "Sickness" score of Mecp2e1 heterozygous females was significantly lower than that of male knockouts. Previous heterozygous female models of RTT showed their most severe symptoms at 12 weeks postnatal (*20*). However, Mecp2e1 deficient mice have only been scored until 9 weeks of age; therefore, comparisons of later developmental dates will be included in future work.

Immuno-staining performed on the cortical brain tissue of Mecp2e1 deficient mice suggests that expression of *Mecp2e1* is absent in the transgenic mice. CSN, an antibody designed to be specific to MeCP2e1, did not produce specific signal above background in Mecp2e1 deficient mice. Because CSN was created based primarily on amino acids derived from exon 1 of *Mecp2*, which is only present in MeCP2e1, and some sequence from exon 3, these results suggest that Mecp2e1 is absent in the Mecp2e1 transgenic mice. MEN8, an antibody targeted for both MeCP2e1 and MeCP2e2 isoforms, had lower intensity signals in Mecp2e1 deficient mice, consistent with the expectation that the total Mecp2 levels would be less in Mecp2e1 knockout mice. Interestingly, JLN, an antibody designed to be specific to MeCP2e1, showed a higher level of nuclear fluorescent signal in Mecp2e1 deficient mice. Since JLN was created using amino acids from exon 3 and some from exon 1, it is not surprising that the staining patterns would differ from CSN antibody staining results.

Because JLN staining contradicts staining patterns from both CSN and MEN8, it is believed that the increased fluorescence is a characteristic of nonspecific JLN antibody staining, not as the result of specific binding to Mecp2e1.

To clarify the results found in this study, future work will include immuno-staining with an MeCP2e2 specific antibody, as well as Western blotting with the same JLN, CSN, and MEN8 antibodies to quantify amounts of each isoform. MeCP2e2 specific antibodies would allow levels of Mecp2 to be directly observed, rather than inferring expression levels from the MEN8 antibody staining, which recognizes total *Mecp2* expression. Western blotting will provide a more quantitative analysis of expression levels of each Mecp2 isoform and confirm results found in the immuno-staining portion of the study. Results of this study show notable overlap with previous studies of Mecp2 knockout mice as well as interesting differences that may eventually illuminate the biological function of the MeCP2e1 isoform. It is hoped that in the future, these novel Mecp2e1 knockout mice may provide a useful model, separate from total MeCP2 knockouts, for exploring Rett Syndrome therapy.

TABLES AND FIGURES

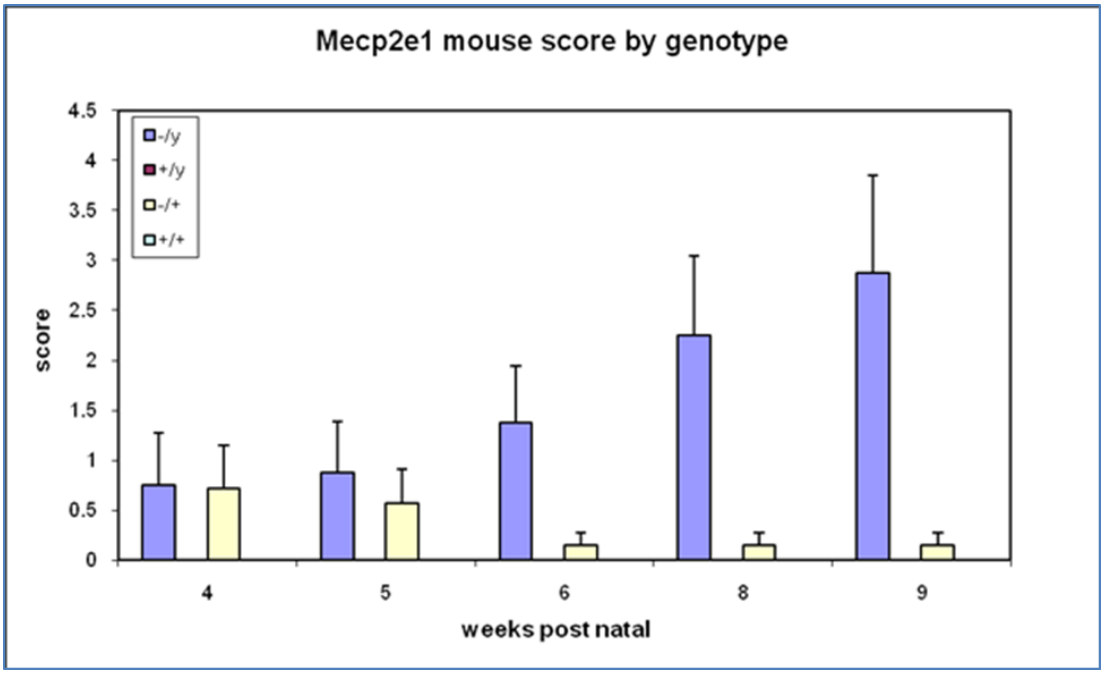

Fig.1 MeCp2e1 knockout mice scored significantly higher on a "Sickness" scale than hemizygous male and wild-type female littermates. The "Sickness" score for knockouts increased from 4 weeks postnatal to 9 weeks postnatal, eventually reaching 2.875, compared to wild-type and hemizygous littermates with scores of 0. MeCP2e1 heterozygous females also scored higher than all littermates besides knockout males. However, "Sickness" scores for heterozygous females decreased by 9 weeks postnatal, stabilizing with a score of 0.143.

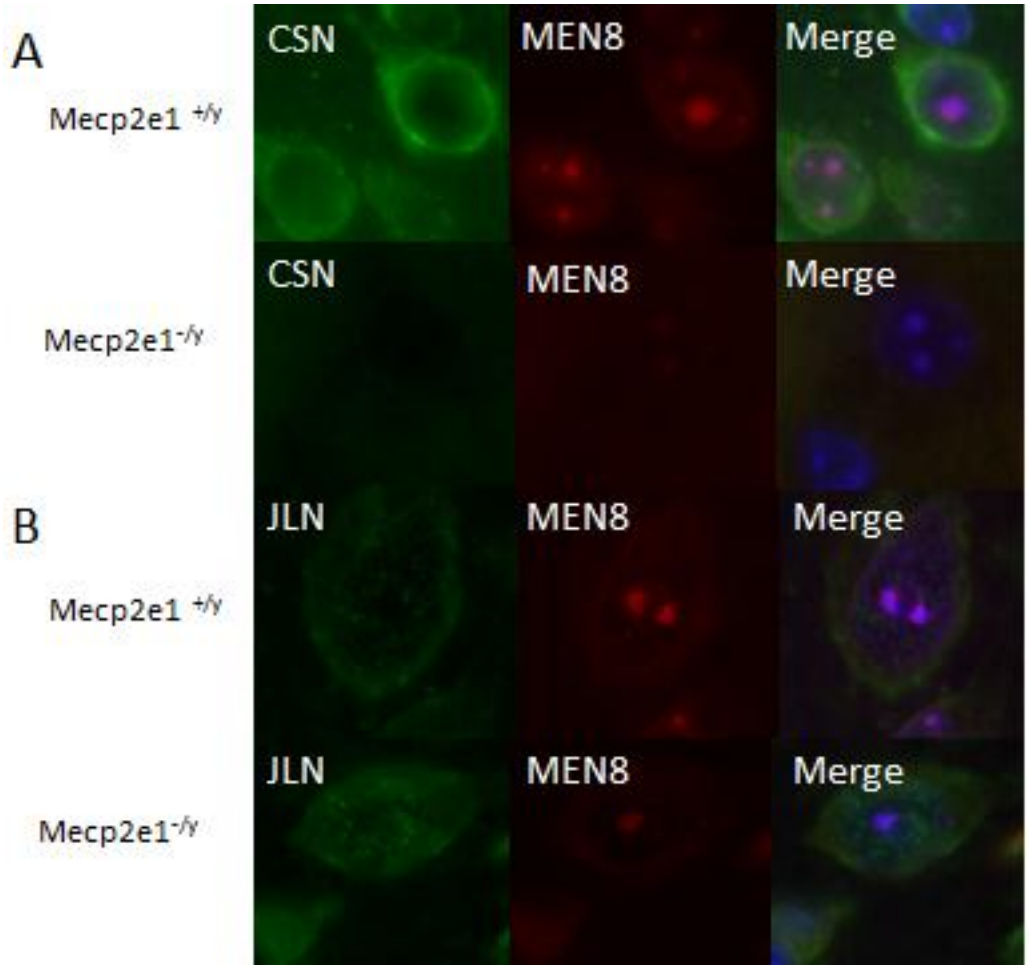

Fig.2 Loss of MeCP2e1 resulted in less MeCP2e1 specific staining in the cortical neurons of mice. A) CSN, an MeCP2e1 specific antibody, showed an absence of staining in knockout mice. MEN8, an antibody specific for both isoforms of MeCP2, showed a lower amount of fluorescence, consistent with expectations. B) JLN, an antibody designed for MeCP2e1 specificity, showed increased amounts of staining in knockout mice. However, MEN8 staining is still reduced in knockouts, indicating an absence of MeCP2e1 expression.

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