Research Report

Ube3a mRNA and protein expression are not decreased in Mecp2R168X mutant mice

Amy Lawson-Yuen, a,b, Daniel Liu, a, Liqun Han, a, Zhichun I. Jiang, c, Guochuan E. Tsai, c, Alo C. Basu, a, Jonathan Picker, a,b, Jiamin Feng, a, Joseph T. Coyle, a,⁎

aDepartment of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478, USA
bDepartment of Genetics, Children’s Hospital Boston, Boston, MA 02115, USA
cDepartment of Psychiatry, Los Angeles Institute at Harbor-UCLA Medical Center, Torrance, CA 90502, USA

ARTICLE INFO

Article history:
Accepted 10 August 2007

Keywords:
Rett syndrome
Angelman syndrome
MeCP2
UBE3A
Mouse model

ABSTRACT

Mutations in the transcriptional repressor methyl CpG binding protein 2 (MeCP2) are responsible for most cases of Rett Syndrome (RS), a severe neurodevelopmental disorder characterized by developmental regression, minimal speech, seizures, postnatal microcephaly and hand stereotypies. Absence of the maternal copy of ubiquitin protein ligase 3A (UBE3A) results in Angelman syndrome, also a severe developmental disorder that shares some clinical features with RS. As MeCP2 regulates gene expression, this has led to the hypothesis that MeCP2 may regulate UBE3A expression; however, there are conflicting reports regarding the expression of Ube3a in Mecp2 null mutant mice. We have generated a novel MeCP2 mutant knock-in mouse with the mutation R168X, one of the most common mutations in patients with RS. These mice show features similar to RS, including hypoactivity, forelimb stereotypies, breathing irregularities, weight changes, hind limb atrophy, and scoliosis. The male mice experience early death. Analysis of Ube3a mRNA and protein levels in the Mecp2R168X male mice showed no significant difference in expression compared to their wild type littermates.

© 2007 Elsevier B.V. All rights reserved.

ARTICLE IN PRESS

1. Introduction

Rett syndrome (RS) is a severe neurodevelopmental disorder characterized by apparently normal initial development followed by slowing of development and head growth (Hagberg et al., 1983, 1985, 2002; Rett, 1966). Purposeful hand skills are lost and replaced by characteristic stereotypies. Spoken language is lost. Patients may also develop seizures, breathing irregularities, sleep disturbance, autistic symptoms and scoliosis. Mutations or deletions in methyl-CpG-binding protein 2 (MECP2) located at Xq28 are detectable in 96% of patients with RS (Amir et al., 1999; Moretti and Zoghbi, 2006). A small percentage of RS patients with early onset seizures have mutations in cyclin dependent kinase-like 5 (CDKL5) (Evans et al., 2005; Scala et al., 2005; Tao et al., 2004). CDKL5 may play a role in phosphorylation and regulation of MeCP2 (Mari et al., 2005).

Because MeCP2 binds to methyl CpG dinucleotides, an early leading hypothesis was that it serves as a global repressor of transcription. However, transcriptional profiling studies have failed to reveal a global de-repression of transcription in the
setting of MeCP2 deficiency (Tudor et al., 2002). An alternate hypothesis that MeCP2 instead regulates transcription in a highly specific and selective manner has thus been raised. Strong evidence for this hypothesis is the finding that MeCP2 regulates BDNF in a calcium- and phosphorylation-dependent manner (Chen et al., 2003; Zhou et al., 2006).

Angelman syndrome (AS) is an imprinting disorder caused by a decrease in or loss of function of the maternal copy of ubiquitin protein ligase E3A (UBE3A) located at 15q11q13 (Kishino et al., 1997; Magenis et al., 1987). This protein, unlike MeCP2, is not a regulator of gene transcription but is involved in the ubiquitination pathway, which targets specific proteins for degradation. AS patients exhibit profound speech deficits, gait ataxia, seizures, characteristic EEG, postnatal acquired microcephaly, sleep disturbance, and an unusually happy demeanor with propensity to paroxysms of laughter (Angelman, 1965; Clayton-Smith and Laan, 2003; Williams et al., 1995). Several of these features are in common with RS, including the speech deficits, acquired microcephaly, sleep disturbance, and seizures. These similarities suggest that UBE3A could be a target for regulation by MeCP2. However, recent studies designed to test this hypothesis yielded conflicting results (Jordan and Francke, 2006; Makedonski et al., 2005; Samaco et al., 2005). The study by Samaco et al. looked at two lines of mutant Mecp2 adult mice, as well as postmortem brain tissue from RS patients (Samaco et al., 2005). One mouse line studied, Mecp2tm1.1Jae, was constructed with an exon 3 deletion and characterized as functionally Mecp2 null (Chen et al., 2001). The other mouse line studied was Mecp2tm1.1Bird, which is Mecp2 null (Guy et al., 2001). They reported a significant reduction in expression of both UBE3A RNA and protein products. Next, Makedonski et al. investigated newborns of one of the same mouse lines studied by Samaco et al. (Mecp2tm1.1Bird) as well as postmortem brain tissue from RS patients and a lymphoblast cell line from an RS patient (Makedonski et al., 2005). They also reported reductions in UBE3A RNA and protein expression. Jordan and Francke then reported studies on both mouse lines, Mecp2tm1.1Jae and Mecp2tm1.1Bird, at 3 and 21 days of age (Jordan and Francke, 2006). However, in direct contrast to the previous two reports, no significant changes in Ube3a RNA or protein expression were detected. This lack of consensus prompted us to investigate Ube3a expression in a novel Mecp2 mutant mouse line designed in our laboratory.

2. Results

2.1. Mecp2R168X mice show RS features

We designed a line of Mecp2 mutant mice, which have the most common mutation associated with RS, R168X, knocked-in the mouse gene (Mecp2R168X) (Bienvenu and Chelly, 2006). This sequence change replaces a codon for arginine with a stop codon. Using site directed mutagenesis, we altered the AGA sequence coding for arginine at codon 168 to a TGA coding for stop (Fig. 1a). The mutant transcript is transcribed and is easily detectable by RT–PCR (Fig. 1b). The amplicon was confirmed to be Mecp2 by sequencing, which also confirmed the presence of the mutation in the mouse line. The mutant transcript is relatively stable compared to the WT transcript (Fig. 1c). The

Fig. 1 – Mecp2R168X mutant mice. (a) An A to T point mutation was engineered to create a premature stop codon in place of an arginine at codon 168. (b) Confirmation of the mutant transcript by RT–PCR. RT–PCR was performed with primers flanking the mutation using cDNA reverse transcribed from WT mouse RNA (lane 1), cDNA reverse-transcribed from mutant mouse RNA (lane 2), WT mouse RNA (lane 3), and mutant mouse RNA (lane 4). The expected 238 bp product was detected in lanes 1 and 2 and confirmed as MeCP2 by sequencing. (c) The Mecp2R168X mutant RNA is relatively stable compared to the WT transcript. Error bars show standard error of the mean. (d) Mecp2R168X mutant mice (right lane) express a small protein which may be a prematurely truncated mutant MeCP2 protein, but do not express the full length WT protein. The WT mice (left lane) express the full length protein.
Mecp2^{R168X} mice do not express full-length wild type Mecp2 (Fig. 1d). These Mecp2 mutant mice differ from the mice previously used to study the impact of Mecp2 on Ube3a expression, which had null mutations.

Male hemizygotes are more severely affected than the female heterozygotes. The males have a shortened on average life span of 85.8 ± 24.2 days (±SD; n=43) (Fig. 2a). We observed forelimb stereotypies, hindlimb atrophy, hypoactivity, and breathing irregularities. The males showed great variability in weight as compared to the wild type littersmates that became more accentuated with age (Figs. 2b and c). While some of the affected males were similar in weight to their unaffected littermates, one subset showed failure to thrive (Fig. 2d) and another showed excessive weight gain (Fig. 2e). By 7 weeks, they showed significant hindlimb clasping (Figs. 2f and g) and breathing irregularities.

The female heterozygotes showed significant symptoms by approximately 6 months. Life span was much longer than that of the males, however, with multiple females surviving more than 1 year. They also showed significant hindlimb clasping and breathing irregularities starting after 6 months of life.

Fig. 2 – Characterization of Mecp2^{R168X} mutant mice. (a) Life span of the Mecp2^{R168X} mutant mice is shortened, with a median life span of 83.5 days (n=45). (b) At 4 weeks of age, greater variance in the weight distribution of the affected males was apparent, with numerous low weight mice. (c) In the mice surviving to 11 weeks, weight variance was also apparent, but many more mice were obese. (d) At 3 weeks, an affected hemizygous Mecp2^{R168X} male (mouse on the right) had difficulty gaining weight while his WT littermate thrived. (e) At 7 weeks, an affected hemizygous Mecp2^{R168X} male (mouse on the right) showed greater weight gain his WT littermate. (f) 7-week-old affected male showing hindlimb clasping. (g) 7-week-old WT male littermate showing normal function of hindlimbs.
2.2. Ube3a and Ube3a-antisense transcript levels are similar in Mecp2R168X mice and wild type (WT) mice

We collected cortical brain tissue samples from seven male Mecp2R168X mice and seven male WT littermates between the ages of 6 and 7 weeks. In this age range, the male mice are severely affected. Using quantitative real-time PCR (qRT-PCR), Ube3a mRNA expression levels showed no significant difference in the affected mice as compared to their WT littermates (Fig. 3a). We also tested expression levels of the Ube3a antisense transcript (Ube3a-ATS) using the same seven pairs of mice (Fig. 3b). UBE3A is imprinted in brain, and so its transcription occurs from only one allele (Rougeulle et al., 1997; Vu and Hoffman, 1997). When UBE3A is not transcribed, an alternate transcript, UBE3A-antisense (UBE3A-ATS), is produced from the antisense strand (Chamberlain and Brannan, 2001; Rougeulle et al., 1998; Runte et al., 2001). Disruption of the maternal Ube3a transcript in murine brain correlates with elevated expression of Ube3a-ATS (Landers et al., 2005). Expression levels between the same WT and the Mecp2R168X groups again showed no significant difference.

2.3. Ube3a protein product levels are similar in Mecp2R168X mice and WT mice

Cortical brain tissue samples from the same seven male Mecp2R168X mice and seven WT littermates were also used to analyze Ube3a protein expression. The samples were analyzed by Western blot (Fig. 4a) and quantified by densitometry relative to a reference protein, β-actin (Fig. 4b). We did not detect any significant difference between the affected males and their WT littermates. In fact, both groups had virtually identical expression levels.

3. Discussion

Conceptually, MeCP2 regulation of UBE3A is an attractive hypothesis, given the similarities between RS and AS. However, the literature is far from a clear consensus. Two groups using mouse models and human post-mortem brain tissue observed decreases in UBE3A RNA and protein in the setting of MeCP2 deficiency (Makedonski et al., 2005; Samaco et al., 2005), while a third group found no change using the same two mouse models as the previous two groups (Jordan and Francke, 2006).

We chose to probe this question using our novel model, which is unique in that it carries a common Mecp2 mutation found in patients with RS. Study of such mutant models with common mutations knocked-in can greatly enrich the information gained by knock-out models. These models may more closely mimic actual disease states and may help to provide information on genotype–phenotype correlations within heterogeneous diagnostic categories. It is possible that some Mecp2 mutations may have dominant negative activity, which could lead to a more complex picture than simple loss of function.

Our studies showed no significant differences between Ube3a mRNA and protein levels between the WT and Mecp2R168X groups. We chose to study males at the 6 to 7 weeks of age because the males are more severely affected than the females, and this is the most affected age range among the males, just prior to death. We hypothesized that if there were a difference in Ube3a expression, it would be best demonstrated in this most severely affected group of mice.

Mecp2 function, however, has recently been demonstrated to be strikingly dynamic (Zhou et al., 2006). Mecp2 is phosphorylated following neuronal activation, which could then lead to very specific time-limited changes in gene expression. One could hypothesize that Mecp2 might truly impact Ube3a expression, but only at discrete and specific times. Mouse model studies, or even post-mortem brain tissue, may have difficulty detecting such time-specific effects. Further studies on neurons in culture may better test the hypothesis that Mecp2 regulates Ube3a. Alternatively, the effect could be for a short time at a very specific age or developmental point, including the prenatal period. Our data at this time, however, do not support the hypothesis.

4. Experimental procedures

4.1. Mouse lines

Site directed mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech). Sequence
alteration was confirmed by sequencing (Massachusetts General Hospital DNA Core Facility). The targeting construct containing a Neomycin resistance (neo) cassette flanked by lox P sites and the mutant sequence was electroporated onto 129SvJ embryonic stem cells. The neo cassette was cloned from pLITMUS-28 (New England Biolabs). Neomycin-resistant colonies were isolated, expanded and characterized by PCR using MeCP2 and Neo cassette-specific primers (sequences available upon request). Cells with the correct recombination were injected into C57BL/6 blastocytes and transferred to pseudo-pregnant females. Chimeric mice were bred with 129S6/SvEv Tac mice. The F1 mice with germ line transmission of R168X mutation were crossed with Cre Recombinase-expressing mice to remove the neo cassette, yielding Mecp2R168X mice. The mice showed no change in Ube3a protein expression in cortical tissue as compared to their WT littermates when analyzed by Western blot. Error bars show standard error of the mean. (b) Western blot showing representative samples from 3 pairs of mice.

Fig. 4 – Ube3a protein expression. (a) Male hemizygous Mecp2R168X mice showed no change in Ube3a protein expression in cortical tissue as compared to their WT littermates when analyzed by Western blot. Error bars show standard error of the mean. (b) Western blot showing representative samples from 3 pairs of mice.

4.3. Western blot analysis

Mouse brain cortex samples were harvested and immediately placed on dry ice, then stored at −80 °C until use. The tissue was disrupted by sonication in lysis buffer (Sigma C3228). Protein was quantified with a bicinchoninic assay (Pierce), and 10 μg was resolved by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Western blots were tested as indicated with a β-actin-specific antibody (Abcam ab8227), a Ube3a-specific antibody (Abcam ab10488) or a Mecp2 N-terminal-specific antibody (Sigma M7443). Secondary antibodies were goat anti-mouse peroxidase conjugate (Sigma A0168) or goat anti-rabbit peroxidase conjugate (Upstate 12-348) as appropriate. All samples were tested at least in duplicate (Fig. 4).

Note added in proof

A BspH1 restriction site was created 3’ of point mutation during the cloning of the mutant construct. The mutant sequence of codons 167 to 172 reads 5′-AGG TGA GAT TCA TGA CCA 3′, while the wildtype sequence reads 5′-AGG AGA GAG CAG AAA CCA 3′. As this is 3′ of the premature stop codon, this will not affect the mutant product.

Acknowledgments

Funding for this study was through NIH grant MH-572901 and from a grant from the American Academy of Pediatrics Section on Genetics and Birth Defects.

REFERENCES


