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Decreased Levels of TMEM70 Observed in Mouse Models of Rett Syndrome

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Abstract

Rett Syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked gene *MECP2*. It predominantly affects females, occurring in approximately 1 in 10,000 births, but has been known to occur in males in rare cases as well. Before mutations in *MECP2* were linked to the occurrence of RTT, it was thought that RTT may be a mitochondrial disease. Several reports have documented altered mitochondrial structure in neuronal tissues collected from patients with RTT and mouse model studies have shown increased oxidative stress in *Mecp2*-null mice. However, the exact cause of mitochondrial dysfunction remains unknown. Given the elevated levels of reactive oxygen species (ROS) found in the neuronal tissues of *Mecp2*-null mice, along with a reduction in coupling, we hypothesised that malfunctioning of ATP synthase is present in neurons expressing mutated *MECP2*. Transmembrane protein 70 (TMEM70) encodes a mitochondrial membrane protein involved in the biogenesis of ATP synthase. To test our hypothesis, we measured levels of TMEM70 in brain slices from RTT mice and compared against wildtype levels using both western blot and immunofluorescence. We found that the concentration of TMEM70 displayed a trend towards decreasing in RTT mice as compared to wildtype. Further work will be needed to determine whether the observed changes are significant.

Introduction

Rett Syndrome (RTT) is a neurodevelopmental disorder known to be caused by mutations in the X-linked gene encoding methyl-CpG binding protein 2 (*MECP2*). Patients with RTT develop normally for the first year, after which they lose acquired speech and develop a variety of neurobehavioral abnormalities, including autism, X-linked mental retardation, and infantile encephalopathy (Chahrour et al. 2008). It predominantly affects females, occurring in approximately 1 in 10,000 births, but has been known to occur in males in rare cases as well. However, due to the X-linked nature of RTT, males with this genetic mutation tend to be affected more severely, with most of them dying before birth or in early infancy.

Before mutations in *MECP2* were linked to the occurrence of RTT, it was thought that RTT may be a mitochondrial disease. Mitochondria are organelles responsible for the production of the majority of a cell's energy. Therefore, disruptions to the mitochondria in the form of gene expression patterns can have direct consequences on normal energy production capacity. Mitochondrial proteins are encoded by both nuclear and mitochondrial genomes, and it has been established that *MECP2* regulates the expression of nuclear genes encoding mitochondrial factors (Kriaucionis et al. 2006; Gibson et al. 2010; Gregersen et al. 2012; Pecorelli et al. 2013). *MECP2* is ubiquitously expressed, so any loss of its function could possibly affect mitochondria in a variety of cells. However, it follows that those cells with high energy demands, such

as muscle and nerve cells, would be most susceptible to damage caused by mitochondrial dysfunction. In fact, mitochondrial diseases often disproportionately affect brain function as compared to other systems. Characteristic symptoms of mitochondrial disease include early symptomatic onset developmental delay, motor and mental regression, dystonia, ataxia, muscle weakness (hypotonia), cardiomyopathy, seizures, gastrointestinal reflux, and impaired function of the respiratory system (Schon and Manfredi 2003). Mitochondrial effects include oxidative damage, including elevated levels of reactive oxygen species (ROS) and a decrease in electron transport chain (ETC) complex activities.

RTT shares many of the clinical presentations, though often not with the same level of severity, in addition to the mitochondrial effects noted in patients with mitochondrial diseases. Tissue samples collected from patients with RTT have shown altered mitochondrial structure and RTT tissues from mouse models have shown evidence of increased oxidative stress (Shulyakova et al. 2017). Studies of tissue in both patients with RTT and *Mecp2*-null mouse models have identified alterations in mitochondrial structure such as abnormally swollen and dumb-bell shaped mitochondria with with vacuolization, abnormally elongated mitochondria with vacuolization and concentric membranous profiles, and oversized mitochondria that had fewer and shorter cristae than normal (Eeg-Olofsson et al. 1988; Ruch et al. 1989; Wakai et al. 1990; Dotti et al. 1993; Cornford et al. 1994; Cardaioli et al. 1999). Similar alterations have also been seen in patients with RTT (Wakai et al. 1990). *Mecp2*-null mouse models have also shown elevated mitochondrial respiration rates and a reduction in coupling, in addition to a decrease in mitochondrial membrane potential (Kriaucionis et al. 2006; Grosser et al. 2012). *Mecp2*-null mice also displayed higher oxidized baseline conditions and reduced activity of the ROS scavenger SOD1 (Grosser et al. 2012). Furthermore, notable mitochondrial factors have shown changes in expression, including several complexes of the ETC (Kriaucionis et al. 2006; Gibson et al. 2010; Pecorelli et al. 2013), factors involved with maintaining functional homeostasis (Forlani et al. 2010; Tanaka et al. 2014), and factors involved with anti-oxidative stress (Grosser et al. 2012; Pecorelli et al. 2013).

Multiple genes related to mitochondrial function have been shown to display altered expression in *Mecp2*-deficient systems (Shulyakova et al. 2017). However, there remain many mitochondrial genes which remain unlinked to regulation by *MECP2*. To help fill this gap, we chose to study transmembrane protein 70 (TMEM70), which encodes a mitochondrial membrane protein involved in the biogenesis of ATP synthase. ATP synthase is responsible for driving protons across the inner mitochondrial membrane, creating ATP in the process. This is known as coupled respiration. Given that *Mecp2*-null mouse models have shown decreased membrane potential and an uncoupling of respiration, we hypothesize that a malfunction of ATP-synthase is occurring in *Mecp2*-null mice.

Materials and Methods

Antibodies

Antibodies utilized in this study are: rabbit anti-TMEM70 (Novus Biologicals), mouse anti-Actin (Novus Biologicals), donkey anti-mouse 555 (Invitrogen), donkey anti-rabbit 488 (Invitrogen), HRP-conjugated donkey anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and HRP-conjugated donkey anti-mouse (Invitrogen).

Western Immunoblots

Whole brain tissues were dissected and homogenized in lysis buffer. Sample concentrations were determined using a spectrophotometer. Equal amounts of protein sample were denatured in loading buffer, heated for 5 minutes, and subjected to SDS/PAGE. Proteins were transferred to PVDF membrane and blocked with 5% nonfat milk in TBST (w/vol; 4g milk, 40ml TBST) for 30 minutes. Membranes were incubated with primary antibodies followed by HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Invitrogen). Quantitation was performed by gathering multiple film exposures, then those film exposures were used for quantitation using ImageJ (Schneider et al. 2012). Antigen expression was compared to β-actin as a loading control.

Immunofluorescence

Brain slices were prepared from adult mice at P50 as previously described (Larimore et al. 2011, 2014, 2017). 60 μm thick brain sections were retrieved from -20°C storage and transferred to PBS. Tissues were incubated in 1% sodium borohydride for 20 minutes. Tissue was washed with PBS, then incubated for 60 minutes in block (5% serum, 1% BSA, 0.3% triton in PBS) then incubated in primary antibody overnight (anti-TMEM70 1:200 with anti-Actin 1:10,000, 1% NHS and 1% BSA). Following primary antibody incubation, samples were incubated for 60 minutes in secondary antibodies (1% NHS and 1% BSA 1:500 anti-mouse 555 and anti-rabbit 488) (Invitrogen Molecular Probes, Carlsbad, CA, USA). Finally, tissue was incubated for 20 minutes in cupric sulfate (3.854 W/V Ammonium Acetate, 1.596 W/V Cupric Sulfate, pH 5). Tissue sections were mounted on slides with Vectashield (Vector Laboratories). Microscopy of immunofluorescent samples were performed using light microscopy with a ZOE Fluorescent Cell Imager (Bio-Rad) using a 20X objective.

Results

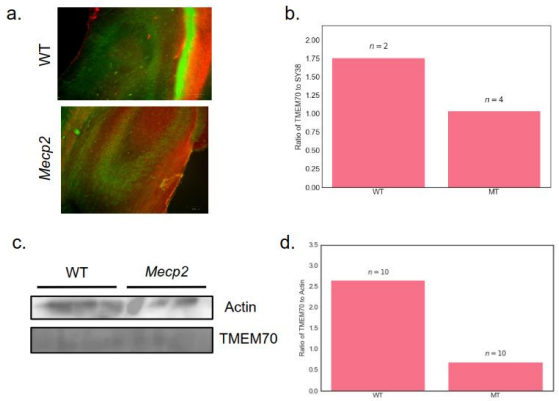

Figure 1. Levels of TMEM70 are lower in *Mecp2*-null mice.

(a) Immunohistochemistry of the hippocampus. Red signifies SY38 and green signifies TMEM70. (b) Average of TMEM70/SY38 samples for wildtype (n = 2) and Mecp2 mutant (n = 4; SEM = 0.206) samples (p = 0.0806, t = 2.33; student's t-test). (c) Levels of TMEM70 as determined by immunoblotting. (d) Average of TMEM70/Actin for wildtype (n=10; SEM = 1.93) and Mecp2 mutant (n=10; SEM = 0.155) samples (p = 0.3083, t = 1.05; student's t-test).

TMEM70 encodes a mitochondrial membrane protein involved in the biogenesis of ATP-synthase. Due to the uncoupling of respiration observed in *Mecp2*-null mice, we hypothesized that there was a malfunction of ATP-synthase in these mice, caused by a decrease in levels of TMEM70. To test this hypothesis, we performed two immunoassays to measure the levels of TMEM70 in brain tissue of wildtype and *Mecp2*-null mice. A student's t-test was employed to analyse the results for both tests.

Immunofluorescence of the dentate gyrus in the hippocampus indicated a trend towards lower levels of TMEM70 in Mecp2 mice as compared to a wildtype control (Figure 1a and 1b), as evident by the larger ratio. The wildtype control has a ratio of 2.63 (n = 2), while Mecp2 mice had a ratio of (n = 4) of TMEM70 to the SY38 control. These results, while not significant (p = 0.0806, t = 2.33), certainly indicate a trend of decreased levels of TMEM70 in Mecp2 mice. Uncoupling increases respiration, leading to an increase in energy production. Lower levels of TMEM70 in the hippocampus are consistent with an uncoupling of respiration, while an increase in energy production is consistent with observed hyperactivity of the hippocampus in RTT mouse models (Calfa et al. 2011).

Immunoblotting of whole-brain lysates indicated a trend towards overall lower levels of TMEM70 in Mecp2 mice as compared to a wildtype control (Figure 1c and 1d). The wildtype control had a ratio of 2.63 (n = 10), while Mecp2 mice had a ratio of 0.681 (n = 10) of TMEM70 to the actin control. Once again, while these results are not significant, as indicated by a student's t-test (p = 0.3083, t = 1.05), there is a clear trend towards lower levels of TMEM70 in Mecp2 mice. Patients with RTT often exhibit motor and mental regression, consistent with defects in normal energy production. A lower level of TMEM70 would directly affect energy production due to the important role it plays in the biogenesis of ATP-synthase. Therefore, these results are consistent with the diminished motor and mental functions observed in these mouse models.

Discussion

In this study, we provide evidence that *Mecp2*-null mice may exhibit a malfunctioning of ATP-synthase. Mutation in *MECP2* is known to lead to RTT. Before RTT was linked to mutations in *MECP2*, it was thought that RTT may be a mitochondrial disease. It has been established that *MECP2* regulates the expression of many nuclear genes encoding mitochondrial factors. Furthermore, while *MECP2* is expressed ubiquitously, and thus would affect mitochondria in all cells, any effects of mitochondrial dysfunction would be most evident in cells with high energy demands, such as those found in the brain. Therefore it is unsurprising that mitochondrial diseases almost disproportionately affect the brain, with characteristic symptoms including early symptomatic onset developmental delay, motor and mental regression, dystonia, ataxia, muscle weakness (hypotonia), cardiomyopathy, seizures, gastrointestinal reflux, and impaired function of the respiratory system. RTT shares many of the clinical presentations, though often not with the same level of severity, as noted in patients with mitochondrial diseases. Furthermore, *Mecp2*-null mouse models have also shown elevated mitochondrial respiration rates and a reduction in coupling, in addition to a decrease in mitochondrial membrane potential.

Due to the uncoupling of respiration observed in *Mecp2*-null mice, we hypothesized that there was a malfunction of ATP-synthase in these mice, caused by a decrease in levels of TMEM70. Using both immunoblotting and immunohistochemistry, we demonstrated that there was indeed a trend to lower levels of TMEM70 in the brain tissue of *Mecp2*-null mice as compared to a wildtype control. These data were not statistically significant, however, suggesting that further work is needed before any definitive statements can be made. Especially in the immunohistochemistry, our sample sizes were small; therefore running more samples may help elucidate whether the observed trend towards lower levels of TMEM70 in *Mecp2*-null mice is truly

significant. A lower level of TMEM70 in *Mecp2*-null mice would support our hypothesis that a malfunctioning of ATP-synthase is occurring in these mice. This could open new avenues in terms of treatment for patients with RTT; drugs targeting ATP-synthase could help ease some of the symptoms these patients have.

It is also possible that lower levels of TMEM70 are an indirect result of Mecp2 loss. Therefore, future work would also include more testing to establish a direct link with Mecp2 knockdown and decrease in TMEM70 levels. It would also be beneficial to directly test mitochondrial activity in live cell cultures to observe ATP-synthase directly. This would be better in terms of determining effects on its functionality in *Mecp2*-null systems. Furthermore, live cell cultures could also be used in testing the effects of TMEM70 knockdown and Mecp2 knockdown in order to determine whether observed mitochondrial effects such as decreased membrane potential and an uncoupling of respiration are a direct result of lower levels of TMEM70, or are somehow more indirectly related to the knockdown of Mecp2.

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