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Research report

Selective preservation of cholinergic MeCP2 rescues specific Rett-syndrome-like phenotypes in *MeCP2*^{stop} mice

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HIGHLIGHTS

- Preserving cholinergic MeCP2 reverses RTT-like hypolocomotion.
- Cholinergic MeCP2 preservation rescues RTT-like anxiety behavior in mice.
- Cholinergic MeCP2 couldn't rescue the hypersocial abnormality of MeCP2-null mice.
- Cholinergic MeCP2 couldn't significantly improve RTT-like poor general condition.
- The rescue effect might not be mediated by the whole brain Chat level alteration.

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ABSTRACT

RTT is a neurodevelopmental disorder characterized by growth regression, motor dysfunction, stereotypic hand movements, and autism features. Typical Rett syndrome (RTT) is predominantly caused by mutations in X-linked *MeCP2* gene which encodes methyl-CpG-binding protein 2 (MeCP2). The brain-abundant MeCP2 protein mainly functions as a transcriptional regulator for neurodevelopment-associated genes. Specific functions of MeCP2 in certain neuron types remain to be known. Although cholinergic system is an important modulating system in brain, how MeCP2 in cholinergic neurons contribute to RTT has not been clearly understood. Here we use a mouse model with selectively activated endogenous MeCP2 in cholinergic neurons in otherwise *MeCP2*^{stop} mice to determine the cholinergic MeCP2 effects on rescuing the RTT-like phenotypes. We found cholinergic MeCP2 preservation could reverse some aspects of the RTT-like phenotypes in mice including hypolocomotion and increased anxiety level, and delay the onset of underweight, instead of improving the hypersocial abnormality and the poor general conditions such as short lifespan, low brain weight, and increasing severity score. Our findings suggest that selective activation of cholinergic MeCP2 is sufficient to reverse the locomotor impairment and increased anxiety-like behaviors at least in early symptomatic stage, supporting future development of RTT therapies associated with cholinergic system.

1. Introduction

RTT is an X-linked neurodevelopmental disorder that predominantly affects girls with the morbidity of about 1 in 10,000. In RTT patients, growth arrest begins to occur after 6–18 months' normal development, followed by regression of acquired skills of locomotion, language and hand use, and the onset of stereotypic hand movements, mental retardation and autistic features of social withdrawal. The patients suffer motor deterioration in late stage of RTT. In addition, seizures and respiratory abnormalities are also common in RTT patients [1]. RTT is also a leading genetic cause of mental retardation in females. Over 95% of typical RTT cases are caused by mutations in the X-linked gene *MeCP2* that encodes the brain-abundant methyl-CpG-binding protein MeCP2 [2], which is involved in transcriptional regulation and chromatin modulation for expression of genes related to neuronal maturation and function maintenance [3].

Abbreviations: RTT, Rett syndrome; MeCP2, methyl CpG-binding protein 2; Chat, choline acetyltrasferase; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band; HDB, nucleus of the horizontal limb of the diagonal band; PPTg, primarily pedunculopontine tegmental nucleus; LDT, laterodorsal tegmental nucleus; PPI, prepulse inhibition.

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Mouse models of *MeCP2* knockout or conditional knockout are used to reproduce aspects of RTT-like phenotypes for studies of the pathogenesis and the therapeutic strategy of RTT [4–6]. Deficiency of MeCP2 in CNS neurons could induce RTT-like phenotypes similar to that of MeCP2-null mice, indicating that the phenotypes are caused by Mecp2 loss in CNS rather than in peripheral tissues [7]. Many studies have reported that MeCP2 in different neuron types play different roles in aspects of RTT-like symptom in mice. Glutamatergic neurons [8], GABAergic neurons [9], aminergic neurons [10] and cholinergic neurons [11] were all proved to be involved in different aspects of the RTT-like behaviors. Functions of brain region-specific MeCP2 have also been explored in basolateral amygdala and hindbrain through conditional knockout [12,13]. However, cell-type-specific mechanisms of MeCP2 deficiency that mediates certain RTT phenotypes still remain to be investigated by selective preservation of MeCP2 in otherwise MeCP2-null mice.

MeCP2$^{lox-stop}$ mice were generated for selective preservation of MeCP2 in specific cells. Using a transgenic Cre-ER expression to allow *MeCP2*$^{lox-stop}$ allele be activated, it has been proved that RTT-like phenotypes are reversible by re-activating endogenous MeCP2 expression [14]. It has also provided a tool for identifying how the cell-specific MeCP2 function affects or rescues the RTT-like phenotypes, as well as the new insights into the contribution of multiple neuron types to the disease and their interplay. Since the rescue effects of neuron type-specific MeCP2 preservations have been previously studied in catecholaminergic neurons and GABAergic neurons [15,16], both of which are modulated by cholinergic system, we wondered whether cholinergic MeCP2 rescue could produce more robust rescue effect than the above 2 down-stream neuron-types or not. Meanwhile, since the loss-of-function mutations of *MeCP2* in RTT patients are global, selective preservation of MeCP2 in a specific neuron population would also be more helpful for developing feasible therapeutic method targeting one certain neuron type such as cholinergic system than using conditional knockout models.

As an important modulating system in brain, cholinergic neurons regulate a variety of neuron types distally and locally while cholinergic MeCP2 loss has been reported to be involved in abnormalities of anxiety-like behavior and social interaction, as well as increased seizure susceptibility in mice. And these dysfunctions could be rescued by the virus-mediated region-specific cholinergic MeCP2 re-expression [11]. However, in cholinergic MeCP2 knockout model, non-cholinergic MeCP2 is preserved, the effect of the regional MeCP2 re-expression might unavoidably involve in functions of non-cholinergic MeCP2. The specific role of cholinergic MeCP2 in RTT is remain to be investigated by selective cholinergic MeCP2 preservation in otherwise MeCP2-null mice. Brain region-specific MeCP2 activation have also been researched in hindbrain, where cholinergic cells also located [13,17], showing effects of improving the short life span and the decreased heart rate caused by global MeCP2 deficiency. Whether MeCP2 in cholinergic neurons including those locate in brainstem was specifically responsible for most of the rescue effects of hindbrain MeCP2 preservation still remain to be explored too.

Given to the important roles of cholinergic neurons in CNS function, further exploring the specific function of cholinergic MeCP2 in RTT through selective preservation of the cholinergic MeCP2 in otherwise MeCP2-null mice, is meaningful for development of cholinergic-related RTT therapy and for determining the hypothesis that if cholinergic MeCP2 deficiency is the primary cause of RTT-like phenotype since cholinergic neurons modulate many non-cholinergic cells like GABAergic and catecholaminergic neurons both are involved in RTT-like symptoms [1]. To study whether activation of MeCP2 in cholinergic system alone could rescue most of the RTT phenotypes in mice, we employed the well-characterized mouse strains *Chat-IRES-Cre* and *MeCP2*$^{lox-Stop}$ mice to produce mice with selective preservation of endogenous MeCP2 in Cholinergic neurons. Behavioral phenotypes of the rescue mice were detected, including locomotion activity, grip strength, anxiety, social interaction, prepulse inhibition and general condition (growth, severity score, heart rate and lifespan). We also attempted to investigate the mechanism underlying the effect of cholinergic MeCP2 rescue.

2. Materials and methods

2.1. Animals

Chat-IRES-Cre mouse strain (Jax. Stock no. 006410) and *MeCP2*$^{lox-Stop}$ mouse strain (Jax. Stock no. 006849) were ordered from Jackson lab. All mice used were housed under 22 ± 1 °C and humidity of 55 ± 5% with food and water *ad libitum*. Both two strains were maintained on C57Bl/6 background. Male *Chat-IRES-Cre*+/− mice were mated with females carrying heterozygous *MeCP2*$^{lox-stop}$ allele to produce males of 4 genotypes: WT, Cre+/−, Stop/y and Cre+/−; Stop/y that was called cholinergic MeCP2 rescue or rescue group for simplicity. All the offspring males were randomly housed 2–5 mice per cage. The investigator remained blind to the genotype of all the tested mice during phenotypic assessment and behavioral tests. All mice were cared and used according to the guidelines of the Animal Advisory Committee of Zhejiang University and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Immunohistochemistry

The WT, Stop, and Rescue mice (8–9 weeks old, n = 2–3) were anesthetized with 4% chloral hydrate and then perfused with 0.9% NaCl solution through heart followed by precooled 4% paraformaldehyde-PBS solution perfusion. The whole brains were fixed with 4% paraformaldehyde for 6 h and then moved to 30% sucrose-PBS solution at 4 °C till sinking. The brains were embedded in OCT for section cutting of 40 μm by a freezing microtome (Leica). Brain slices were collected in PBS with 0.2% sodium azide for storage at 4 °C. When staining was done, slices were washed once in PBS for 5 min and then blocked with 5% normal donkey serum in PBS containing 0.3% Triton X-100 for 1 h at room temperature. Incubation in primary antibodies shaking at 4 °C for 24 h was followed. Primary antibodies were anti-MeCP2 (1:400 in blocking solution, rabbit, Cell Signaling Technology, #3456s) and anti-Chat (1:200 in blocking solution, goat, Millipore, #AB144P). After rinsing with PBST (PBS containing 0.3% Triton X-100), slices were incubated in fluorophore-conjugated secondary antibodies (1:400, Alexa Fluor 488 donkey anti-goat IgG and 1:400 Alexa Fluor 633 donkey anti-rabbit lgG, diluted with the blocking buffer) for 1 h at room temperature. DAPI (1:1000 in PBS) was used to stain cell nuclei for 5–10 min after rinsing with PBST. Slices were moved onto charged glass slides and sealed for confocal imaging of 10 × or 40 × objective (Nikon A1R or Olympus FV-1000).

2.3. Behavioral tests

All the behaviors tested were measured during the light cycle at 8:00-12:00. The tested mice were habituated for 30 min at the room with the same environment as the test room before the tests. The investigator was blinded to all the genotypes after data collection.

2.3.1. Open field test

For study the locomotion activity and anxiety-related behavior, the open field test was conducted. Each mouse was randomly placed to an open field arena (50 cm × 50 cm × 60 cm, zones defined by MED behavioral analysis software) and allowed to explore the