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Article

Choline ameliorates disease phenotype in an iPSC model of Rett syndrome

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Author Contributions

EC characterised the iPSC-derived NPCs and neurons, performed lipids profiling experiments and analysis, Western blots and analysis for ChAT, prepared all figures, and wrote the manuscript. GM reprogrammed and characterised the iPSCs, and the iPSC-derived NPCs and neurons. SY did all electrophysiological recordings and analyses. DM characterised differentiation properties of iPSCs and did Western blots and analysis for ChAT. FR provided critical inputs to the design of the project. GA designed electrophysiology experiments, and provided critical input to data analysis and manuscript. EG initiated and directed the entire study, designed experiments, analysed data, and wrote the manuscript.

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Conflict of interest:

The authors declare that they have no conflict of interest.

Abstract

Rett syndrome (RTT) is a postnatal neurodevelopmental disorder that primarily affects girls. Mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene account for approximately 95% of all RTT cases. To model RTT *in vitro*, we generated induced pluripotent stem cells (iPSCs) from fibroblasts of RTT patients with mutations in their *MECP2* gene. We found that these iPSCs were capable of differentiating into functional neurons. Compared to control neurons, the RTT iPSC-derived cells had reduced soma size and a decreased amount of synaptic input, evident both as fewer Synapsin1-positive puncta and a lower frequency of spontaneous excitatory postsynaptic currents. Supplementation of the culture media with choline rescued all of these defects. Choline supplementation may act through changes in the expression of choline acetyltransferase, an important enzyme in cholinergic signaling, and also through alterations in the lipid metabolite profiles of the RTT neurons. Our study illustrates the potential for using choline as a nutraceutical to treat RTT.

Introduction

Rett syndrome (RTT) is a postnatal neurodevelopmental disorder and is the second leading cause of mental retardation in girls, with an incidence rate of approximately one in 10,000 (Hagberg *et al.*, 1983; Kerr and Stephenson, 1985). Mutations in the gene *MECP2* encoding the X-linked protein methyl-CpG-binding protein 2 (MeCP2) have been found in more than 95% of classical RTT cases, with the severity of the symptoms dependent on the location and type of mutations within the gene (Amir *et al.*, 1999, 2000; Wan *et al.*, 1999; Bienvenu *et al.*, 2000; Huppke *et al.*, 2000; Lee *et al.*, 2001). Approximately 70% of all *MECP2* mutations stem from eight specific missense and nonsense mutations, while C-terminal deletions and sequence rearrangements account for approximately 10 % and 6 % respectively (Chahrour and Zoghbi, 2007).

In order to develop cellular models of complex human diseases, such as RTT, conventional disease modeling techniques may not be the most suitable of platforms. Induced pluripotent stem cells (iPSCs) offer a novel way to investigate mechanisms underlying complex neurobehavioural disorders (Muotri, 2008; Marchetto *et al.*, 2010b; Christian *et al.*, 2012). iPSCs have been generated for several neurological disorders, including Parkinson's disease, amyotrophic lateral sclerosis, spinal muscular atrophy, and RTT (Dimos *et al.*, 2008; Ebert *et al.*, 2009; Soldner *et al.*, 2009, Marchetto *et al.*, 2010a). However, thus far, few such studies take advantage of such model systems to demonstrate a phenotypic rescue of disease-specific defects (Marchetto *et al.*, 2010b). In this study, we aimed to develop an iPSC model for RTT that could recapitulate some of the phenotypic defects of the disorder. In addition, we attempted to rescue the observed defects via a nutraceutical approach, through the use of choline supplementation.

Choline is an essential micronutrient for normal brain development (Fischer *et al.*, 2001; Antony, 2007). Dietary choline deficiency can cause a variety of systemic abnormalities (Zeisel *et al.*, 1991; Zeisel and Blusztajn, 1994). A major pathomorphological abnormality in RTT is the decrease in brain weight and volume by approximately 12% to 34% (Armstrong, 2005). Individual neurons from RTT brains have structural defects, including smaller soma size, reduced dendritic arborizations as well as reduced synapse number and functions (Bauman *et al.*, 1995; Casanova *et al.*, 2003; Armstrong, 2005; Ma *et al.*, 2015). In addition, the cognitive and motor impairments exhibited by RTT patients suggest abnormalities in their central cholinergic system, which is known to modulate these functions (Woolf, 1991; Chahrour and Zoghbi, 2007). Given the physiological roles of choline as the precursor of major cellular membrane components and acetylcholine, the neurotransmitter of cholinergic neurons (Blusztajn and Wurtman, 1983), it is possible that this micronutrient can compensate for the pathophysiology of RTT. We used the iPSC-derived cellular model of RTT that we developed to show that choline supplementation to RTT iPSC-derived neurons can restore the synaptic defects associated with RTT. We further showed that this rescue could in part be mediated via increases in the levels of choline acetyltransferase and/or modulating the membrane lipid composition of RTT neurons.

Materials and methods

Generation and characterization of iPSC lines

The generation of iPSCs from RTT patient fibroblasts has been described previously in Su *et al.* (2015). The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). When the cultures have reached approximately 80 % confluence, the cells were detached using 0.25 % trypsin-EDTA (Gibco). DMEM with 10 % FBS was used to quench the trypsin digestion. The cell suspension was then centrifuged at 200 x g for five minutes, after which the supernatant was removed and the resultant cell pellet was resuspended in transfection solution from Nucleofector™ Kit for human stem cells (Lonza). The cells were electroporated with episomal vectors containing Oct4, c-Myc, Klf4 and Sox2 human cDNAs (Addgene) according to the manufacturer's instructions. After electroporation, the cells were plated in DMEM supplemented with 10 % FBS. The medium was then replaced with mTeSR™1 medium (Stemcell Technologies) 48 hours later. The cell culture medium was changed daily until iPSC colonies were observed (approximately 28 to 35 days later).

When ready for isolation, Dispase (1 U/ml) (Stemcell Technologies) was added to the cultures to gently detach the iPSC colonies. The colonies were then manually cut out and reseeded onto Matrigel-coated plates (BD Biosciences) in mTeSR™1 medium with 10 µM Rho-associated protein kinase (ROCK) inhibitor (Y-27632), or used for further analyses.

Karyotyping analysis was carried out by the Cytogenetics Lab in the Department of Pathology and Laboratory Medicine at KK Women's and Children's Hospital, Singapore. To assess the *in vivo* pluripotency of the iPSC lines, teratoma formation assays were performed by injecting iPSCs subcutaneously into the dorsal flanks of SCID mice. Five to six weeks post-injection, teratomas were dissected and fixed in 10 % buffered formalin phosphate solution. Sections were stained with hematoxylin and eosin for further analysis.

Neural induction of iPSCs and neuronal differentiation

The neural induction of iPSCs and the subsequent differentiation of the neural progenitor cells to neurons have been previously described in Su *et al.* (2015). Briefly, when the iPSCs cultures reached approximate confluence of 20 %, the mTeSR™1 medium was removed and replaced with neural induction media containing DMEM/F12 and Neurobasal media in 1:1 ratio, 1x N2 supplement, 1x B27 supplement, 1x Glutamax, 1x penicillin/streptomycin, 5 µg/mL BSA, 4 µM CHIR99021 (Cellagentech), 3 µM SB431542 (Cellagentech), 0.1µM Compound E (γ-Secretase Inhibitor XXI, EMD Chemicals Inc.), and 10 ng/mL human LIF (Millipore). The cultures were maintained for a week before passaging. The human NPCs (hNPCs) were cultured on Matrigel-coated plates in hNPC maintenance medium consisting of DMEM/F12 and Neurobasal media in 1:1 ratio, 1x N2 supplement, 1x B27 supplement, 1x Glutamax, 1x penicillin/streptomycin, 5 µg/mL BSA, 3 µM CHIR99021, 2 µM SB431542, and 10 ng/mL human LIF. Media was replenished every three to four days.

For neuronal differentiation, hNPCs were dissociated with Accutase™ (Stemcell Technologies) and plated on poly-L-lysine/laminin-coated glass coverslips and plated in neuronal differentiation medium containing DMEM/F12 and Neurobasal media in 1:1 ratio, 1x N2 supplement, 1x B27

supplement, 1x Glutamax, and 1x penicillin/streptomycin. 10 ng/ml BDNF and 10 ng/ml GDNF were added to the culture media every two days. Half of the media was replaced every three to four days during maintenance. In the electrophysiological studies, hNPCs were co-cultured with primary rat cortical neurons and allowed to differentiate, as reported in Su *et al.* (2015). For cells cultured with choline, Choline chloride (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) to make a stock solution of 1 mM. This was then added to the culture media at a final working concentration of 100 μ M every two days for the entire period of culture.

Immunocytochemistry and image analysis

Cells were washed twice with PBS and fixed with 4 % paraformaldehyde for 15 minutes at room temperature. Samples were permeabilized and blocked with 0.1 % Triton X-100 and 5 % donkey serum in Tris-buffered saline (TBS) for an hour at room temperature. Samples were then incubated overnight with the desired primary antibodies at 4 °C. Primary antibodies used were: anti-AFP (1:500; Dako), anti-ChAT (1:500; Abcam), anti-DCX (1:500; Santa Cruz), anti-H3K27me3 (1:500; Millipore), anti-MAP2 (1:500; Sigma), anti-Nanog (1:500; Santa Cruz), anti-Nestin (1:1000; Millipore), anti-Oct4 (1:500; Santa Cruz), anti-SMA (1:500; Sigma), anti-SOX2 (1:500; Santa Cruz), anti-Synapsin1 (1:500; Abcam), anti-Tra160 (1:500; Santa Cruz), and anti-Tuj1 (1:1000; Abcam). This was followed by three washes with TBS with 0.1 % Triton X-100, and incubation with secondary antibodies for two hours at room temperature. Images were acquired using a Zeiss LSM 710 confocal microscope, and analyzed with ImageJ. Quantification of soma size was done by outlining the perimeter of each cell body within a 2-dimensional image of each neuron, and then measuring the area within the outlined area. For counting of Synapsin1 puncta density, segments of Tuj1-positive neurites were imaged and their lengths measured. The number of Synapsin1-positive puncta immunostaining on each of the segments was recorded and expressed per 100 μ m length of neurite. Approximately 100 – 150 cells for each condition were analyzed. At least three independent batches were used for each experiment.

Electrophysiological analysis

Whole-cell patch clamp recordings were performed on iPSC-derived neurons. Recordings were made at room temperature (25°C) in an external solution containing (in mM): 127 NaCl, 2.6 KCl, 23.8 NaHCO₃, 0.77 NaH₂PO₄, 2 MgCl₂, 2.5 CaCl₂, and 10 glucose. Whole-cell voltage clamp recordings were made at a holding potential of -70 mV using a Multiclamp 700B amplifier, filtered at 2 kHz and digitized at 10 kHz with a Digidata 1440A, and acquired/analyzed with PCLAMP software (all from Molecular Devices). Recording pipette resistance ranged from 4-6 M Ω ; series resistance ranged from 10-20 M Ω and was monitored throughout the recordings.

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the presence of the GABA_A receptor antagonist bicuculline (20 μ M). For such recordings, the internal solution was low in chloride ions and contained (in mM): 120 K-gluconate, 9 KCl, 10 KOH, 4 NaCl, 10 HEPES, 1 EGTA, 2 Mg₂ATP, 0.4 Na₃GTP (pH 7.4, 295 mOsm). sEPSCs were identified and isolated using Minianalysis software (Synaptosoft, GA, USA). Events were identified by using a threshold of 5 pA. These events were then manually inspected to ensure that the baseline and peak were accurately determined.

Western blot analysis

After 30 days of differentiation, the cells were washed twice with ice-cold PBS and lysed in cell lysis buffer. Samples were vortexed three times, with a ten minute interval each time, and then centrifuged at maximum speed for 15 minutes at 4 °C. The supernatants were retained and their protein concentrations were measured using the DC™ Protein Assay (Bio-Rad). Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % SDS-PAGE gels under reducing conditions. The proteins were then transferred to polyvinylidene difluoride membranes and blocked with 5 % BSA in TBS with 0.1 % Tween-20. After which, the membranes were blotted with the desired primary antibodies overnight at 4 °C. Primary antibodies used were: anti-ChAT (1:1000; Abcam), anti-Gapdh (1:5000; Cell Signaling), and anti-Tuj1 (1:1000; Abcam). On the following day, the membranes were washed thrice with TBS with 0.1 % Tween-20 and blotted with the secondary antibodies – IgG-horseradish peroxidase conjugates (GE Healthcare). For detection of immunoreactive bands, ECL Western blotting substrate (Thermo Scientific Pierce) was used. For quantification of Western blot bands, the Gel Analyzer function in ImageJ was used.

Sample preparation for lipid metabolomics

Cell cultures were scrapped in ice-cold PBS and pelleted via centrifugation at 3000 rpm for 5 minutes at 4 °C. The cell pellets were then resuspended in 100 µl PBS, with 5 µl kept for protein concentration measurement, and the remainder sent for analysis. Lipid profiling was performed on a fee-for-service basis by the Metabolomics Facility at Duke-NUS Medical School.

Methods of extraction and metabolic profiling of lipids were adapted from Siddique *et al.* (2013) and Sinha *et al.* (2014). The samples were spiked with 20 µl lipid internal standard mix (C₁₇-sphingosine, C₁₇-sphinganine, C₁₇-sphingosine 1-phosphate, C₁₇-sphinganine 1-phosphate, C₁₂-ceramide, C₁₂-ceramide 1-phosphate, C₁₂-glucosylceramide, C₁₂-lactosylceramide, C₁₂-sphingomyelin, and 14:0 phosphocholine; Avanti Polar Lipids). The samples were then de-proteinated by precipitation with methanol. Supernatants were separated from the precipitated proteins via centrifugation and dried under nitrogen gas. The dried extracts were reconstituted in 100 µl methanol before analysis via liquid chromatography-mass spectrometry (LC-MS).

Lipids were separated using an Agilent 1260 liquid chromatography system and a Thermo Scientific Accucore HILIC column (100 x 2.1 mm; particle size 2.6 µm). Mobile phase A consisted of acetonitrile/water (95:5) with 10 mM ammonium acetate, pH 8.0. Mobile phase B consisted of acetonitrile/water (50:50) with 10 mM ammonium acetate, pH 8.0. For the separation, the column was equilibrated with 100 % mobile phase A, increasing to 20 % mobile phase B in five minutes, then held for five minutes. The column was finally re-equilibrated with 100 % mobile phase A for five minutes. Mass spectrometry was done using an Agilent 6430 triple quadrupole mass spectrometer (Agilent Technologies, CA, USA).

Statistical analysis

All experiments were repeated at least three times. Statistical tests were performed using GraphPad Prism 5 software. One-way ANOVA with *post hoc* Bonferroni test was used for comparison between groups. Statistical significance was set at $p < 0.05$.

Results

Reprogramming and characterization of iPSC cell lines from RTT patients

Using a non-integrating episomal approach, we established iPSC lines from two RTT patients, one with a missense MECP2 mutation at nucleotide 916 (C→T) (RTT1), and the other with a 32-nucleotide deletion at nucleotide number 1155, resulting in a frameshift MECP2 mutation (RTT2) (**Fig. 1A**). We also established an isogenic control line from RTT2, denoted as CTRL. This isogenic line is a clone from RTT2 line that has the same genotype as RTT2 but does not express mutant MeCP2. We performed quality control analyses to verify pluripotency: the clones showed positive staining for pluripotency markers such as OCT4, Nanog, Tra1-60, and SSEA4 (**Fig. 1B**, top row), and also for differentiation markers such as AFP, SMA, and DCX (**Fig. 1B**, bottom row). Teratomas containing derivatives from all three embryonic germ layers confirmed that the iPSCs were able to differentiate *in vivo* (**Fig. 1C**). The clones exhibited no chromosomal defects; they had normal karyotypes (**Fig. 1D**), as well as the ability to undergo X-inactivation. Further, the cells immunostained positively for the X-inactivation marker H3K27me3 (**Fig. 1E**).

Characterization of iPSC-derived NPCs and neurons

To characterize the differentiation properties of the RTT iPSC lines, we first induced the iPSCs into hNPCs, which were further differentiated into neurons over a period of approximately 30 days (**Fig. 2A**). Standard markers typically used for identifying NPCs (e.g. Nestin and SOX2) and neurons (e.g. DCX and MAP2) were used for immunostaining of differentiated cells at seven days after NPC induction, and two weeks after differentiation respectively. At Day 7 after induction, all cells in the three cell lines were positive for Nestin and SOX2 (**Fig. 2B**), indicating that they were NPCs. At two weeks after differentiation from hNPCs, the iPSC-derived neurons showed positive immunostaining for DCX and MAP2 (**Fig. 2C**), documenting their neuronal identity. However, there were no significant differences in the number of DCX⁺ neurons produced from the three cell lines, indicating that these cell lines have similar efficiencies in neuronal differentiation (**Fig. 2D**).

Morphological and functional analysis of iPSC-differentiated neurons

We first examined the structural properties of iPSC-derived RTT neurons; it is known that neurons with RTT mutations have smaller cell bodies (Bauman *et al.*, 1995; Ma *et al.*, 2015). We similarly observed smaller soma area in the iPSC-derived RTT neurons – the somatic area of cells in the RTT1 and RTT2 lines were significantly smaller (by 20 – 25 %) relative to the CTRL cells (**Fig. 3A**).

We next used whole-cell patch clamp recording techniques to determine the state of functional maturation of the iPSC-derived cells. As primary neurons have been shown to facilitate the maturation of iPSC-derived neurons (Weick *et al.*, 2011; Verpelli *et al.*, 2013; Su *et al.*, 2015), we cultured iPSC-derived hNPCs together with primary rat cortical neurons. To identify iPSC-differentiated neurons among the co-culture system, we selectively expressed a fluorescent protein, tdTomato, behind a neuron-specific Synapsin1 (Syn1) promoter in the hNPCs. While hNPCs can differentiate to various cell types, only neurons express Syn1. Thus, this strategy allowed us to use tdTomato expression to specifically identify neurons differentiated from the iPSCs (**Fig. 3B**). Whole-cell patch clamp recordings were made from these cells four weeks after neural induction, a time at which the cells had already formed synapses with the rat neurons.

We first assessed the passive electrical properties of these cells by measuring their input resistance and membrane capacitance. Mean input resistance was 1.35 ± 0.12 G Ω for RTT1 and 1.44 ± 0.17 G Ω for RTT2, both of which were higher than the value of 0.94 ± 0.1 G Ω measured for CTRL (**Fig. 3C**). The membrane capacitance of RTT neurons was also lower (32.0 ± 1.8 pF for RTT1 and 22.8 ± 1.6 pF for RTT2) compared to CTRL neurons (32.1 ± 1.8 pF) (**Fig. 3D**). Both the higher input resistance and smaller capacitance indicate that RTT neurons were smaller than their CTRL counterparts, which is consistent with our morphological observations.

To determine if the altered input resistance of RTT neurons affected their excitability, we applied depolarizing current pulses (1 s duration) to evoke action potentials in these neurons. Action potentials were evoked by such current pulses in both iPSC-derived CTRL and RTT cells (**Fig. 3E**). Thus, all three lines of iPSC-derived cells were excitable, as expected for neurons. However, there were differences between the lines in terms of excitability: for a given current pulse amplitude, RTT neurons fired action potentials at a higher frequency than did CTRL neurons (**Figs. 3E and F**). In addition, the peak action potential frequency was higher in RTT neurons, indicating that RTT neurons were intrinsically more excitable than CTRL neurons. In summary, RTT neurons were more excitable than CTRL neurons because both their input resistance and their intrinsic excitability were higher.

RTT iPSC-derived neurons exhibited defects in synaptic transmission

We next examined synaptic transmission by measuring spontaneous excitatory postsynaptic currents (sEPSCs) in the iPSC-derived neurons. Such responses were recorded in the presence of bicuculline (20 μ M), to block inhibitory synaptic transmission, and cells were held at a holding potential of -70 mV to reduce electrochemical driving force on any residual inhibitory synaptic currents. Under these conditions, sEPSCs were observed as brief inward currents, which were blocked by the bath application of CNQX (10 μ M) and AP-5 (50 μ M) (**Fig. 4A**). We found that sEPSC frequency was significantly lower in RTT neurons compared to CTRL neurons (**Fig. 4B**). In contrast, the amplitude of sEPSCs was not significantly different in RTT neurons (**Fig. 4C**). To demonstrate that the decreased sEPSC frequencies were due to the *MECP2* mutations of the RTT lines causing a loss of MeCP2 functions, the MeCP2 protein was knocked down in the CTRL cells. Reduction of MeCP2 in the CTRL neurons similarly resulted in reduced sEPSC frequency, while expression of WT MeCP2 in the RTT1 neurons resulted in increased sEPSC frequency (**Fig. 4D**). There is no significant difference in amplitude of neurons in all the four groups (**Fig. 4E**). Taken together, these data suggest a reduction in the number of synapses as a result of mutations in the *MECP2* gene. We then examined synapse formation by immunostaining with an antibody directed against Synapsin1 (Syn1), a synaptic vesicle protein that is a well-established presynaptic marker (Hilfiker *et al.*, 1999). Consistent with our functional data, we also found a significant reduction in the number of Syn1⁺ puncta in both RTT1 and RTT2 cells as compared to CTRL cells indicating a reduction in the number of synapses (**Figs. 5A and B**).

Choline supplementation ameliorated synaptic transmission defects in RTT iPSC-derived neurons

Structural defects in neurons of RTT brains and abnormalities in the central cholinergic system in RTT patients have been previously reported (Chahrour and Zoghbi, 2007). Given the physiological roles of choline as the precursor of major cellular membrane components and acetylcholine, the neurotransmitter of cholinergic neurons, it is possible that choline can compensate for the pathophysiology of RTT. Mouse and cellular models of RTT, including human RTT iPSCs shown here,

have reported fewer synapses in the RTT neurons (Fukuda *et al.*, 2005; Chao *et al.*, 2007; Marchetto *et al.*, 2010a; Ma *et al.*, 2015). Therefore, we examined if choline supplementation can ameliorate these defects in synapse formation observed in the RTT neurons by counting the density of Syn1⁺ puncta. In the presence of choline, the density of Syn1⁺ synapses in the RTT1 and RTT2 neurons increased to CTRL level (**Figs. 5A and B**). Furthermore, whole-cell patch clamp recordings showed that choline supplementation rescued the changes in sEPSC frequency observed in RTT1 and RTT2 neurons, whereas choline treatment had no effect on sEPSC frequency in CTRL neurons (**Figs. 5C and D**). While sEPSC amplitudes in CTRL neurons were unaffected by choline, there was a trend towards larger sEPSC amplitudes in RTT neurons in response to choline (**Figs. 5C and E**). In summary, our data indicate that choline supplementation is able to ameliorate the defects in synaptic transmission found in iPSC-derived RTT neurons.

Choline supplementation increased choline acetyltransferase (ChAT) expression in iPSC-derived neurons

To gain insight into the molecular mechanisms underlying the ability of choline treatment to improve the synaptic defects of RTT neurons, we examined several possible ways in which choline can exert its physiological functions. Free choline is the immediate precursor of the neurotransmitter acetylcholine, whose synthesis is mediated by the enzyme choline acetyltransferase (ChAT). ChAT is produced in the cell bodies of cholinergic neurons and transported to the nerve terminals, where it exerts its function (Oda, 1999).

To determine the presence of cholinergic neurons in our culture system, we immunostained the cultures with an antibody against ChAT. We observed positive immunostaining of cells in the CTRL, RTT1, and RTT2 lines for ChAT, particularly in the cell bodies (**Figs. 6A and B**). Upon quantification, we found that approximately 12 – 16 % of Tuj1⁺ cells were positive for ChAT in the three cell lines, thus indicating that the NPCs were capable of differentiating into cholinergic neurons under the stated culture conditions (**Fig. 6C**). Culturing CTRL and RTT cells with choline supplementation for three weeks did not affect the percentage of ChAT⁺ cells differentiated (**Fig. 6C**).

Rather than increasing cholinergic differentiation, choline supplementation may increase the expression level of the enzyme that utilizes it. Thus, we next examined the expression of ChAT in the three cell lines. We found a trend of increased levels of ChAT expression in CTRL and both RTT cell lines upon choline supplementation (**Figs. 6D and E**). Treatment with choline increased ChAT expression by approximately 40 – 74 % in CTRL, RTT1, and RTT2 cells, when normalized to the expression of pan-neuronal marker Tuj1 (**Figs. 6D and E**). Therefore, choline could exert its physiological functions in RTT cells, at least in part, by enhancing ChAT expression.

Choline supplementation modulated levels of choline-phospholipid species in RTT iPSC-derived neurons

Choline is predominantly present in cells in the form of the membrane lipids phosphatidylcholine, sphingolipids, and ceramide. Thus, we investigated the levels of these choline-phospholipid species using LC-MS. Overall, we observed an increased trend in the levels of the various groups of phosphatidylcholine, sphingomyelin, sphinganine, sphingosine, and ceramides in the RTT neurons

supplemented with choline (Figs. 7A, B, C, D, and E). When the three different cell lines were compared, we found significant decreases in the sphingomyelin (Fig. 7B), sphinganine (Fig. 7C), sphingosine (Fig. 7C), and ceramide groups (Figs. 7D and E), but not the phosphatidylcholine groups (Fig. 7A), in RTT1 cells, but not RTT2 cells as compared to CTRL cells. However, there were no significant changes in most of these species upon choline supplementation (Figs. 7B, D, and E), except in a few species such as sphinganine (Fig. 7C), and the phosphatidylcholine groups (Fig. 7A). Choline supplementation seemed to have differential effects on the different lipid species. Sphinganine was significantly increased in RTT1 and RTT2 cells treated with choline, but choline treatment of CTRL cells did not show any change (Fig. 7C). On the other hand, choline treatment to RTT2 cells showed an increase trend in all species of the phosphatidylcholine groups, but not in CTRL or RTT1 cells (Fig. 7A).

Discussion

In this study, we generated and characterized an iPSC model of RTT using fibroblasts from patients carrying mutation or deletion in *MECP2* gene. We found that the neurons derived from these RTT iPSCs showed defects in morphology and synaptic transmission. These results are consistent with the deficiencies seen in the neurons in the brains of RTT animal models and RTT patients. Because *MeCP2* is a gene transcription regulator, it is expected that *MeCP2* mutations would adversely affect various organ systems and processes. Moreover, due to its mosaic pattern of expression and dosage-dependency problems (too much *MeCP2* expression leads to another disorder with a clinically similar neurological phenotype – the *MECP2* duplication disorder), it is currently not feasible to re-express the wild-type protein in human RTT patients and attain results similar to those seen in mice by Guy *et al.* (2007). Thus, our strategy was to provide a broad-targeting intervention that would ameliorate the downstream effects caused by mutations in the *MECP2* gene. By doing so, it hopefully can affect multiple pathways and hence alleviate the wide-ranging symptoms of the disorder. Making changes to the nutritional content of the diets of RTT patients seems to be an efficient method for realizing this strategy.

Choline supplementation to the culture medium of iPSC-derived RTT neurons was found to alleviate the synaptic transmission defects associated with RTT. We investigated two possible mechanisms of action of choline and observed that both ChAT expression and levels of choline-related lipid metabolites were increased in the RTT neurons supplemented with choline. The findings thus far present a case for choline as a nutraceutical in the therapy for RTT.

Cholinergic deficits may underlie some of the behavioural abnormalities in RTT, because the central cholinergic system is associated with cognitive and motor functions (Woolf, 1991). Moreover, in RTT patients the number of basal forebrain cholinergic neurons that send diffuse projections throughout the cortex is decreased; further, reduced levels of cholinergic markers have been reported in the basal forebrain, basal ganglia, and midbrain tegmentum of RTT patients (Kitt and Wilcox, 1995; Wenk, 1995; Wenk and Hauss-Wegrzyniak, 1999). This is in conjunction with altered expression of GABA and glutamate receptors (Blue *et al.*, 1999; Johnston *et al.*, 2005; Medrihan *et al.*, 2008). Increased ChAT levels due to choline supplementation may facilitate the synthesis of more acetylcholine, thereby leading to enhanced cholinergic neurotransmission. This is possible either through an increase in the differentiation of cholinergic neurons, or an increase in the amount of ChAT enzyme produced by the cells. Our data suggests that the latter – increasing ChAT expression –

could be how choline supplementation achieves its effects. This potentiates the feasibility of using choline as a nutraceutical in RTT patients, as it is able to work within the existing neuronal network to alleviate the defects.

Other studies have reported decreased phosphatidylcholine turnover in RTT mouse models. In vivo magnetic resonance imaging and spectroscopy of the brains of *Mecp2*^{-y} mice revealed alterations in the profile of choline-containing phospholipids. Decreased lysophosphatidylcholine was found amid increased phosphatidylcholine levels, indicating reduced phosphatidylcholine turnover in *Mecp2*^{-y} mice compared to controls. An increased ratio of phosphocholine to glycerophosphocholine in *Mecp2*^{-y} mice further substantiated this point (Viola *et al.*, 2007). Such a metabolic fingerprint could indicate less phosphatidylcholine production during the growing state and hence, a reduced potential for cellular growth, accounting for the diminished morphology of RTT neurons (Baburina and Jackowski, 1999; Zhang *et al.*, 2006). In this study, we observed a series of minute increases in the levels of various choline-related lipid metabolites – phosphatidylcholine, sphingomyelin, sphinganine, sphingosine, and ceramide. Given that the pool of lipids in a cell is dynamic, it would be most difficult to detect dramatic changes in lipid metabolite levels even with the use of an acute detection technique such as radiolabeling. It is also possible that the different *MECP2* gene mutations carried by the different cell lines contribute to the different compositions of cellular phospholipid pools. Subsequent studies using different clones of iPSC-derived cells reprogrammed from somatic cells of RTT patients carrying a larger array of mutations or deletions will help to establish signature profiles of phospholipids in RTT. These profiles will be useful in further understanding of how phospholipids are affected in RTT, and can be correlated to the severity of the disorder, as well as for investigating how nutraceuticals such as choline can ameliorate the pathogenesis of RTT.

There are many possible ways in which the examined panel of lipid metabolites and their downstream products may contribute to the amelioration of RTT defects. Phosphatidylcholine, sphingolipids, and ceramides are major components of the cellular membrane. Small soma size is a characteristic of RTT neurons, and it is plausible that choline supplementation helps replenish the pool of membrane components needed for larger soma. Furthermore, a downstream product of phosphatidylcholine, glycerophosphocholine, is an important organic osmolyte in neurons (Gallazzini *et al.*, 2006; Fisher *et al.*, 2010). It has been speculated that dysfunctional osmoregulation features in RTT neurons (Viola *et al.*, 2007). Hence, choline supplementation to RTT neurons may aid in their osmoregulation, and thereby improving their morphology. Another way could be through the regulation of synaptic vesicles. The cycle of phosphatidylcholine synthesis and degradation is a driver for vesicular trafficking, whereby altering the rate of either process leads to changes in vesicle secretion (Kearns *et al.*, 1997; Henneberry *et al.*, 2001; McMaster, 2001). This may have implications on the rate of secretion of neurotransmitters, thereby affecting neurotransmission. Thus, changes in lipid metabolites could be an explanation for the increase in the number of synapses and increase in sEPSC frequency detected in the choline-supplemented RTT neurons.

In conclusion, we have generated and verified an iPSC model of RTT, and showed that it was able to recapitulate some of the neuronal defects observed in the disorder. We have shown that choline supplementation can improve the morphology and synaptic transmission of iPSC-derived RTT neurons. We have also provided two possible mechanisms of action through which these effects can be achieved – via increasing ChAT expression levels, and through increasing the different species of

434 lipid metabolites in the neurons. A human iPSC model was used here in hopes of better facilitating
435 the translation of the results to actual RTT patients. Future studies investigating choline
436 supplementation in human subjects can have major public health implications.

Figure legends

Figure 1. Generation and characterization of iPSCs. (A) A schematic diagram showing the location of the mutation (*) in the RTT1 cell line, and deletion (Δ) in the RTT2 cell line, of the *MECP2* gene in the RTT patient fibroblasts used for reprogramming. MBD, methyl-binding domain; TRD, transcription regulation domain; WW, WW-containing domain for modulating interactions with protein ligands. (B) Representative images of iPSCs expressing different pluripotency (top panel) and germ layer differentiation markers (bottom panel). (C) Representative H&E-stained teratoma sections showing the three germ layers – mesoderm (top panel), ectoderm (middle panel) and endoderm (bottom panel). (D) Representative karyogram of iPSCs reprogrammed from fibroblasts of RTT patients showing no karyotypic abnormalities. (E) Representative immunofluorescence image showing iPSCs immunopositive for H3K27me3, a marker for X-inactivation. Scale bars represent 20 μ m (B), 50 μ m (C), 5 μ m (E), or 2 μ m (E - inset).

Figure 2. Characterization of RTT iPSC-derived NPCs and neurons. (A) Schematic overview of the neural differentiation protocol. iPSC colonies were obtained approximately 30 days after reprogramming from fibroblasts. Neural progenitor cells were then induced from the iPSCs and maintained for seven days before further differentiation into neurons. Functionally mature neurons were attained after about 30 days of culturing in neuronal differentiation medium. (B) Neural progenitor cells from all three cell lines (CTRL, RTT1, and RTT2) were positive for neural precursor markers, Nestin and SOX2. (C) Representative images of iPSC-derived neurons showing positive staining for neuronal markers DCX and MAP2. (D) Quantification of the percentage of neuronal differentiation of the CTRL and RTT cell lines, as determined by the percentage of the number of DCX⁺ cells over the total number of cells (DAPI⁺). Scale bars represent 20 μ m. Data shown as mean $\pm$ s.e.m.

Figure 3. Morphological and electrophysiological alterations in RTT neurons. (A) Quantification of the soma area of the iPSC-derived neurons, expressed as a percentage relative to the CTRL cells. (B) Representative fluorescence images of iPSC-derived CTRL and RTT neurons expressing Synapsin1-tdTomato. The reporter fluorescence was used to identify neurons for electrophysiological recordings. Scale bar represents 20 μ m. (C, D) Graphs show input resistance (C) and membrane capacitance (D) of the CTRL and RTT neurons measured at various time points over a period of six weeks. At 3, 4, and 6 weeks respectively, n = 7, 45, 21 (CTRL), n = 8, 33, 24 (RTT1), n = 8, 15, 7 (RTT2). (E) Representative traces show action potentials evoked by currents in CTRL and RTT neurons. (F) Graph shows the relationship between depolarizing current pulse amplitude and action potential frequency for all three types of iPSC-derived neurons. Data shown as mean $\pm$ s.e.m. * p < 0.05; ** p < 0.01; *** p < 0.001; One-way ANOVA, Bonferroni test *post hoc*.

Figure 4. Defects in synaptic transmission of RTT iPSC-derived neurons (A) Representative spontaneous excitatory postsynaptic current (sEPSC) recordings from CTRL and RTT neurons. (B, C) Graphs show mean sEPSC frequency (B) and amplitude (C) in CTRL and RTT neurons. n = 13 (CTRL), 16 (RTT1), and 18 (RTT2). (D, E) Graphs show mean sEPSC frequency (D) and amplitude (E) in CTRL or RTT1 neurons expressing control vector or *Mecp2* cDNA. n = 8 (CTRL + Vector), 6 (CTRL + shMECP2), 10 (RTT1 + Vector), and 9 (RTT1 + MeCP2). Data shown as mean $\pm$ s.e.m. ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001; One-way ANOVA, Bonferroni test *post hoc*.

Figure 5. Amelioration of RTT neuronal phenotype with choline supplementation. (A) Representative images of Synapsin1 immunopositive (Syn1⁺) puncta on Tuj1 immunopositive (Tuj1⁺) neurites of CTRL and RTT neurons, with and without choline supplementation. Scale bar represents 5 μ m. (B) Quantification of the number of Synapsin1 puncta per 100 μ m of Tuj1⁺ neurite. (C) Representative sEPSC recordings from CTRL and RTT neurons untreated, or supplemented with choline. (D, E) Graphs show sEPSC frequency (D) and amplitude (E) of CTRL and RTT neurons cultured with or without choline. For CTRL, n = 6; for CTRL + Choline, n = 5; for RTT1, n = 7; for RTT1 + Choline, n = 8; for RTT2, n = 8; and for RTT2 + Choline, n = 7. Data shown as mean $\pm$ s.e.m. * p < 0.05; ** p < 0.01; *** p < 0.001; One-way ANOVA, Bonferroni test *post hoc*.

Figure 6. Effect of choline supplementation on cholinergic differentiation and ChAT enzyme expression in RTT neurons. (A) Representative images of CTRL, RTT1, and RTT2 iPSC-derived neuronal cultures immunostained for the enzyme ChAT (magenta), pan-neuronal marker Tuj1 (green), and DAPI (blue). White boxes outline the areas enlarged in (B). Scale bar represents 20 μ m. (B) Higher magnification images of Tuj1⁺ cells from CTRL, RTT1, and RTT2 lines with positive immunostaining for ChAT. Scale bar represents 10 μ m. (C) Percentage of cholinergic neurons in CTRL, RTT1, and RTT2 cell cultures untreated, or supplemented with choline, as determined by the proportion of ChAT⁺ cells to Tuj1⁺ cells. n = 3. (D) Representative Western blots of ChAT, Tuj1 and Gapdh expression in CTRL and RTT neurons untreated, or supplemented with choline. (E) Relative expression ChAT/Tuj1 levels in CTRL and RTT neurons cultured with choline. n = 3. Data shown as mean $\pm$ s.e.m.

Figure 7. Lipid profiling of iPSC-derived RTT neurons. CTRL and RTT neurons were cultured in the presence of choline and harvested for lipid metabolite analysis via LC-MS. (A-E) Graphs show levels of the indicated lipid metabolites: phosphatidylcholine (A), sphingomyelin (B), other sphingolipids – sphinganine and sphingosine (C), 1-ceramide (D), and 2-ceramide (E) in CTRL and RTT neurons untreated, or cultured with choline supplementation. The lipid species are grouped according to their degrees of saturation and carbon (C) chain lengths. n = 4. Data shown as mean $\pm$ s.e.m.

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