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Endosomal Trafficking is disrupted in Neurodevelopmental Disorders

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Abstract

Endosomal trafficking has been implicated in several neurodevelopmental disorders including Rett Syndrome (RTT) and Schizophrenia (SZ). Endosomal proteins have been identified in genomic studies in both disorders, suggesting a common molecular mechanism. To characterize endosomal proteins in these disorders, we analyzed protein expression in the cortex and dentate gyrus of the hippocampus as well as in synaptosome

preparations from whole-brain lysates. Immunofluorescent staining demonstrated alterations in key endosomal proteins in coronal brain sections from Mecp2 deficient mice and BLOC-1 deficient mice in the cortex and hippocampus. In addition, biochemical analysis of whole-brain homogenate and synaptosome fractions demonstrated an alteration in endosomal markers. Neurite outgrowth was also significantly decreased in PC12 cells lacking dysbindin and Mecp2. Lastly, to characterize alterations in endosomal trafficking, we used a pulse-chase assay in Mecp2 KD cells and observed altered kinetics of endosomal trafficking. Together, these data further characterize the role of endosomal trafficking mechanisms that underlie neurodevelopmental disorder pathogenesis, highlighting potential biomarkers or therapeutic targets for these disorders.

Keywords: Endosomal Trafficking • Neurodevelopmental • Schizophrenia • Synaptosome

Introduction

Endosomal trafficking has been implicated as a common molecular pathway disrupted in several neurodevelopment disorders such as Autism Spectrum Disorders (ASDs), schizophrenia (SZ), and Rett Syndrome (RTT) [1-4]. Endosomal trafficking regulates the transport of vesicles from the donor membrane to the acceptor membrane within the endosomal organelles including the Golgi, the early endosome, the recycling endosome, and the lysosome [5]. Trafficking within the endosomal pathway is regulated at the organelle by GTPases as well as their activating proteins, coat proteins like Adaptor Protein 3 (AP3), and the biogenesis of lysosome related organelles complex 1 (BLOC-1), as well as SNARE complexes that are specific for that organelle. Within a neuron, endosomal trafficking is necessary for proper dendritic spine outgrowth, synaptic formation, and the recycling of receptors [6-8].

Neurodevelopmental disorders alter emotion, cognition, communication, and learning due to aberrant development of the central nervous system. RTT is a neurodevelopmental disorder characterized by impairments in speech, learning and memory, sensory processing, movement, loss of effective hand use, social anxiety, sleep disturbances, and seizures [8-16]. Because of shared phenotypes, RTT was previously categorized as an ASD (DSM IV) [15,16]. Physical and occupational therapy can improve movement, balance and purposeful hand use, and language therapy can assist with nonverbal communication. Medications can manage some symptoms of RTT including breathing, sleep, GI, and cardiac problems [1,17]. However, a gap remains in our ability to greatly improve the quality of life for patients with RTT due to insufficient understanding of how RTT alters brain function during development.

Research conducted by Amir et al. was among the first to demonstrate that RTT was due to mutations in MECP2 [18]. Since then, over 60 mutations in the MECP2 gene have been identified to result in RTT, with most of these mutations occurring de novo at CpG dinucleotides [19-21]. Even with mutations identified, the precise cellular and molecular mechanisms underlying synaptic defects observed in RTT are not completely understood. To better understand the role of MeCP2, Chahrour et.al. evaluated the transcripts altered in the hypothalamus of MeCp2 deficient mice. They found that Mecp2 regulates the expression of over 2,000 genes in the hypothalamus functioning as a transcriptional activator or repressor [22]. Among those 2,000 mRNAs, endosomal trafficking mRNA's were altered, including GTPases Arf2, Rab3b, Rab13, and Rab15; coat and coat-associated proteins COG, Gga2, Adaptor Protein 1 (AP-1), Adaptor Protein 2 (AP-2) subunits, palladin and cappuccino (subunits of the BLOC-1 complex); and the Arf GAP, Arfgap1 [22]. In addition to the mRNA data which indicates that endosomal trafficking may be altered in RTT, studies in mouse models have also described a potential role of endosomal trafficking in RTT pathology. Studies have demonstrated that Mecp2-deficient neurons lack synaptic plasticity because of reduced levels of EEA1 which is an early endosome maker that docks, fuses, and recycles vesicles [23].

SZ, another neurodevelopmental disorder, is characterized by impairments in speech, learning and memory, sensory processing, disorganized or abnormal motor movement, social processing, sleep disturbances and seizures, similar to RTT [24]. SZ is also characterized by apathy, depression, disorganized thinking, and hallucinations. Genome-wide association studies of patients with SZ have implicated endosomal proteins as well, including subunits of BLOC-1 [25-28]. While there is no singular genetic cause for SZ, the DTNBP1 gene is among the foremost genes associated with SZ risk. DTNBP1 encodes dysbindin, a subunit that forms part of an octameric protein complex, BLOC-1. This octameric complex consists of palladin, snapin, cappuccino, muted, Blos-1 and dysbindin [29]. The reduction of dysbindin within the hippocampus has

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been reported in the post-mortem tissue of patients with SZ [25,26,30,31]. Mecp2 has also been linked to SZ through its interactions with the BLOC-1 complex subunit dysbindin [32].

Endosomal vesicle formation is driven by the coat and coat-associated protein recruitment from the cytosol, which initiates the curvature of the donor membrane to form a vesicle. BLOC-1 is an endosomal coat-associated protein located at the early endosome [33-36]. It is responsible for trafficking vesicles from the endosome to the lysosome or, in neurons, synaptic vesicles targeted to the axon terminal [37]. Mice deficient in BLOC-1 subunits demonstrate a failure to traffic BLOC-1 dependent cargo to the axon terminal, similarly indicating a role for endosomal trafficking in SZ. In order to better understand the role of endosomal trafficking in neurodevelopmental disorders, we analyzed the protein expression of key markers as well as endosomal kinetics in models of RTT and SZ. This work is crucial to clarify the relationship between endosomal trafficking and neuronal development.

Materials and Methods

Mice

Mecp2 mice (Mecp2$^{tm1.1Jae/y}$, "Jaenisch" strain) and Bloc1s8$^{sdy/sdy}$ (sandy) are maintained in C57BL/6 background were a gift from Dr.Victor Faundez, Emory University. Mice were housed at Emory University according to approved IACUC protocols.

Stable infected PC12 cells

PC12 adherent cells were purchased from ATCC. Cells were maintained in 5% CO2 and complete media according to the manufacturer's instructions. Lentiviral shRNAs were in pLKO.1 vector with a resistance to puromycin (Dharmacon). Cells were infected with RNAi MAX (Invitrogen) following manufacturer protocol. Puromycin was added to the media 48 hours after infection. Cells were maintained in the presence of puromycin. Cells were plated on poly-D-lysine coverslips and imaged on ZOE cell imager (BioRad).

Antibodies

Mouse anti-actin, mouse anti-AP-3 (delta subunit), mouse anti-chromogranin A and mouse anti-LAMP1 monoclonal antibody were purchased from Developmental Studies Hybridoma Bank (DSHB, University of Iowa, Iowa City, IA). Goat anti-EEA1 polyclonal antibody was purchased from Thermo Fisher Scientific (PA5-17228). Rabbit anti-Rab11 polyclonal antibody was purchased from Invitrogen (71-5300). Secondaries anti-goat 555, anti-mouse 488, and anti-mouse 568 were purchased from Invitrogen. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoblot analysis

Lysates were separated by SDS–PAGE and transferred to PVDF membranes (BioRad, Hercules, CA). Membranes were probed with primary antibodies followed by Horseradish Peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were detected using Supersignal West Dura Extended Duration Substrate (Pierce Chemical, Rockford, IL). Films were analyzed with NIH Image J and protein expression is analyzed as a ratio to the actin loading control.

Immunofluorescence microscopy

Brain sections were prepared as previously described [37]. Briefly, brain slice preparations were obtained from mice between 6 to 8 weeks of age. Following deep anesthesia with ketamine, animals were transcardially perfused with Ringer's solution followed by fixative of 4% paraformaldehyde with 0.1% glutaraldehyde. Following postfixation, brains were cut into 60 µm-thick sections. 60 µm thick brain sections were rinsed with PBS and incubated for 20 minutes in 1% Sodium Borohydride (PBS) at room temperature. Samples were incubated in preincubation/blocking solution (5% normal horse serum, 1% bovine serum albumin, and 0.3% Triton in PBS) for one hour at room temperature. Then, primary antibodies (1% NHS and 1% BSA in PBS) were incubated overnight at room temperature. Samples were rinsed with 3 × 5 minutes with PBS and incubated in secondary antibodies (1% NHS and 1% BSA in BSA) for 60 minutes at room temperature. Alexa-conjugated secondary antibodies were used at a 1:1000 dilution: anti-goat 555, anti-mouse 488, and anti-mouse 568. Tissues were washed 3 × 5 minutes and incubated in cupric sulfate (3.854 W/V ammonium acetate, 1.596 W/V cupric sulfate in distilled water, pH 5) for 30 minutes at room temperature. Tissues were rinsed with PBS and mounted onto slides with Vectashield mounting medium (Vector Laboratories). Tissue samples were observed and imaged with a ZOE Fluorescent Cell Imager (BioRad). Fluorescent intensity was analyzed with NIH ImageJ and expressed as a ratio to a control stain, Vamp2.

Immunocytochemistry

PC12 cells were fixed and permeabilized using saponin (0.02%) containing 3.7% formaldehyde for 2 min on ice, followed by 3.7% formaldehyde on ice 30 min, washed 3 X with PBS then blocked for 60 min in 5% in BSA with 1% Tween at RT. Primary and secondary antibodies were diluted in 3% normal horse serum in PBS. Primary antibodies were incubated overnight at 4ºC and then washed three times for 10 min at RT. Secondary antibodies were incubated for 120 min at RT. Coverslips were washed 3 X for 10 min each and once with ddH20, and mounted with Vectashield without DAPI (Vector Laboratories, CA). Images were acquired using a BioRad Zoe Cell Imager. Percent colocalization or neurite outgrowth was measured using NIH Image J.

Synaptosome

Synaptosomes were prepared as previously described from P50 mice [37]. Briefly, mice were anesthetized and brains quickly transferred to PBS on ice. Tissues were homogenized using a Potter-Elvehjem homogenizer at 800 rpm in 0.32 M sucrose, 5 mM HEPES and 0.5 mM EDTA supplemented with complete anti-protease inhibitor (Roche Molecular Biochemicals; Indianapolis, IN). Homogenates were spun for 10 minutes at 1,000 x g and S1 supernatants were further sedimented for 20 minutes at 12,000 x g. The P2 pellet was resuspended in 8.5% Percoll (Sigma-Aldrich). The resuspended P2 pellet was added to a discontinuous gradient comprised of 10% and 16% Percoll. Gradients were spun for 20 minutes at 15,000 x g.

Transferrin recycling assay

Transferrin uptake and trafficking was assayed as previously described in neurons. Stably transfected PC12 cells were cultured on coverslips and then serum-starved in imaging buffer for 1 h at 0ºC and then incubated with 50 µg/ml Alexatransferrin (Molecular Probes, Carlsbad, CA) in 1% BSA for 30 min at 37ºC to label the recycling endosome pool. PC12 cells were washed and incubated with 100 µg/mL unlabelled holo-transferrin (Sigma Aldrich, St. Louis, MO) Cells were labeled (pulse) for 2 hours at 0ºC with an alexa-conjugated cargo (transferrin) of the endosomal pathway. Cells were transferred to 37º C (chased). At 5 minutes transferrin should mark the early endosome (EEA1 positive compartment), at 15 minutes transferrin should mark the recycling endosome (Rab11 positive compartment), and at 30 minutes transferrin should reach the plasma membrane and be released.

Stats

Using Kaleidagraph, we utilized a Wilcoxon test to measure statistical significance for our data.

Results

Endosomal protein expression in coronal brain sections from mouse models neurodevelopmental disorders

Endosomal trafficking regulates the movement of various neurotransmitters, receptors, and integral membrane proteins through several sub-compartments within a cell. In order to determine the

role of endosomal trafficking in neurodevelopmental disorders, immunohistochemistry was performed on 60 µm coronal sections of the cortex (CX) and the dentate gyrus (DG) region of the hippocampus to evaluate the levels of endosomal markers compared to a control stain, the synaptic vesicle marker Vamp2 (Figures 1 and 2). The CX was selected as a region of importance because of its altered function in decision making, memory, and personality in neurodevelopmental disorders. Similarly, the DG was selected as a region of importance, as learning and memory are affected in neurodevelopmental disorders.

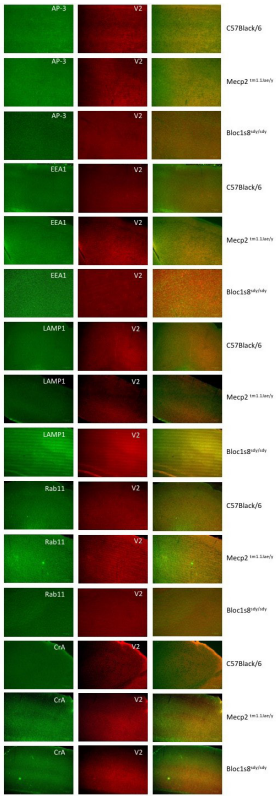

Figure 1A. Endosomal markers in the cortex: Immunofluorescent micrographs of the cortex from C57Bl/6, Mecp2$^{tm1.1Jae/y}$, and Bloc1s8$^{sdy/sdy}$ mice. Brain sections were stained with antibodies against the endosomal markers AP-3, EEA1, LAMP1, Rab11, and secretory marker CrA (green channel) as well as the synaptic vesicle SNARE Vamp2 (red channel).

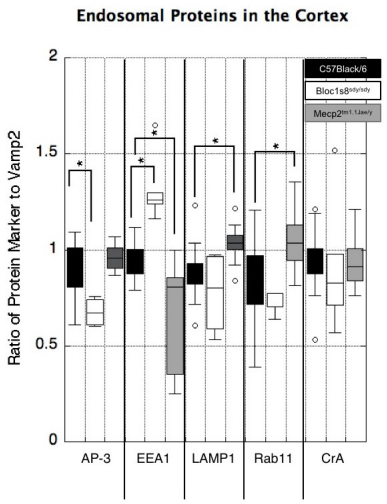

Figure 1B. Endosomal markers in the cortex: Quantification of the cortex immunofluorescence imaging. The trafficking markers are expressed as a ratio to Vamp2. In the CX of Bloc1s8$^{sdy/sdy}$ mice, there is a significant decrease in AP-3 fluorescent intensity compared to control (p=0.007) and a significant increase in EEA1 (p=0.001). In the Mecp2$^{tm1.1Jae/y}$ there is a significant decrease in EEA1 (p=0.001) and a significant increase in LAMP1 (p=0.0001) and Rab11 (p=0.006). Groups were compared using a Wilcoxon test; *<0.05. For each genotype, n=5.

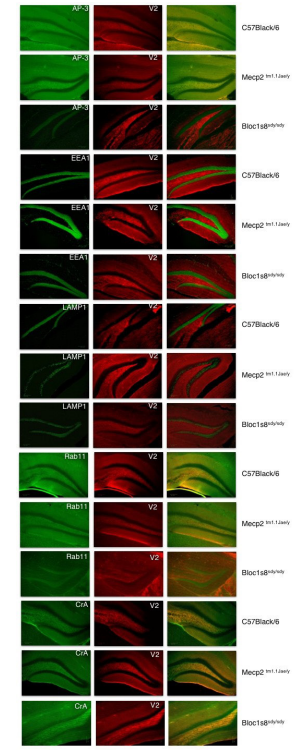

Figure 2A. Endosomal markers in the dentate gyrus: Immunofluorescent micrographs of the dentate gyrus from C57Bl/6, MeCP2$^{tm1.1Jae/y}$, and Bloc1s8$^{sdy/sdy}$ mice. Brain sections were stained with antibodies against the endosomal markers AP-3, EEA1, LAMP1, Rab11, and CrA (green channel) and the synaptic vesicle SNARE Vamp2 (red channel).

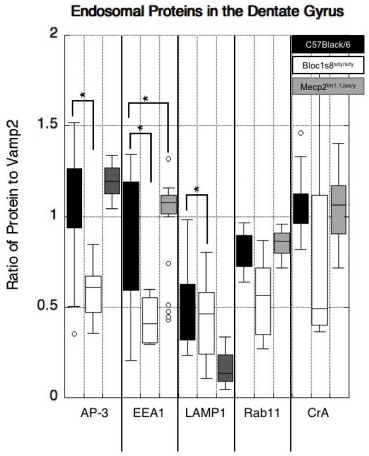

Figure 2B. Endosomal markers in the dentate gyrus: Quantification of the dentate gyrus immunofluorescence imaging. The trafficking markers are expressed as a ratio to Vamp2. In the DG of Bloc1s8$^{sdy/sdy}$ mice, there is a significant decrease in AP-3 (p<0.0001), EEA1 (p=0.033), and LAMP1 (p=0.008). In the DG of Mecp2$^{tm1.1Jae/y}$ mice, there is a significant decrease in EEA1 (p=0.01). Groups were compared using a Wilcoxon test; *<0.05. For each genotype, n=5.

When examining the protein levels for the endosomal coat protein, AP-3 we observed a significant decrease in AP-3 fluorescent intensity compared to control in the CX (p=0.007) and DG (p<0.0001) of Bloc1s8$^{sdy/sdy}$ mice (Figures 1 and 2). The reduction in AP-3 is in keeping with previous demonstration of a reduction of AP-3 in BLOC-1 deficient mice [36,38]. In the CX and DG of Mecp2$^{tm1.1Jae/y}$ mice, there is no significant change in AP-3 levels (Figures 1 and 2). Protein levels for the marker of the early endosome, EEA1, (Figures 1 and 2) demonstrate a significant increase of EEA1 (p=0.001) in the CX (p=0.001) and a significant reduction in the DG (p=0.033) in Bloc1s8$^{sdy/sdy}$ mice. There is a significant decrease in EEA1 in the CX (p=0.001) and the DG (p=0.01) in the Mecp2$^{tm1.1Jae/y}$ mice. The data in the Mecp2 deficient mice is in keeping with the EEA1 reduction observed

in primary cultured hippocampal neurons isolated from Mecp2$^{tm1.1Jae/y}$ mice previously reported [23].

When observing the protein levels for the lysosome (Figures 1 and 2), there is a decrease of LAMP1 in the DG (p=0.008) but no change in the CX of Bloc1s8$^{sdy/sdy}$ mice. In the CX of Mecp2$^{tm1.1Jae/y}$ mice, there is a significant increase in LAMP1 levels (p=0.0001) with no alteration observed in the DG.

Measurements for the marker of the recycling endosome, Rab 11 (Figures 1 and 2), indicated an increase in Rab 11 levels in the CX (p=0.006) with no alteration observed in the DG for Mecp2$^{tm1.1Jae/y}$ mice. There is no change in Rab11 protein expression for the Bloc1s8$^{sdy/sdy}$ mice.

There is no significant difference in CrA levels for the Bloc1s8$^{sdy/sdy}$ mice or the Mecp2$^{tm1.1Jae/y}$ mice. We observed a decrease in both regions in the endosomal coat protein AP-3 in the Bloc1s8$^{sdy/sdy}$ mice but no change in the Mecp2$^{tm1.1Jae/y}$ mice. We observed an increase in the CX and a decrease in the DG of the early endosome marker, EEA1 in the Bloc1s8$^{sdy/sdy}$ mice and a decrease in both regions in the Mecp2$^{tm1.1Jae/y}$ mice. Alterations in EEA1 levels could indicate a loss of the organelle or an increase in volume of the organelle due to failure of cargo to traffic out of the organelle. For the recycling endosome marker, Rab11, there was no change in the Bloc1s8$^{sdy/sdy}$ mice but there was an increase in the CX of the Mecp2$^{tm1.1Jae/y}$ mice. Again, this increase could indicate an increase in the number or an increase in the volume of the organelle. Finally, for the lysosome marker, LAMP1 there was a decrease of LAMP1 in the DG of the Bloc1s8$^{sdy/sdy}$ mice and an increase in the CX of the Mecp2$^{tm1.1Jae/y}$ mice. Taken together, these data demonstrate an alteration in endosomal proteins and indicate a potential alteration in endosomal function in the cortex and hippocampus of Mecp2$^{tm1.1Jae/y}$ mice and Bloc1s8sdy/sdy mice. It also demonstrates a difference in the expression patterns of endosomal proteins between the two disorders and a difference in the regional expression of endosomal proteins in these disorders. These differences can aid in understanding the specific role of endosomal organelles in development.

Endosomal protein levels at the synapse

Using a homogenate of whole-brain lysates (H fraction), heavy synaptosomes (HS fraction) which contain the pre- and post-synaptic regions of a synapse were prepared (Figure 3). We probed for PSD95 to demonstrate enrichment of a synaptic protein in the HS fraction compared to the H fraction. Then, we measured the levels of endosomal proteins in the H (whole brain homogenate). In the Bloc1s8$^{sdy/sdy}$ H fraction and the Mecp2$^{tm1.1Jae/y}$ H, there is a significant decrease in AP-3 (p=0.0009, p=0.004 respectively). In the Bloc1s8$^{sdy/sdy}$ H fraction but not in the Mecp2$^{tm1.1Jae/y}$ there is a significant reduction in EEA1 (p=0.03). There was no significant difference in Rab11 or CrA (Figure 3). Data from the homogenate fraction further describe alterations of endosomal protein expression in whole brain lysates from mouse models of neurodevelopmental disorders.

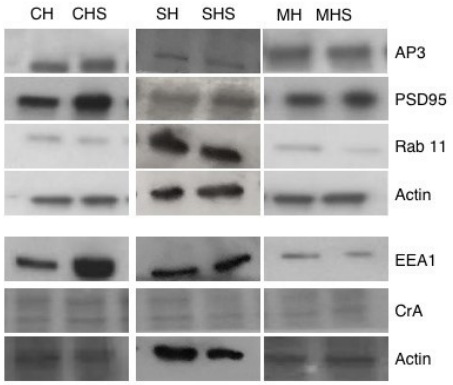

Figure 3A. Endosomal markers at the synapse: Immunoblot of the homogenate (H) and heavy synaptosome (HS) fractions C57Bl/6, Mecp2$^{tm1.1Jae/y}$, and Bloc1s8$^{sdy/sdy}$ mice. Membranes were probed for AP-3, synaptic marker PSD-95, Rab11, EEA1, and CrA. For each genotype, there are 3 samples that were each run in triplicate.

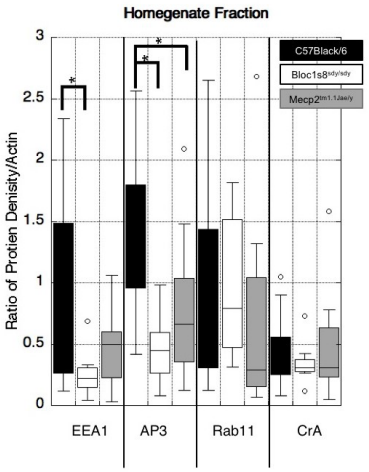

Figure 3B. Graph of the homogenate fraction: There is a significant decrease in EEA1 in Bloc1s8$^{sdy/sdy}$ (p=0.03). There is a significant decrease in AP-3 in Bloc1s8$^{sdy/sdy}$ (p=0.0009)and in Mecp2$^{tm1.1Jae/y}$ (p=0.004).

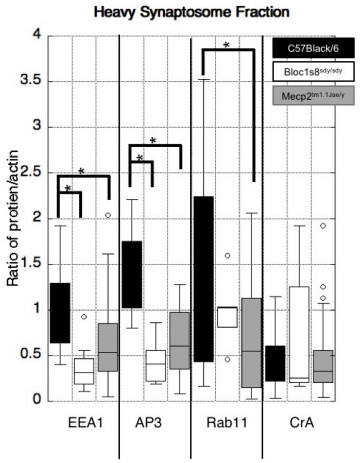

Figure 3C. Graph of the heavy synaptosome fraction: There is a significant decrease in EEA1 in Bloc1s8$^{sdy/sdy}$ (p=0.002) and in Mecp2$^{tm1.1Jae/y}$ (p=0.03) and in AP-3 in Bloc1s8$^{sdy/sdy}$ (p<0.0001) and in Mecp2$^{tm1.1Jae/y}$ (p<0.0001). There is a significant decrease of Rab11 in Mecp2$^{tm1.1Jae/y}$ (p=0.01).

To better understand how endosomal proteins impact neuronal function, we characterized endosomal protein levels at the level of the synapse in the HS fraction. In the Bloc1s8$^{sdy/sdy}$ HS fraction and the Mecp2$^{tm1.1Jae/y}$ HS, there is a significant decrease in EEA1 (p=0.002, p=0.03, respectively) and in AP-3 (p<0.0001, p<0.0001 respectively). In Mecp2$^{tm1.1Jae/y}$ HS but not in Bloc1s8$^{sdy/sdy}$ HS, there is a significant decrease in Rab11 (p=0.01). There is no significant difference in the levels of CrA (Figure 3). These data are significant as they describe endosomal differences at the level of the synapse and indicate a reduction in the machinery necessary for local recycling of neurotransmitters, receptors, and local membrane proteins. These data are supported by previous studies that demonstrate the early endosome and recycling endosome is necessary for dendritic spine outgrowth and that dendritic spine density is decreased and spine structure is immature in mouse models of RTT [7,8,39-41].

Neurite outgrowth in PC12 cells stable for Dysbindin KD and Mecp2 KD

Previous data demonstrate a reduction in dendritic outgrowth in both Mecp2 deficient individuals and mice as well as in dysbindin deficient mice [39-45]. As we will be using a cellular model for endosomal kinetics, we wanted to determine if there was a phenotypic similarity in PC12 cells with a stable expression of dysbindin siRNA or with a stable expression of Mecp2 siRNA. Before we examined phenotype, we utilized western blot to confirm KD expression (data not shown). Overall, we observed a 64.61% KD of

dysbindin and an 80.58% KD of Mecp2.

To analyze neurite length, stably infected PC12 cells were used (Figure 4). Compared to control PC12 cells with an average neurite length of 35.6 microns, PC12 cells lacking dysbindin had a significant decrease in neurite length (22.5 microns, p=<0.0001, Figure 4). PC12 cells lacking Mecp2 also had a significant decrease in neurite length (27.35 microns, p=0.001, Figure 4). Both of these results are in keeping with data from primary culture neurons, slice cultures, and post-mortem tissues. These data demonstrate that cellular models resemble the phenotypes of primary cultured neurons with reduced dysbindin or Mecp2.

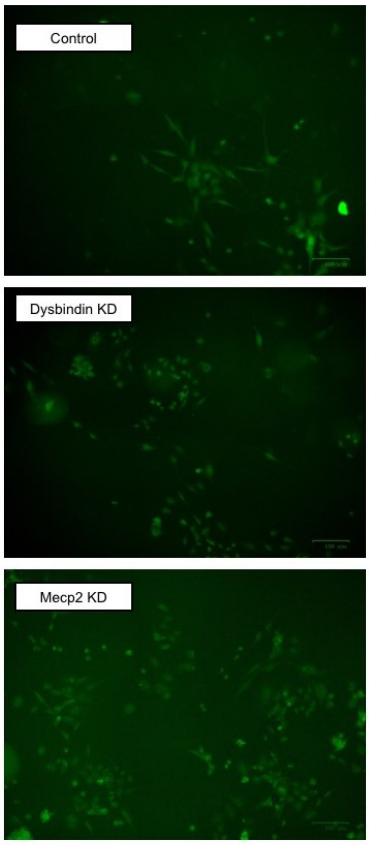

Figure 4A. Neurite length reduced in Dysbindin KD and Mecp2 KD deficient PC12 cells: Micrographs of PC12 cells with stable infections with shRNA for Dysbindin and Mecp2.

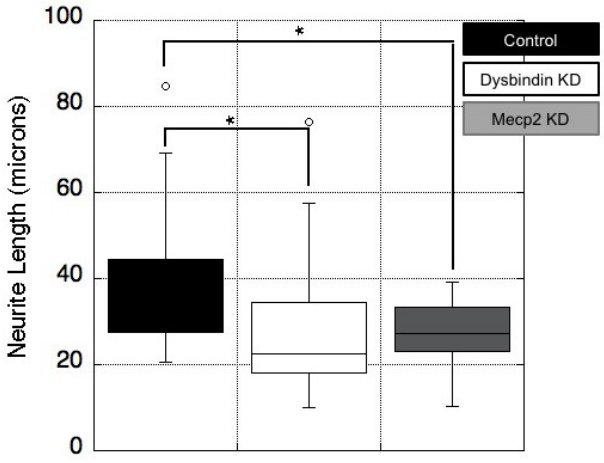

Figure 4B. Neurite length reduced in Dysbindin KD and Mecp2 KD deficient PC12 cells: Neurite length was measured in each group using Image J. Neurite length was reduced in dysbindin KD (p=<0.0001) and Mecp2 KD (p=0.001).

Endosomal trafficking kinetics in cellular models of neurodevelopmental disorders

The data from the IHC, immunoblots, and neurite outgrowth measurements indicate a potential role for endosomal trafficking in neurodevelopment. Alterations in endosomal trafficking in BLOC-1 deficiencies have been described at the level of the early endosome. Both BLOC-1 and its binding partner AP-3 have well-established roles in sorting proteins into vesicles that are targeted to the lysosome, lysosome-related organelles, and synaptic vesicles [34,35]. Previous groups have reported that the absence of BLOC-1 subunits results in a failure of specific cargo to leave the early endosome and traffic to melanosomes, again indicating a role of trafficking at the early endosome [29,46]. Work from our lab has demonstrated that cargo dependent in part on AP-3 trafficking from the early endosome to the cell membrane fails to traffic in the dysbindin deficient mice [37,47]. Work from our lab has also demonstrated BLOC-1deficiencies that reduce the targeting to target membranes [47-49]. As previous studies have documented alterations in endosomal trafficking at the level of the early endosome in BLOC-1 deficiencies, we focused on Mecp2 deficiencies.

To determine if endosomal trafficking kinetics are also altered, we utilized stably transfected PC12 cells and a pulse-chase assay. To analyze the trafficking of transferrin, we measured colocalization with compartment markers (Figure 5). At 0 minutes, the transferrin is at the plasma membrane and we observe 0% co-localization.

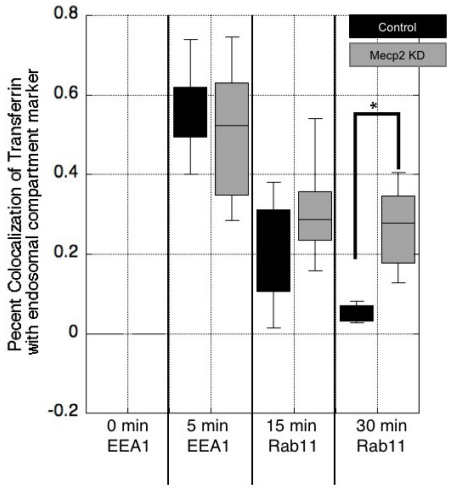

Figure 5. Transferrin trafficking in PC12 cells: Average percentage of transferrin colocalizing with endosomal markers at 0 and 5 minutes (transferrin and EEA1) and at 15 and 30 minutes (transferrin and Rab11) for both the control PC12 cells and PC12 cells with Mecp2. There was a significant increase in the amount of Rab11 and transferrin at the 30 minute time point in Mecp2 KD cells compared to control (p=0.0006). N=3 pulse-chase assay.

with EEA1 in both the control and in the Mecp2 KD. At 5 minutes, there is 57.8% colocalization with EEA1 and transferrin for the control group and 52.3% colocalization for Mecp2 KD with no significant difference (p=0.23). At 15 minutes, there is between 18% colocalization with Rab11 (a recycling endosome marker) and the transferrin for the control and 28.7% for the Mecp2 KD (p=0.122). At 30 minutes, when the alexatransferrin should have been released from the cell, in control cells, we observed 5.3% colocalization with Rab11 and transferrin. In the Mecp2 KD cells, we observe a 27.9% colocalization with Rab11 and transferrin. The difference between the control and the MeCP2 KD is significant (p=0.0006). This suggests that there is an alteration in trafficking out of the recycling endosome in cells with Mecp2 KD.

Discussion

Previous studies have demonstrated that various endosomal proteins are implicated in RTT and SZ, and this study serves to further elucidate the

endosomal pathways impacted in neurodevelopment. In this study, we used immunohistochemistry to analyze the expression of EEA1, Rab11, LAMP1, and AP-3 in the cortex and dentate gyrus of C57, Mecp2$^{tm1.1Jae/y}$ mice, and Bloc1s8$^{sdy/sdy}$ mice. We analyzed biochemical fractions from these mice as well. Finally, we utilized a cellular model to analyze endosomal kinetics in Mecp2 KD. Our results reinforce the findings that, in mouse models of neurodevelopment, endosomal trafficking is interrupted in unique patterns for each disorder.

AP-3 is an endosomal coat protein that is necessary for the proper trafficking of specific cargo. Previous work in our lab has demonstrated that AP-3 and BLOC-1 work together for the trafficking of some cargo from the endosome to the plasma membrane [37]. There was a significant decrease in AP-3 in the CX and DG of the BLOC-1 deficient mice. In both mouse models, there was a significant decrease in AP-3 in the homogenate and heavy synaptosome fractions. These data demonstrate key endosomal coat proteins are altered in neurodevelopment.

EEA1's main role within the endosomal pathway is to sort and provide directionality to vesicles that fuse to the early endosome [50-54]. The mechanism by which EEA1 allows for vesicular/endosomal binding is through interaction with the SNARE complexes on vesicles that promote the docking and subsequent fusion of both membranes [55,56]. EEA1 exhibited significant decreases in expression in the DG of both mouse models. The reduction of EEA1 was also observed in the CX of the Mecp2 deficient mice while an increase in EEA1 was observed in the CX of BLOC-1 deficient mice. In both mouse models, there was a significant decrease in EEA1 levels in the synaptosome. While overall, there was a reduction of EEA1 observed, better understanding the regional effect of altered EEA1 levels is necessary to understand the role of EEA1 in neurodevelopment. With EEA1 being a key component crucial for vesicle fusion activity, alterations in EEA1 content can lead to problems directing cargocontaining vesicles to their acceptor membranes.

Our immunofluorescent staining revealed increased levels of LAMP1 in the CX of Mecp2 deficient mice and a decrease in the DG of BLOC-1 deficient mice. LAMP1 has been previously described as a hub for degradation within endosomal pathways in neurons [57,58]. In neurotypical cases, LAMP1 maintains equilibrium with a number of other cellular components including endosomes, lysosomes, and autophagic machinery. As observed in our experiments, LAMP1 content was significantly increased in the cortex of the mouse models of RTT. On the cellular level, increased LAMP1 alters the distribution of late endosomes and lysosomes in the cell. This shift in distribution phenotypically presents as an aggregation of the late endosomes and lysosomes, without fusion, as well as the reduced movement of these organelles within the cell along microtubules [59]. To further elucidate, dysregulation of intracellular organelle distributions interrupts proper endosome-lysosome fusion, a process important to degrading unnecessary cellular components, thereby increasing the risk for aggregation of unwanted material within the cell. With optimal cellular function being necessary during neurodevelopment, the interruptions to the mechanisms that maintain LAMP1/endosomal organelle equilibrium could be detrimental to proper neurodevelopment.

There was also a significant increase in Rab11 content in the CX of Mecp2 deficient mice and a significant decrease in Rab11 content in the synaptosome fraction. Research demonstrates that dendritic growth, spine growth, and spine maintenance are regulated by endosomal trafficking. In accordance with the previous research on dendritic spines in neurodevelopmental conditions, we measured the average length of neurites in dysbindin and Mecp2 KD cells compared to controls. Significant reductions in average neurite length were observed for both experimental cell groups, suggesting that the mechanisms that drive proper neurite outgrowth were disrupted in these cells. Rab11's role in the endosomal pathway has been previously described as one that mediates the recycling of endosomes to the trans-Golgi network [60,61]. Reductions in Rab11 have been associated with impaired recycling of endosomes [61]. Dendritic spine morphology is regulated in part by recycling endosomes located at the base of dendritic spines [8]. Receptor recruitment through these endosomes regulates spine growth and maintenance in an LTP-dependent manner [7]. Interestingly, thin, immature, filamentous spines have been observed in numerous neurodevelopmental disorders such as ASDs, SZ, Down syndrome FRX, and RTT [39-42,45,62-67]. As demonstrated in Chapleau et. al. spine density is decreased in the CA1 of post-mortem brain samples from patients with RTT compared to non-MR age-matched controls [40]. The data demonstrated a decrease in mushroom (type 2) or stubby spines (type 1) and an increase in thin filamentous spines (type 3), a loss of proper dendritic outgrowth, and altered levels of the brain-derived neurotrophic factor (BDNF) [39-41]. Our observed reduction of Rab11 in the synaptosome fraction was to be anticipated based on these previous findings. Future studies should consider the underlying mechanisms implicated in the disruption occurring in the recycling endosome and the involvement of dendritic spines in neurodevelopment.

Finally, we know based on previous research that BLOC-1 deficiencies result in a dysfunctioning early endosome. To determine if a sub-compartment of the endosomal pathway was not functioning properly in Mecp2 KD, we utilized a pulse-chase assay. The results of this assay demonstrated an increase in transferrin colocalized with Rab11 at the 30 minute time point, when the transferrin should have exited the cell. Based on this result, it is possible that the vesicle has trouble detaching from or exiting the recycling endosome. The pulse-chase assay shows that there is a normal movement with the marker until the 30-minute mark, showing a notable difference between the control and the Mecp2 KD. This signifies that the complication lies within the recycling endosome.

Conclusion

Taken together, there are altered compartment markers, altered neurite outgrowth, and altered trafficking kinetics in models for two neurodevelopmental disorders, RTT and SZ. The alterations for each of the sub-endosomal compartments was different for each neurodevelopmental disorder and reflects where alterations in phenotypes or phenotype severity may be observed. Comparing these results with models of other neurodevelopmental disorders will broaden our understanding of how endosomal trafficking impacts neurodevelopment. This understanding will allow us to broaden our targets for potential therapeutics among several neurodevelopmental disorders.

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