MeCP2 Regulates the Synaptic Expression of a Dysbindin-BLOC-1 Network Component in Mouse Brain and Human Induced Pluripotent Stem Cell-Derived Neurons

Jennifer Larimore1, Pearl V. Ryder2, Kun-Yong Kim3, L. Alex Ambrose1, Christopher Chapleau4, Gaston Calfa4*, Christina Gross2, Gary J. Bassell2, Lucas Pozzo-Miller4, Yoland Smith5,6, Konrad Talbot7, In-Hyun Park3, Victor Faundez2*

1 Department of Biology, Agnes Scott College, Decatur, Georgia, United States of America, 2 Cell Biology, Emory University, Atlanta, Georgia, United States of America, 3 Department of Genetics, Yale Stem Cell Center, Yale School of Medicine, New Haven, Connecticut, United States of America, 4 Department of Neurobiology, The University of Alabama, Birmingham, Alabama, United States of America, 5 Department of Neurology, Emory University, Atlanta, Georgia, United States of America, 6 Yerkes National Primate Center, Emory University, Atlanta, Georgia, United States of America, 7 Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America

Abstract

Clinical, epidemiological, and genetic evidence suggest overlapping pathogenetic mechanisms between autism spectrum disorder (ASD) and schizophrenia. We tested this hypothesis by asking if mutations in the ASD gene MECP2 which cause Rett syndrome affect the expression of genes encoding the schizophrenia risk factor dysbindin, a subunit of the biogenesis of lysosome-related organelles complex-1 (BLOC-1), and associated interacting proteins. We measured mRNA and protein levels of key components of a dysbindin interaction network by, quantitative real time PCR and quantitative immunohistochemistry in hippocampal samples of wild-type and MeCP2 mutant mice. In addition, we confirmed results by performing immunohistochemistry of normal human hippocampus and quantitative qRT-PCR of human inducible pluripotent stem cells (iPSCs)-derived human neurons from Rett syndrome patients. We defined the distribution of the BLOC-1 subunit palladin in human and mouse hippocampus and contrasted this distribution with that of symptomatic MeCP2 mutant mice. Neurons from mutant mice and Rett syndrome patients displayed selectively reduced levels of palladin transcript. Palladin immunoreactivity decreased in the hippocampus of symptomatic MeCP2 mutant mice, a feature most prominent at asymmetric synapses as determined by immunoelectron microscopy. Palladin immunoreactivity decreased concomitantly with reduced BDNF content in the hippocampus of MeCP2 mice. Similarly, BDNF content was reduced in the hippocampus of BLOC-1 deficient mice suggesting that genetic defects in BLOC-1 are upstream of the BDNF phenotype in MeCP2 deficient mice. Our results demonstrate that the ASD-related gene MeCP2 regulates the expression of components belonging to the dysbindin interactome and these molecular differences may contribute to synaptic phenotypes that characterize MeCP2 deficiencies and ASD.

Introduction

Autism spectrum disorder (ASD) and schizophrenia are disorders with some intersecting clinical characteristics such as their shared impairment of social cognition [1–4]. Phenotypic similarities between these disorders suggest common molecular roots [5]. This hypothesis has recently received substantial support from epidemiological, bioinformatic, and genetic studies [6]. Epidemiological evidence points to non-genetic and genetic risk factors. Among the non-genetic factors, obstetric complications as well as migrant status increase the risk of both schizophrenia and autism [7] while among genetic risk factors a parental history of schizophrenia increases the risk for ASD and advanced paternal
age is a risk factor for both schizophrenia and ASD [8–10]. Advanced paternal age can be explained by the increased rate of de novo mutations during spermatogenesis in older subjects [9]. Genome-wide association studies further support common molecular roots between schizophrenia and ASD. An increasing number of copy number variations that span multiple genes associate with both schizophrenia and ASD [11,12]. This supportive genetic evidence extends to monogenic defects such as those in GRIN2A [13], ANK3 [14], NRXN1 or MECP2. NRXN1 encodes a presynaptic neuronal cell adhesion molecule [15,16] and NRXN1 genetic defects robustly associate with schizophrenia and ASD [17–21]. Similarly, mutations in the X-linked MECP2 gene, which encodes the transcriptional regulator methyl-CpG-binding protein 2 (MeCP2), result in one of the ASDs, the Rett syndrome, and are associated with childhood schizophrenia [22–26]. Genetic manipulation of MeCP2 in mice causes well-characterized synaptic phenotypes and a transcriptional signature, which is defined from mRNA expression profiles in loss- and gain-of-function mouse mutations [27,28]. Mutations to MeCP2 affect the neuronal expression of 12–15% of the mouse genome [29,29], suggesting that some of these MeCP2-regulated transcripts could encode unrecognized synaptic proteins associated with schizophrenia pathogenesis. Here, we focus on a MeCP2-dependent mechanism that regulates the expression of subunits of the biogenesis of lysosome related organelles complex 1 (BLOC-1), a synaptic complex that contains the schizophrenia-associated protein, dysbindin.

Dysbindin and its protein interaction network regulate mechanisms implicated in schizophrenia pathogenesis pathways. Polymorphisms in DTNBP1, the gene encoding dysbindin, associate with schizophrenia [30–32]. Moreover, a significant reduction of synaptic dysbindin expression has been reported in nearly 80% of schizophrenia cases [33–36]. Dysbindin is part of a protein interaction network spanning a minimum of 24 experimentally identified proteins. Nearly one-third of the genes encoding these dysbindin network components are affected by copy number variation defects in schizophrenia subjects [37]. Functions ascribed to dysbindin further support its participation in schizophrenia disease mechanisms. Dysbindin is part of the octameric protein complex BLOC-1, which is involved in the targeting of membrane proteins from endosomes to diverse organelles and the synapse [31,32,38,39]. Dysbindin localizes to pre- and post-synaptic compartments. At the pre-synaptic level, its deficiency affects synaptic homeostasis, synaptic vesicle composition and vesicle fusion, while at the post-synaptic level, its deficiency alters the surface content of glutamatergic and dopaminergic receptors, which are strongly implicated in schizophrenia pathogenesis [40–49]. The synaptic mechanisms in which the dysbindin network participates as well as the experimentally verified nature of this protein-protein interaction network make it a good target for exploring mechanistic convergence between ASD and schizophrenia. Here we test this idea by investigating if MeCP2 regulates the expression of components of the dysbindin protein interaction network. To this end, we first defined the normal anatomical and ultrastructural distribution of the BLOC-1 subunit pallidin in human and mouse hippocampus. Next, we demonstrate that MeCP2 and BLOC-1 deficiencies share a reduced content of brain-derived neurotrophic factor (BDNF), suggesting that BLOC-1 is upstream of the BDNF phenotype in MeCP2-deficient brain. These findings reveal a novel molecular link between an ASD causative gene, MeCP2, and the protein interactome of the schizophrenia susceptibility gene product, dysbindin. We speculate that defects on the dysbindin interactome may contribute to synaptic and circuit defects that characterize MeCP2 mutations.

Materials and Methods

Reagents

Mouse-anti pallidin was a gift from Dr. Estate Dell’Angelica (UCLA, Los Angeles, California) [50] and rabbit anti-VAMP-2 was purchased from Synaptic Systems (Gottingen, Germany). Synaptophysin (SY38) antibody was from Chemicon International/Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against BDNF were from Santa Cruz (Dallas, Texas, SC-5456) and human recombinant BDNF was from Promega (Madison, WI, G1491). The monoclonal antibody specificity in brain immunohistochemistry was determined in this study using pallidin-null B16.C57BL/6J brain. B6.Cg-Tg(J) breeding pairs were obtained from Jackson Labs (Bar Harbor, Maine). Breeding pairs of MeCP2 mice [51] were purchased from the Mutant Mouse Regional Resource Center at the University of California, Davis (B6.Cg-Mecp2tm1.Jae). “Jaenisch” strain maintained in C57BL/6 background, and a colony established at UAB. Mice were bred in-house following Emory and UAB IUCAC approved protocols.

Quantitative Real Time PCR (qRT-PCR)

Control and mutant cortical and hippocampal regions were dissected from P7 animals, which are asymptomatic, and young adult animals between P42–P52 sacrificed by CO2 narcosis. Tissue was then flash frozen. Following TRIzol (Invitrogen Life Technologies, Grand Island, NY) extraction, isolated mRNA was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis (Invitrogen Life Technologies, Grand Island, NY). PCR amplifications were performed on a LightCycler480 Real Time plate reader using LightCycler 480 SYBR Green reagents (Roche, Indianapolis, IN). Table 1 describes the primers used in this study.

Brain Sections, Immunohistochemistry and Microscopy

Detailed procedures for mouse tissue preparation, immunoperoxidase light microscopy, indirect immunofluorescence microscopy, immunoperoxidase electron microscopy and quantification procedures were described in our previous work [37,43,44]. 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 (4% paraformaldehyde with 0.1% glutaraldehyde). Following postfixation, their brains were cut into 60 µm thick sections and processed to localize pallidin at the light and electron microscopic level using mouse anti-Pallidin antibody (1:400), the peroxidase-anti-peroxidase (PAP) method and 3,3′- diaminobenzidine (DAB) as chromogen for the peroxidase reaction, according to procedures described in detail in our recent studies [37,43,44].

For light microscopy, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. Light microscopy analysis was performed with a Leica DMIRB microscope (Leica Microsystems, Inc., Bannockburn, IL, USA) and images were captured with a CCG camera (Leica DCM500). Images were acquired with a 10x/0.3 DIC objective. Images were acquired with Leica IM50 software.
Electron microscopy sections were further postfixed in osmium tetroxide, dehydrated and embedded in resin on microscope slides according to procedures described in our recent studies [37,43,44]. From this EM-prepared tissue, blocks of the dentate gyrus were mounted on resin blocks, faced with a glass knife, and sectioned with a diamond knife into 60 nm sections with an ultramicrotome (Leica Ultracut T2). Sections were collected on Pioloform-coated copper grids and stained with lead citrate for 5 minutes to enhance contrast on the electron microscope. Electron microscopy was performed with a Zeiss EM-10C and a JEOL electron microscopes equipped with CCD cameras (DualView 300 W; Gatan, Inc., Pleasanton, CA, USA). Images were acquired with Gatan Digital Micrograph Software (v. 3.10.1; Gatan, Inc.) Ultrathin sections from the surface of the blocks of the dentate gyrus corresponding to regions where the neuropil and cell bodies intersect were chosen. Random fields of view including asymmetric synapses were imaged at 30,000–50,000X. A total of 25 fields were examined in each animal or a total of 70–100 fields was analyzed per genotype. The proportion of terminals with pallidin-immunoreactive active zones and spines with labeled PSDs was estimated in control and mutant mice.

The human hippocampus tissue was obtained from non-psychiatric individuals. Human hippocampus data is representative data from 19 normal human cases (9 males, 10 females) studied. The mean age +/- the SD was 81.11 +/- 9.8 years. The mean postmortem interval (PMI) +/- the SD was 11.31 +/- 9.2 hours. After removal of the hippocampal formation, the tissue was fixed for 12–24 hours in either neutral buffered formalin or ethanol in saline. Following embedding in paraffin, the tissue was sectioned at 10 µm, mounted on adhesive-coated slides, air dried, and then processed immunohistochemically for pallidin using a monoclonal antibody described above (1:150), heat-induced antigen retrieval with EDTA, and a standard avidin-biotin peroxidase with light silver intensification following the protocol of Talbot et al. (2004) [33].

Immunofluorescence Microscopy

Confocal microscopy was performed with an Axiovert 100 M microscope (Carl Zeiss) coupled to an Argon and HeNe1 lasers. Images were acquired using Plan Apochromat 10x/0.5 dry, 20x/0.5 dry, and 40x/1.3 and 63x/1.4 DiC oil objectives. Emission filters used for fluorescence imaging were BP 505–530 and LP 560. Images were acquired with ZEN and LSM 510 software (Carl Zeiss). Hippocampal formation 60 µm-thick brain sections were rinsed with PBS and then incubated in 1% sodium borohydride in PBS for 20 minutes at room temperature. Samples were preincubated in a solution of PBS with 5% normal horse serum and 1% BSA and 0.3% Triton X-100 for 60 minutes at room temperature. Samples were incubated overnight at 4°C in primary antibody solutions of PBS with 1% NHS and 1% BSA. After rinsing in PBS, sections were incubated for 60 minutes in a secondary antibody PBS solution with 1% NHS and 1% BSA and 1:50 dilutions of the following Alexa-conjugated secondary antibodies: anti-mouse 555 and anti-rabbit 488 (Invitrogen Molecular Probes, Carlsbad, CA, USA). Following PBS rinses, sections were incubated in cupric sulfate (3.854 W/V Ammonium Acetate, 1.596 W/V Cupric Sulfate in distilled water, pH 5) for 30 minutes and mounted on slides with Vectashield (Vector Laboratories).

Light Microscopy Quantitations

Quantitations of peroxidase light microscopy staining and immunofluorescence were performed as previously described using Metamorph software [43–45].

Human iPSCs Cells and Neuronal Differentiation

The monoallelic expressing Rett syndrome iPSCs cells RTT3 and RTT4 were previously characterized [52]. iPSCs cells were differentiated into neural rosettes and neurons as described by Li and Zhang [53]. Neurons were generated from these cells by interference with bone morphogenetic protein signaling [54].

Mouse and Human Subjects

Animal Procedures were approved by the IACUC committee at Emory University and the University of Alabama. Data on human postmortem tissue derived from samples of U.S. citizens autopsied at the Hospital of the University of Pennsylvania as approved by the Institutional Review Board at that university. Autopsy consent from next-of-kin or legal guardian was obtained in all cases. For most cases, consent was granted in writing before death and always confirmed after death. Ethics committee at the University of Pennsylvania approved consent procedures. To keep postmortem delays to a minimum when written consent had not been obtained before death, verbal consent was obtained as witnessed by a third party and documented by the physician making the request. Written records of the consent for autopsy were archived. These procedures for written and verbal consent are standard medical practice in the U.S.A.

Statistical and Bioinformatic Analyses

Statistical analyses were performed with Synergy KaleidaGraph v4.03 (Reading, PA) or the Vassar Web Engine (http://vassarstats.net/). BLOC-1 interacting proteins were compiled from our quantitative dysbindin interactome and a previously published curated list of putative candidate interactors [37,55]. A BLOC-1 network interaction map was built using GeneGo Metacore (version 6.11 build 4105) and visualized in Cytoscape (version 2.8.3). Node color and sizes were mapped to changes in mRNA expression level in MECP2-overexpressing and Mep2-null mouse neuronal cells as previously reported [29].

Results

Bioinformatic Analysis Identify BLOC-1 Components among MeCP2-regulated Transcripts

The similarities between ASD and schizophrenia could result from a gene product affected in ASD. Such a gene could in turn modify the expression of proteins associated with pathways implicated in schizophrenia. We tested this hypothesis by investigating if the expression of genes encoding components of the dysbindin protein interaction network is susceptible to MeCP2 levels. We built a comprehensive dysbindin network by merging our dysbindin protein interaction network of 24 candidates with a prioritized list of putative protein-protein interactions for BLOC-1 subunits [37,55]. The resultant dysbindin network contained 119 proteins. Six of these dysbindin interactome components were common to gene products whose mRNA expression is sensitive to MeCP2 content as previously determined by microarrays (Fig. 1) [29]. Pallidin (Pldn), peroxiredoxin 1 (Prdx1), COG7, pleiotrophin (PTN), ADP-ribosylation factor interacting protein 2 (ARFIP2), huntingtin-associated protein 1 (HAP1), and discoidin domain receptor tyrosine kinase 1 (DDR1) mRNAs are increased in MECP2 over-expressing mouse neurons (Fig. 1A, Mep2+/+ vs. Mep2−/−) and decreased in Mep2-null mouse neurons (Fig. 1B, Mep2−/− vs. Mep2+/+). We previously demonstrated that three of these six proteins, pallidin, peroxiredoxin 1, and COG7, interact with dysbindin and their brain expression is sensitive to a dysbindin-null allele (Bloc1s8sdy/sdy) previously referred as Dnlp1+/−/−) [37]. Our bioinformatic
analysis suggests that MeCP2 regulates expression of key components of the dysbindin interaction network.

We used quantitative real time PCR to determine the mRNA levels of transcripts encoding dysbindin and other BLOC-1 subunits in samples from the hippocampus and cortex from symptomatic young adult Mecp2tm1.1Jae/y mice and littermate controls. The mRNA levels of pallidin decreased by 26% in the hippocampus of symptomatic Mecp2tm1.1Jae/y mice as compared to controls (p<0.002 Mann-Whitney U test). In contrast, Bloc1s1–3, dysbindin, and muted transcripts were not affected in Mecp2tm1.1Jae/y hippocampus. The reduced content of pallidin was restricted to the hippocampus since all BLOC-1 subunit transcripts remained unaffected in Mecp2tm1.1Jae/y cortex (Fig. 2A). Since Rett syndrome affects the brain during development, we explored if BLOC-1 subunit mRNA levels were susceptible to Mecp2 deficiency before becoming symptomatic [56,57]. We examined

Figure 1. MeCP2 Regulates mRNA Levels of BLOC1-Interacting Proteins. BLOC1-interacting proteins were compiled from previous reports and mapped using the GeneGo Metacore pathway. The network was visualized in Cytoscape. Node sizes and colors were mapped to mRNA expression level changes in MECP2-overexpressing (A, Mecp2Tg/y) and Mecp2 null mouse (B, Mecp2−/−) neurons as reported in Chahrour et al., 2008 [29]. We observed that six components of the BLOC1 interactome were modified by Mecp2 gene dosage. The protein products of three of these affected genes, PLDN, COG7, and PRDX1, are decreased in mice null for the schizophrenia susceptibility factor dysbindin [37].

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mice at postnatal day seven, P7, a period that coincides with enhanced hippocampal synaptogenesis [58] and when alterations in spine formation occur in the Mep2 mutant mice [59]. The BLOC-1 pallidin subunit transcript was 50.5% lower in Mep2<sup>2m1.1Ja</sup>/P7 hippocampi, a statistically significant decrease. Mouse mutants carrying one copy of a null allele of pallidin (Bloc1<sup>s6pa/pa</sup>) showed a 50% reduction of pallidin mRNA (data not shown), indicating that the effect of MeCP2-mutant upon pallidin mRNA expression is equivalent to the loss of one copy of the gene encoding pallidin. Strikingly, muted mRNA was also diminished in Mep2 mutant hippocampi (Fig. 2B, 70% of control, p<0.0004 Mann-Whitney U test). P7 cortical transcripts encoding BLOC-1 subunits were not affected by the Mep2 mutant allele (Fig. 2B). These data demonstrate that MeCP2 regulates the expression of transcripts encoding BLOC-1 subunits in an anatomic and temporal-specific manner.

**Pallidin Transcript Content Decreases in Human MECP2-deficient Neurons**

We sought to determine if the pallidin mRNA phenotypes observed in Mep2<sup>2m1.1Ja</sup>/mouse hippocampus were observed in Rett syndrome human neural tissue. We took advantage of neuronal cells differentiated from human iPSCs cells generated from skin fibroblasts of Rett syndrome patients. Fibroblasts were reprogrammed into iPSCs by infecting them with a retrovirus expressing OCT4, SOX2, KLF4, and MYC. MECP2 is encoded in the X-chromosome, the status of which is maintained after reprogramming (Fig. 3A) [60]. This chromosomal inactivation allowed us to generate iPS cell clones from the same patient where either the wild-type or the MeCP2 mutant alleles were selectively expressed [52]. This approach facilitates transcript expression analysis since control and experimental samples are from the same individual, thus eliminating contributions by inter-individual genome differences [52].

We focused on patients carrying single MECP2 alleles, E235fs or the R306C, and generated monoallelic expressing iPS cell lines that we differentiated into neurons. We validated these cells by recapitulating a neuronal maturation defect we have previously reported [52]. This phenotype is characterized by a 50% decrease in the neuronal specific markers NeuroD1 and the neuron specific beta tubulin III, TuJ, encoded by TUBB3 (Fig. 3B). Pallidin mRNA was significantly reduced by 35% in neurons monoallelically expressing mutant MeCP2 (Fig. 3B). However, the mRNA levels of the BLOC-1 subunit, Bloc1s1, were not significantly different compared to neurons expressing the wild type allele of MECP2 or a control transcript (GFAP). These results demonstrate that defects in the expression of pallidin mRNA observed in Mep2<sup>2m1.1Ja</sup>/mice are common to those of patients carrying two different mutant alleles of MECP2.

**Anatomical Distribution of MeCP2 in Mouse and Human Brain**

In order to assess the consequences of a Mep2 mutation upon pallidin hippocampal protein expression, we first determined the anatomical localization of pallidin, which has not been reported in the mammalian brain. To this end, we used a monoclonal antibody against pallidin in adult mouse and human hippocampal tissue (Fig. 4) [61]. We established the specificity of this antibody in tissue sections from wild-type and pallidin-null Bloc1<sup>s6pa/pa</sup> brain, as demonstrated by the loss of immuno-peroxidase labeling in pallidin-null brain (Fig. 4, compare A-B). We then examined the distribution of pallidin in human hippocampal tissue. Strong pallidin immunoreactivity was observed throughout the neuropil of the human hippocampal formation (Fig. 4C, HF). Similar to the distribution of dysbindin we previously reported [33], the highest neuropil levels of pallidin were found in axon terminal fields of glutamatergic neurons intrinsic to the hippocampal formation, most conspicuously in the inner molecular layer of the dentate gyrus (Fig. 4C, DGilm) and among CA2 and CA3 pyramidal cells (Fig. 4C). Figures 4D-G also show that neurons producing these axon terminal fields (hippocampal pyramidal cells in CA3 and polymorph cells in the dentate gyrus hilus [DGhl]) are also rich in pallidin (Fig. 4 D, F), as are pyramidal cells in the subiculum (Fig. 4E). Pallidin in dentate gyrus granule cells are clearly seen in Fig. 4G. The distribution of pallidin in mouse brain was similar to the human hippocampus, with a prominent staining in the neuropil of the dentate gyrus hilus and synaptic fields such as the molecular layer of the dentate gyrus (Fig. 4A and H).

Dysbindin is localized to presynaptic and postsynaptic compartments yet if other subunits of the BLOC-1 complex are localized to the synapse remains unknown [40]. Immunofluorescence microscopy revealed the presence of pallidin concentrated in cell bodies of dentate gyrus neurons and as a diffuse staining in the adjacent neuropil (Fig. 4J–J’). A similar pattern was observed by immuno-peroxidase staining (Fig. 4F). The low neuropil pallidin signal attained precluded colocalization studies with a universal presynaptic marker, the synaptic vesicle protein VAMP2 (Fig. 4J–J’).
Thus to address the subcellular localization of pallidin, we performed quantitative immuno-electron peroxidase microscopy of the dentate gyrus and determined the ultrastructural distribution of pallidin in wild-type and pallidin-nullBloc1s6pa/pahippocampal tissue. Pallidin immunoreactivity was present in axon terminals, axons, and dendritic spines in the dentate gyrus of wild-type animals (Figs. 5A–D, F–G, I–J). Pallidin immunoreactivity decorated synaptic vesicles closely apposed to the active zone (Fig. 5A–D), coated vesicles or cisternae present in dendritic spines (CCV and Cast, respectively, Fig. 5C), and internal vesicles of multivesicular bodies located in spines (MVB, Fig. 5F). None of these structures were labeled in pallidin-nullBloc1s6pa/patissue (Fig. 5, I–J). These results indicate that pallidin is present in pre- and postsynaptic compartments consistent with a role of BLOC-1 and its protein interaction network modulating synaptic physiology.

This anatomical and ultrastructural distribution of pallidin in human and mouse hippocampus was contrasted between wild type andMecp2mutant mouse tissue. Notably, we observed a marked reduction of pallidin immunoreactivity in the dentate gyrus, CA1...
and CA3 as well as their associated synaptic fields in Mecp2<sup>tm1.1Jae/y</sup> hippocampus (Fig. 4, compare H–I). We performed quantitative confocal microscopy of the dentate gyrus labeled with antibodies directed against pallidin and VAMP2 and the total VAMP2 fluorescence intensity in the dentate gyrus, respectively. Wild type (Mecp2<sup>+/y</sup>, Blue, n = 4) and Mecp2 mutant tissue (Mecp2<sup>tm1.1Jae/y</sup>, Red, n = 4) were analyzed. P values were obtained by Mann-Whitney U test. The VAMP2 content is similar between genotypes. Images C–G correspond to sections from human hippocampal tissue stained with pallidin antibody. In C, HF denotes the hippocampal formation, consisting of the hippocampus proper (CA1–3), the dentate gyrus (DG), and the subiculum (Sub). The inner molecular layer of the DG (C, DGiml), the DG hilus (DGh) and the dentate gyrus granule cells layer (DGg) are indicated. Pallidin immunoreactivity is present in cell bodies of CA3 cells (D), subicular pyramidal cells (E), dentate gyrus hilus (F), and dentate gyrus granule cells (G). The presence of pallidin in ectopic granule cells (arrow heads in G) verifies that granule cells, as opposed to terminal fields among them, contain the protein. Note that pallidin is a cytoplasmic, not a nuclear protein. The scale bar in C is 1 mm; that in G is 50 µm.

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Figure 4. Light Immunomicroscopy of Pallidin in Mouse Hippocampus. Images A, B, H-K’ depict sections from mouse hippocampus. Sections correspond to immunoperoxidase microscopy with a pallidin monoclonal antibody in wild type (Bloc1s6<sup>+/+</sup>, A or Mecp2<sup>+/y</sup>, H), pallidin null (Bloc1s6<sup>+/-</sup>, B) and Mecp2 mutant hippocampi (Mecp2<sup>tm1.1Jae/y</sup>, I). J–K’ depict indirect immunofluorescence microscopy of Pallidin and VAMP2. Quantitative imaging was performed by confocal microscopy of wild type (Mecp2<sup>+/y</sup>, J–J’) and Mecp2 mutant hippocampus (Mecp2<sup>tm1.1Jae/y</sup>, K, K’). VAMP2 was used as a control to normalize staining between animals and experiments. L–M) Box plots depict relative fluorescence intensity expressed as a ratio between pallidin and VAMP2 and the total VAMP2 fluorescence intensity in the dentate gyrus, respectively. Wild type (Mecp2<sup>+/y</sup>, Blue, n = 4) and Mecp2 mutant tissue (Mecp2<sup>tm1.1Jae/y</sup>, Red, n = 4) were analyzed. P values were obtained by Mann-Whitney U test. The VAMP2 content is similar between genotypes. Scale bars A = 0.5 mm, H = 1 mm, J’ = 25 µm. DGh, dentate gyrus hilus. Images C–G correspond to sections from human hippocampal tissue stained with pallidin antibody. In C, HF denotes the hippocampal formation, consisting of the hippocampus proper (CA1–3), the dentate gyrus (DG), and the subiculum (Sub). The inner molecular layer of the DG (C, DGiml), the DG hilus (DGh) and the dentate gyrus granule cells layer (DGg) are indicated. Pallidin immunoreactivity is present in cell bodies of CA3 cells (D), subicular pyramidal cells (E), dentate gyrus hilus (F), and dentate gyrus granule cells (G). The presence of pallidin in ectopic granule cells (arrow heads in G) verifies that granule cells, as opposed to terminal fields among them, contain the protein. Note that pallidin is a cytoplasmic, not a nuclear protein. The scale bar in C is 1 mm; that in G is 50 µm.

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and CA3 as well as their associated synaptic fields in Mecp2<sup>tm1.1Jae/y</sup> hippocampus (Fig. 4, compare H–I). We performed quantitative confocal microscopy of the dentate gyrus labeled with antibodies directed against pallidin and a control marker not affected by BLOC-1 or Mecp2 mutant alleles, the synaptic vesicle protein VAMP2 (VAMP2, Fig. 4 compare J–K) [43,62–64]. Similar to the immuno-peroxidase results, pallidin content was reduced in the dentate gyrus of Mecp2<sup>tm1.1Jae/y</sup> mice (Fig. 4, L) without affecting the VAMP2 content (Fig. 4, M). We next asked if the reduction of pallidin content affected the synapse. We quantified the number of asymmetric synapses in wild type and symptomatic adult Mecp2<sup>tm1.1Jae/y</sup> mice that were positive for pallidin immunoreactivity by immunoelectron microscopy. Total synaptic counts per unit area showed no significant difference between control and Mecp2 or Bloc1s6 null genotypes (Fig. 5J). However, symptomatic Mecp2<sup>tm1.1Jae/y</sup> mice demonstrated a 50% reduction in the number of pallidin-positive presynaptic elements in asymmetric synapses of the dentate gyrus (Fig. 5F–G compare to H, and I–J). Our results
show that pallidin protein expression phenotypes exceed those predicted by the content of pallidin transcripts in adult Mecp2<sup>tm1.1Jae/y</sup> hippocampus and demonstrate that MeCP2 regulates the synaptic expression of a BLOC-1 network component.

Effects of Mecp2 and BLOC-1 Genetic Deficiencies on BLOC-1 Cargo Content in the Dentate Gyrus

Reduced pallidin immunoreactivity in Mecp2<sup>tm1.1Jae/y</sup> dentate gyrus suggests impaired BLOC-1-dependent trafficking in Mecp2<sup>tm1.1Jae/y</sup> hippocampus. We and others have established that BLOC-1 and its binding partner, the adaptor complex AP-3, generate vesicles that target the SNARE VAMP7 and phosphatidylinositol-4-kinase type II<sub>a</sub> (PI4KII<sub>a</sub>) to nerve terminals [43–45,65]. Mutations in BLOC-1 subunits lead to reduced AP-3, PI4KII<sub>a</sub>, and/or VAMP7 immunoreactivity in axons and/or asymmetric axospinous nerve terminals in the dentate gyrus [44,45,64,66,67]. Thus, we tested if Mecp2 mutations would lead to reduced AP-3, PI4KII<sub>a</sub>, and/or VAMP7 immunoreactivity in the dentate gyrus secondary to decreased pallidin content. The immunoreactivity of PI4KII<sub>a</sub> (Fig. 6A to 6B), VAMP7 (Fig. 6C and C), and the delta subunit of AP-3 (AP-3<sub>d</sub>, Fig. 6E and E), were not significantly modified in Mecp2<sup>tm1.1Jae/y</sup> dentate gyrus. In contrast, VAMP7 immunoreactivity in dysbindin-null mice (Bloc1s<sup>d6</sup>/dyd6, Fig. 6D–D) was robustly decreased in Bloc1s<sup>d6</sup>/dyd6, much like the phenotype previously observed in BLOC-1 null mice Bloc1s<sup>d6</sup>/d6 or Bloc1s<sup>mut/mut</sup> [44]. These results
suggest that genetic defects in Mecp2 do not affect the delivery of BLOC-1 cargoes to synaptic vesicles.

Defective BLOC-1 function in Bloc1s8sdy/sdy mice results in alterations in the structure and fusion of large dense secretory granules [46]. Thus, we tested the hypothesis that neuropeptides implicated in the pathogenesis of ASD and/or schizophrenia could be commonly affected in Mecp2 and BLOC-1 genetic defects. We focused on BDNF, a neurotrophic factor packaged in large dense core vesicles present in pre- and post-synaptic terminals whose content is reduced in diverse brain regions of Mecp2-deficient mice [68–71]. We measured the immunoreactivity of BDNF and the synaptic vesicle protein synaptophysin by quantitative confocal immunofluorescence microscopy in dentate gyruses from Mecp2tm1.1Jae/y, the BLOC-1-null mice Bloc1s8sdy/sdy, and Bloc1s6pa/pa (Fig. 7). BDNF antibody signal localized in the pre-synaptic terminal as indicated by overlap with the synaptic vesicle marker synaptophysin (Fig. 7A, Sphysin). BDNF immunoreactivity specificity was confirmed by out-competition of the dentate gyrus BDNF immunoreactivity with recombinant human BDNF (Fig. 7A–B and in C compare blue and light blue boxes, rhBDNF). These changes in BDNF immunoreactivity in Mecp2 and BLOC-1 deficient dentate gyrus were not due to changes in BDNF mRNA content as determined by qRT-PCR (Fig. 7D compare blue to red boxes). These results demonstrate that genetic defects in Mecp2 and BLOC-1 protein function generate a common BDNF phenotype, a growth factor packaged into large secretory granules present in nerve terminals.

Discussion

Phenotypic overlap between ASD and schizophrenia is likely rooted in shared molecular pathways between both disorders [5]. This concept is supported by recent epidemiological evidence suggesting that parental history of schizophrenia is a risk factor for ASD and advanced paternal age is a risk factor for both schizophrenia and ASD [8–10,72]. Moreover, genetic data reaffirm common molecular roots to both disorders either at the copy number variations level [11] or the disease phenotypes observed in monogenic defects [17–21,23,26]. Here we expand the mechanisms that could explain similarities between these disorders testing the idea that molecular phenotypes downstream of an ASD monogenic defect would encompass synaptic gene products associated with schizophrenia. Our results reveal a novel null alleles of the BLOC-1 complex, Bloc1s8sdy/sdy and Bloc1s6pa/pa (Fig. 7A–B and in C compare blue to red boxes). These changes in BDNF immunoreactivity in Mecp2 and BLOC-1 deficient dentate gyrus were not due to changes in BDNF mRNA content as determined by qRT-PCR (Fig. 7D compare blue to red boxes). These results demonstrate that genetic defects in Mecp2 and BLOC-1 protein function generate a common BDNF phenotype, a growth factor packaged into large secretory granules present in nerve terminals.
regulatory pathway linking MeCP2, an epigenetic transcriptional regulator and an ASD-Rett syndrome causative gene, with the interactome of the schizophrenia susceptibility gene product dysbindin. We demonstrate that a mutation in Mecp2 in mice and humans alters pallidin hippocampus transcript levels and in mouse, protein content at the synapse. The mRNA phenotype in mice is more pronounced in asymptomatic postnatal day 7 animals, a time associated with synapse formation in hippocampal ontogenesis [50]. These findings suggest that altered function of pallidin and/or the dysbindin pathway could contribute to part of the synaptic defects that characterize MeCP2 deficiencies in asymptomatic and symptomatic stages of disease [27,63,73–75]. This hypothesis is consistent with the reported functions of BLOC-1 complex subunits in neuronal cells. Pallidin- or dysbindin-deficient neurons or neuroblastoma cells have impaired neurogenesis in vitro [76–78]. In addition, dysbindin-null neurons and chromaffin cells have defective fusion of synaptic vesicles and large dense core granules, respectively [46]. These cellular defects likely contribute to impaired paired-pulse facilitation in dysbindin-null neurons [79] and the defective synaptic scaling observed in dysbindin- or snapin-deficient Drosha[phila synapses [41,42]. Strikingly, MeCP2 regulates synaptic scaling, suggesting that BLOC-1 subunits may underlie part of the scaling phenotypes in Mecp2 mutant neurons [80].

Dysbindin localization to pre- and post-synaptic compartments in mammalian neurons, yet before our study there were no reports of the subcellular localization of pallidin in mammalian brain [40]. Pallidin immunoreactivity is present in synaptic vesicles in human and mouse hippocampus. This light microscopy observation is confirmed by immunoelectron microscopy where we detect pallidin on synaptic vesicles in terminals forming asymmetric synapses of the dentate gyrus as well as on cisternae and multivesicular bodies in dendritic spines. This ultrastructural subcellular distribution confirms biochemical studies of BLOC-1 subunits co-purifying with synaptic vesicles and synaptosomes [34,44,81]. Moreover, pallidin localization to MVBs supports the role of BLOC-1 complexes controlling surface expression and lysosomal delivery of post-synaptic receptors [40,82]. Mecp2 mutation decreases the mRNA levels of pallidin by 26% in the hippocampus of symptomatic animals and by ≈30% in iPSCs-derived neurons from patients carrying MECP2 pathogenic mutations. Pallidin protein immunoreactivity by light and electron immunomicroscopy is even more reduced in the neuropil and cell bodies of neurons in the dentate gyrus. This enhanced pallidin reduction suggests additional factors downstream of MeCP2 control the protein expression of pallidin. The identity of such mechanisms remains unknown. Immunoelectron microscopy indicates that 70% of asymmetric axosynaptic synapses in the Mecp2 mutant mice have no detectable pallidin in the dentate gyrus. Other synaptic fields in the hippocampus such as the stratum lucidum in CA3 also have decreased pallidin immunoreactivity, suggesting that the synaptic defect observed in the dentate gyrus might be common to other synapses. The number of synapses per unit area of the dentate gyrus is not affected in Mecp2-deficient mice as assessed by either ultrastructural synapse counting or staining with the synaptic vesicle marker VAMP2 (Fig. 3–4). This result is similar to previous electron microscopy findings in CA1 [57]. Thus, we interpret this decreased content of pallidin-positive asymmetric axosynaptic synapses as decreased expression of pallidin in synapses rather than decreased synapse density. Importantly, loss of just one copy of dysbindin is sufficient to trigger synaptic and behavioral phenotypes in mice [49,79], supporting the contention that a 50% reduction in the levels of pallidin observed by immunoelectron microscopy in Mecp2 mutant

Figure 7. Mecp2 and BLOC-1 Deficiency Affect BDNF Immunoreactivity in the Dentate Gyrus. Images depict indirect quantitative immunofluorescence confocal microscopy of BDNF and the synaptic vesicle marker synaptophysin in the dentate gyrus from Mecp2−/− (A–B), the BLOC-1-null mice Bloc1s6pa/pa and Bloc1s8sdy/sdy (A–B). BDNF immunoreactivity was abrogated by preincubation of antibodies with recombinant human BDNF (rhBDNF, A–B). Box plots in C depict the quantitation of BDNF immunoreactivity normalized to synaptophysin. P values were obtained by One Way Anova with Dunnett’s Multiple comparisons correction, n = 4 animals per genotype. D, BDNF transcripts from symptomatic adult mice hippocampi were analyzed by qRT-PCR. Box plot depicts relative mRNA content for wild type (Mecp2+/+), Blue), Mecp2 mutant tissue (Mecp2−/−, Red), as well as the BLOC-1-null mice Bloc1s6pa/pa and Bloc1s8sdy/sdy. P values were obtained by One Way Anova with Dunnett’s Multiple comparisons correction, n = 4 animals per genotype. doi:10.1371/journal.pone.0065069.g007
hippocampus may contribute to synaptic and circuit defects that characterize MeCP2 mutations [27,63,73–75].

The reduced pallidin immunoreactivity in MeCP2-deficient hippocampus suggested that BLOC-1 sensitive cargo delivered to nerve terminals would be affected in the dentate gyrus of MeCP2<sup>+/−</sup> <i>D绿水青山</i> mice. We tested this hypothesis by analyzing the expression of three proteins present in regulated secretory vesicles: VAMP7 and P-HKII, which reside in synaptic vesicles and whose targeting is dependent on BLOC-1 and AP-3 complexes, and BDNF, which is present in large dense core granules [83–85]. We focused on BDNF because its brain content is reduced in MeCP2 brains [68–71]. However, if brain BDNF is affected by BLOC-1 deficiency was unknown. Of these three secretory vesicle markers, only BDNF immunoreactivity was reduced to the absence of BLOC-1 in the dentate gyrus. In the past we have interpreted reduced levels of an antigen in BLOC-1-null dentate gyrus as an indication of defective membrane traffic [43]. However, our findings with BDNF need additional evidence to determine if the reduced BDNF immunoreactivity in the dentate gyrus is a reflection of a BLOC-1-defective trafficking mechanism.

Mutations of dysbindin are associated with a destabilization of other components of the BLOC-1 interactome [37,86,87]. For example, dysbindin-null hippocampi possess reduced levels AP-3, peroxiredoxin 1, and COG7 [37,43,44]. It is possible that MeCP2 deficiency may also influence other components of the BLOC-1 interactome as indicated in Fig. 1. In fact, the levels of mutated are reduced in MeCP2 deficient P<sub>7</sub> hippocampi. We postulate that diverse components of the BLOC-1 interactome may be targets for transcriptional regulation by MeCP2 and/or may be directly affected in de novo cases of ASD or other neurodevelopmental disorders.

**Author Contributions**

Conceived and designed the experiments: VF LPM YS GB KT IP. Performed the experiments: JL PVR GG CC LG AA KK. Analyzed the data: JL PVR KT VF YS PK. Wrote the paper: VF.

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