Autism-Associated Chromatin Regulator Brg1/SmarcA4 Is Required for Synapse Development and Myocyte Enhancer Factor 2-Mediated Synapse Remodeling

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Synapse development requires normal neuronal activities and the precise expression of synapse-related genes. Dysregulation of synaptic genes results in neurological diseases such as autism spectrum disorders (ASD). Mutations in genes encoding chromatin-remodeling factor Brg1/SmarcA4 and its associated proteins are the genetic causes of several developmental diseases with neurological defects and autistic symptoms. Recent large-scale genomic studies predicted Brg1/SmarcA4 as one of the key nodes of the ASD gene network. We report that Brg1 deletion in early postnatal hippocampal neurons led to reduced dendritic spine density and maturation and impaired synapse activities. In developing mice, neuronal Brg1 deletion caused severe neurological defects. Gene expression analyses indicated that Brg1 regulates a significant number of genes known to be involved in synapse function and implicated in ASD. We found that Brg1 is required for dendritic spine/synapse elimination mediated by the ASD-associated transcription factor myocyte enhancer factor 2 (MEF2) and that Brg1 regulates the activity-induced expression of a specific subset of genes that overlap significantly with the targets of MEF2. Our analyses showed that Brg1 interacts with MEF2 and that MEF2 is required for Brg1 recruitment to target genes in response to neuron activation. Thus, Brg1 plays important roles in both synapse development/maturation and MEF2-mediated synapse remodeling. Our study reveals specific functions of the epigenetic regulator Brg1 in synapse development and provides insights into its role in neurological diseases such as ASD.

Received 25 May 2015 Returned for modification 24 June 2015 Accepted 5 October 2015 Accepted manuscript posted online 12 October 2015


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Supplemental material for this article may be found at http://dx.doi.org/10.1128/MCB.00334-15.

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tion cofactors, and Ca²⁺ signaling-induced exchange from the corepressor complex to coactivator complex is important for MEF2 transcription activity (30, 31). However, it is unclear how these cofactors coordinate with MEF2 to activate gene expression in response to neuronal activities.

In this report, we specifically deleted Brg1 in developing neurons and revealed essential functions of Brg1 in synapse formation, maturation, and remodeling. Brg1 specifically regulates a significant number of genes encoding synaptic proteins and proteins implicated in ASD. We found that Brg1 is required for dendritic spine/synapse elimination mediated by MEF2C and that Brg1 regulates the activity-induced expression of a number of MEF2 target genes. Our analysis showed that MEF2C is required for Brg1 recruitment to MEF2 targets upon neuronal activation. Thus, Brg1 regulates synapse formation, maturation, and MEF2-mediated synapse remodeling. Our study revealed the specific mechanisms through which the epigenetic factor Brg1 regulates synapse development and provides insights into its role in neurological diseases.

MATERIALS AND METHODS

Mice. Brg1flo×flo (here called Brg1F/F) mice (32), Syn1-Cre mice (33), and MEF2Cflo×flo (here called MEF2CΔM) mice (34) were kindly provided by Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire [IGBMC], France), Luis Parada (University of Texas [UT] Southwestern Medical Center), and Eric Olson (UT Southwestern Medical Center), respectively. BAF53b-Cre mice were generated by transgenic injection of a bacterial artificial chromosome (BAC) construct containing a Cre gene under the control of the neuron-specific BAF53b promoter and regulatory elements (35). These mice were maintained on a mixed genetic background at UT Southwestern Medical Center Animal Facility. All procedures were performed in accordance with the IACUC-approved protocols. In all animal experiments, both males and females were used, and there was no significant difference found between genders.

Plasmid and constructs. The construct for expression of Cre is PMCCcreN (36). The MEF2 reporter construct MEF2 response element (MRE)-Luc contains three MRE sequences upstream of the luciferase reporter. The MEF2C, MEF2C-VP16, and MEF2A-VP16 expression constructs in the pCDNA3 vector and the green fluorescent protein (GFP) construct containing an expression cassette for both GFP and myristylated GFP were described previously (29) and were provided by Chris Cowan (Harvard). pSin-Brg1 (37) was used for expression of Brg1 in cultured cells.

Behavior tests. All experiments in this study were performed in the Behavior Core Facility and approved by the IACUC at UT Southwestern Medical Center. Mice were housed with food and water available ad libitum with a 12-h light/dark cycle, and all behavior testing occurred during the light cycle. For the open-field activity test, mice were placed in the periphery of a novel open-field environment (44 cm by 44 cm with walls 30 cm high) in a dimly lit room and allowed to explore for 15 min. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision, version 3.0; Noldus) to determine the time, distance moved, and number of entries into two areas, the periphery (5 cm from the walls) and the center (area of 14 cm by 14 cm). The open-field arenas were wiped and allowed to dry between mice.

Immunofluorescent staining. Immunostaining experiments were performed on paraffin sections (7-μm) or vibratome thick sections (50-μm) of brain tissues and on cultured hippocampal slices and neurons cultured on cover glasses. Antibodies used were against Brg1 (G7 or H88; Santa Cruz Biotechnology), NeuN (Abcam), GFP (Molecular Probes), and HuC/D (Molecular Probes). The images were visualized using an Olympus BX50 microscope.

Dendritic spine analyses of dentate gyrus granule neurons with Lucifer yellow injection. Brg1F/F or Syn1-Cre Brg1F/F mice at postnatal day 21 (P21) were intracardially perfused with 1.5% paraformaldehyde (PFA) solution, and brains were postfixed in 1.5% PFA solution for 6 h and then sectioned into 300-μm thick slices. Lucifer yellow injection into single dentate gyrus (DG) neurons was performed with a microelectrode amplifier (Multiclamp 700B; Molecular Devices). Neurons in the DG granule cell layer were selected visually under the microscope and patched with the electrodes filled with Lucifer yellow solution (L-12926; Invitrogen). Secondary dendrites (50 to 200 μm from the cell body) were imaged using a Zeiss LSM780 two-photon microscope (40× water immersion lens). Spine density was measured with the NeuronStudio software package with default settings (38). Z-stack confocal images of dendrite segments were reconstructed and analyzed in NeuronStudio for dendritic spine identification and classification as mushroom-, stubby-, or thin-shaped spines. In classification of spine shapes we used the following cutoff values: aspect ratio for thin spines, 2.5; head-to-neck ratio, 1.3; and head diameter, 0.45 μm. Two segments (50 to 100 μm) per neuron (n = 16 to 22 neurons under each condition) were chosen for quantitative analysis. Dendritic spine volume was further analyzed with Imaris software (39).

Hippocampal slice culture, biologic transfection, and dendritic spine analyses. Organotypic hippocampal slice cultures were prepared from P6 Brg1F/F mice (29). Hippocampi were dissected and sliced to a 300-μm section with a tissue slicer. Hippocampal slices were cultured on a membrane at the interface between the medium and the air. The culture medium included minimal essential medium (MEM) with horse serum, t-glutamine, CaCl₂, MgSO₄, dextrose, NaHCO₃, HEPES (pH 7.2), and insulin. Gold bullet preparation and biologic DNA transfection to hippocampal slices cultured for 1 day in vitro (DIV) were performed with a Helios gene gun system (Bio-Rad) according to the manufacturer’s protocols. Five days after transfection, dendritic spines were imaged and analyzed as described above.

Electrophysiological measurement of synapse activities. Organotypic hippocampal slice cultures were prepared from P6 wild-type or Brg1F/F mice. Cultures were biologically transfected with plasmids for expression of GFP and Cre or control plasmids at 3 DIV. Ten days later, simultaneous whole-cell recordings were obtained from CA1 pyramidal neurons in slice cultures visualized using infrared-differential interference contrast (IR-DIC) and GFP fluorescence to identify transfected and nontransfected neurons. Recordings were made at 32°C in a submersion chamber perfused with artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 11 mM glucose, 3 mM CaCl₂, 2 mM MgCl₂, 0.1 mM picrotoxin, 0.002 mM 2-chloro-adenosine, and 0.1% dimethyl sulfoxide (DMSO) at pH 7.28 at 305 mosM and saturated with 95% O₂–5% CO₂. Neurons were voltage clamped at −60 mV through whole-cell recording pipettes (−4 to 6 MΩ) filled with an intracellular solution containing 0.2 mM EGTA, 130 mM K-glutamate, 6 mM KCl, 3 mM NaCl, 10 mM HEPES, 2 mM QX-314, 4 mM ATP-Mg, 0.4 mM GTP-Na, and 14 mM phosphocreatine-Tris, pH 7.2, adjusted using KOH, at 285 mosM.

For miniature excitatory postsynaptic current (mEPSC) measurements, the ACSF was supplemented with 1 μM tetrodotoxin (TTX). Series and input resistance were measured in a voltage clamp with a 400-ms, 10-ms step from a −60-mV holding potential (filtered at 30 kHz, sampled at 50 kHz). Cells were used for analysis only if the starting series resistance was less than 30 MΩ and was stable throughout the experiment. Input resistance ranged from 50 to 900 MΩ. Data were not corrected for junction potential. No significant difference was observed between transfected and untransfected neurons in resting membrane potentials, indicating that overall neuronal health was unaffected by expression of Cre. Synaptic currents were filtered at 3 kHz, acquired, and digitized at 10 kHz using custom software (Labview; National Instruments). mEPSCs were filtered at 1 kHz and detected off-line using an automatic detection program (MiniAnalysis; Synaptosoft, Inc.) with a detection threshold set at a value greater than at least 5-fold of the root mean square noise levels, followed by a subsequent round of visual confirmation. Significance of differences between results for transfected and untransfected neurons was determined using a paired t test.
Cortical and hippocampal neuron culture and transfection. Embryonic day 18.5 (E18.5) hippocampal and E16.5 to E18.5 cortical cells were cultured as previously described (19). Dissociated cells were plated on poly-λ-cornathine- and fibronectin-coated coverslips. Culture medium contained Dulbecco’s modified Eagle’s medium supplemented with F-12 medium with putrescine, 2-mercaptoethanol, transferrin, insulin, selenium, progesterone, MEM vitamin additive, and 5% fetal bovine serum (FBS). At 7 DIV, hippocampal cultures were transfected with GFP and MEF2 expression constructs using Lipofectamine 2000. Cultures were fixed and imaged at 14 DIV for dendritic spine analyses as described above. Cortical cultures were transfected with reporter and MEF2 constructs at 5 DIV and analyzed at 6 DIV. For depolarization, 50 mM KCl was added to the cultures for 1 to 6 h as described previously (25). Luciferase reporter assays were performed using a Dual-Luciferase assay kit (Promega). The mating to produce BAF53b-Cre Brg1F/F mutant embryos for primary neuron cultures was between BAF53b-Cre Brg1F/F and Brg1F/F. Neurons from Brg1F/F, Brg1F/F, or BAF53b-Cre Brg1F/F heterozygous littersmates displayed no significant differences from each other in all experiments and were used as controls.

Reverse transcription-PCR (RT-PCR) and qPCR. RNA from cells or ground tissues was extracted with TRIzol (Invitrogen). cDNAs were synthesized by reverse transcription using Iscript (Bio-Rad), followed by PCR or quantitative PCR (qPCR) analysis. A Bio-Rad real-time PCR system (C1000 Thermal Cycler) was used for quantitative PCR. Levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase gene) mRNA were used to normalize input RNA. Graphics shown are representative of experiments performed in triplicate. The experiments were repeated at least three times. Standard errors were calculated according to a previously described method (37). The sequences of all the primers are listed in Table S3 in the supplemental material.

RNA-seq and data analyses. DG of P13 or P21 Brg1F/F and Syn1-Cre Brg1F/F mice were used for transcriptome sequencing (RNA-seq) analyses. One pair each of P13 and P21 control and mutant samples was used; each sample was pooled from two mice. In addition, cultured BAF53b-Cre Brg1F/F and control cortical neurons with or without KC1 stimulation (each sample was pooled from three mice) were subjected to RNA-seq analyses. Total RNAs were extracted, and RNA-seq libraries prepared using an Illumina RNA-Seq Preparation Kit were sequenced on a HiSeq 2500 sequencer at UT Southwestern Sequencing Core Facility. RNA-seq reads were mapped using TopHat with default settings (http://ccb.jhu.edu/software/tophat/index.shtml). The mapped reads with a Phred quality score of <20 were filtered out, whereas the duplicates were marked but not removed using SAMTOOLS (40) and Picard (http://broadinstitute.github.io/picard/). Transcript assembly and transcript abundance quantification were carried out using CUFFLINKS, and then differential expression analysis between control and Brg1 mutants was performed using CUFFDIFF (41). The differentially expressed genes with fold changes larger than 1.5 and P values of <0.05 were selected as Brg1-regulated genes. Gene ontology analysis was performed using DAVID (http://david.abcc.ncifcrf.gov/). Fisher’s exact test was used to determine the significance of overlapping data sets.

ChIP experiments. Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (37, 42). Tissue disrupted by Dounce homogenization or dissociated cells were cross-linked with PFA and sonicated to fragments (200 to 500 bp). Antibodies used were against Brg1/Brm (J1) (43) or rabbit IgG control, J1 antibody has been used previously for Brg1 ChIP sequencing (ChIP-seq) analyses (44, 45). Precipitated DNA was purified and subjected to real-time PCR. The percentage of input or fold of enrichment relative to the IgG ChIP level was measured.

Immunoprecipitation and Western blotting. Cultured cortical neurons were treated with or without KC1 at 7 DIV for 1 h. Cells were harvested and lysed with coimmunoprecipitation (co-IP) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, with protease inhibitor freshly added). After centrifugation, rabbit polyclonal antibodies against Brg1/Brm (J1) were added to precleared cell extracts, and samples were incubated at 4°C overnight. Samples were incubated with protein A beads (GE Healthcare) for 1 h; beads were washed with co-IP buffer four times. Precipitated proteins were eluted by boiling in 2× sample buffer (Bio-Rad) before SDS-PAGE and Western blot analysis. For immunoblotting, cell lysates or immunoprecipitates were separated on SDS-PAGE gels. Antibodies used were against Brg1 (G7; Santa Cruz Biotechnology), MEF2C (Cell Signaling), and MEF2D (BD Biosciences). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

RESULTS
Deleting Brg1 in hippocampal neurons impairs synapse formation and maturation. The association of Brg1 with ASD prompted us to evaluate synapse development in Brg1 mutant neurons. To determine the cell-autonomous functions of Brg1 in synapse formation, we deleted Brg1 in individual hippocampal neurons and determined the effects on excitatory synapse activities. Using a gene gun biolistic particle delivery system, we introduced plasmids expressing Cre or control empty vector into cultured postnatal day 6 (P6) Brg1F/F (32) hippocampal slices. The cotransfection efficiency using this method is more than 95%, and we observed Cre-mediated Brg1 deletion in GFP-positive (GFP+) neurons (Fig. 1A). Since very few neurons can be transfected with this method, any effects of Brg1 deletion on postsynaptic development should be cell autonomous. We focused on CA1 pyramidal neurons as their stereotypical position and morphology make them easily identifiable in the hippocampal cultures. Ten days after transfection, miniature excitatory postsynaptic currents (mEPSCs) were measured from simultaneous recordings of transfected GFP+ and neighboring untransfected CA1 neurons. Brg1-deleted neurons displayed a decreased mEPSC frequency relative to that of untransfected neurons, whereas mEPSC amplitudes were unchanged (Fig. 1B and C). The reduction of mEPSC frequency was not caused by Cre expression but by Brg1 deletion because transfection of CA1 neurons from wild-type hippocampal slice cultures with the vector for expression of Cre had no effect on mEPSCs (Fig. 1D). mEPSC frequency is correlated with synapse number (29), whereas mEPSC amplitude represents the strength of individual synapses. These data indicate that Brg1 is required for the development of functional excitatory synapses.

Most excitatory synapses are built on dendritic spines that contain the postsynaptic signaling machinery and receive synaptic inputs. Spine densities and morphologies faithfully reflect synapse numbers and levels of maturation (46–48). Mushroom-shaped and stubby spines with larger volumes usually indicate mature synapses, whereas long and thin spines with small volumes indicate no or immature synapses. To assess the role of Brg1 in development of dendritic spine, we measured spine densities, volumes, and shapes from CA1 neurons of cultured P6 Brg1F/F hippocampal slices biolistically transfected with constructs for expression of Cre or control and membrane-targeted GFP. Six days after transfection, two-photon confocal microscopy images of GFP+ dendritic spines were taken and analyzed with NeuronStudio software for quantification and spine classification (38, 49). We observed a significant decrease in dendritic spine densities in Brg1F/F CA1 neurons expressing Cre relative to the number of neurons transfected with empty vectors (Fig. 1E and F), indicating impaired synapse formation in the absence of Brg1. Analyses of the spine shapes showed a significant reduction in mushroom-shaped spines and an increase in thin spines in Brg1-deleted neurons.
FIG 1 Deleting Brg1 in hippocampal neurons impairs synapse/dendritic spine formation and maturation. (A) Organotypic hippocampal slice cultures from P6 wild-type or Brg1F/F mice were biolistically transfected with Cre-expressing plasmids or empty vector controls together with GFP. Immunostaining showed Brg1 deletion in Cre-expressing GFP-labeled cells but not in the control cells 5 days after transfection (arrows). (B to D) In the organotypic hippocampal slice culture system, synaptic function was measured using whole-cell patch clamp recordings of Cre-transfected CA1 pyramidal neurons or neighboring untransfected neurons. Representative traces of mEPSCs are shown (B). Average mEPSC frequency and mEPSC amplitude from Cre-expressing (Brg1F/F) and from Cre-expressing (n = 18) or untransfected (n = 18) wild-type neurons (D) are shown. (E to H) Organotypic hippocampal slice cultures from P6 Brg1F/F mice were biolistically transfected with GFP- and Cre-expressing plasmids or with a vector control. Representative pictures of dendritic spines of CA1 pyramidal neurons are shown in panel E (scale bar, 5 μm). Average dendritic spine densities (F), classifications (G), and spine volumes (H) of control (n = 16) and Brg1-deleted (n = 20) CA1 neurons are shown. Values in the graphs represent averages plus standard errors. **, P < 0.01; *, P < 0.05 (Student’s t test). DAPI, 4',6'-diamidino-2-phenylindole.

compared to results in control neurons (Fig. 1G). Consistently, there was a significant decrease in spine volumes in Brg1-deleted neurons compared to volumes in control neurons (Fig. 1H). These data demonstrate that Brg1 is required for dendritic spine/synapse formation and maturation in postsynaptic neurons and indicate that Brg1 promotes synapse formation and maturation in a cell-autonomous manner.

Brg1 deletion in neurons led to neurological defects in mouse. To determine the function of Brg1 in synapse development in vivo, we crossed Brg1 conditional knockout mice (32) with several neuron-specific Cre transgenes. A Brg1 deletion in forebrain neurons mediated by a widely used Camk2a-Cre line (50) led to hydrocephalus, mainly due to non-cell-autonomous effects (51). A newly developed BAF53b-Cre line (35) deleted Brg1 in all developing neurons, which caused lethality at birth due to respiratory defects (data not shown). Therefore, these mice cannot be used to study postnatal synaptogenesis. A Synapsin1-Cre (Syn1-Cre) transgene (33) is expressed exclusively in neurons beginning in the late embryonic stage, but its expression pattern is mosaic in most brain areas. Syn1-Cre Brg1F/F mice survive, which enabled us to study Brg1 function in synapse development in vivo. In hippocampus, Syn1-Cre is not expressed in the CA1 region but has strong expression in the dentate gyrus (DG) and CA3 regions (Fig. 2A) (33). In Syn1-Cre Brg1F/F mice, Brg1 deletion from the NeuN+ DG granule neurons was detected at P7; at P14 and P21, Brg1 deletion was clearly observed in neurons in the DG and CA3 regions (Fig. 2A). Control and Brg1-deleted dentate gyrus showed similar morphologies (Fig. 2A). The gross examination of Syn1-Cre Brg1F/F brain at different ages did not reveal obvious structural defects (Fig. 2B). The brain weights of these mice were also normal (Fig. 2C).

Although born with normal weight, Syn1-Cre Brg1F/F mutant mice were smaller during development than control mice.
Brg1 mutants displayed locomotor and behavior abnormalities beginning in the early postnatal stage. They exhibited significantly increased hindlimb clasp frequency compared to that of Brg1/F/F controls which was more severe prior to weaning than after (Fig. 2E). Young Brg1 mutant pups were overactive and unbalanced. At P7, in the righting reflex test none of the Brg1 mutant pups righted after 1 min, whereas all control mice righted within 30 s (Fig. 2F and data not shown). These severe behavioral abnormalities at a young age suggest that Brg1 deletion affects neuronal and synapse development. Adult Syn1-Cre Brg1F/F mice had significantly increased hindlimb clasp (Fig. 2E) but had largely recovered from locomotor abnormalities and displayed normal activity/anxiety levels in the open-field activity test in the first 5 min (data not shown) or in the full 15 min (Fig. 2G). During a foot shock test, we observed that adult Brg1 mutant mice had significantly increased sensitivity to foot shock stimulation, as indicated by the significantly decreased jump threshold compared to that of control Brg1F/F mice (Fig. 2H). These neurological defects indicate that Brg1 deletion in developing neurons impairs neuronal development and function.

**Neuronal Brg1 deletion impairs synapse maturation in vivo.** To evaluate the dendritic spine morphology in Brg1 mutant neu-
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The gene expression profiles in control and mutants synapse development, we performed RNA-seq to compare synaptic gene network is a key step to control synaptogenesis in regulators as autism risk genes suggests that the regulation of the neuronal functions of Brg1 in synaptic gene regulation, we compared the Brg1-regulated genes in developing DG with known synaptic genes (52) and with human genes linked to autism as shown in the SFAI (Simons Foundation Autism Research Initiative) gene database (www.sfari.org). The P13 DEG data set was also used for comparison as P13 is a stage when synaptic and neurological defects are apparent in Brg1 mutant mice. There are significant overlaps between Brg1-regulated genes and synaptic autism genes (Fig. 4E). Thus, Brg1 specifically regulates synaptic genes in developing neurons; the abnormal expression of these genes may contribute to autism pathogenesis.

BAF53b-Cre-mediated pan-neuronal Brg1 deletion in cultures. Many synaptic genes are regulated by neuronal activities that help convert transient stimuli into long-term changes in neuronal morphology and synapse activities. Neuronal BAF complexes regulate activity-dependent dendrite growth, suggesting a signaling pathway from Ca²⁺ influx to chromatin regulation (19). Brg1 was found to repress the basal expression of the c-fos gene (53). However, activity-induced nBAF target genes are not known, and it is not clear how nBAF complexes regulate gene activation in response to neuronal activities. To understand the function of Brg1 and nBAF complexes in activity-dependent gene regulation, we deleted Brg1 in cultured neurons. The mosaic expression pattern of Snt1-Cre in the cortex and hippocampus prevented us from using this line for neuronal culture studies. Therefore, we took advantage of the newly generated pan-neuron-specific BAF53b-Cre transgene (35) to delete Brg1 in all neurons in the culture (Fig. 5). The BAF53b subunit of the nBAF complexes is expressed exclusively in neurons and in all neurons examined (19). BAF53b-Cre BAC transgene activities were detected in all neurons by E18.5. BAF53b-Cre Brg1F/F mice died at birth due to respiratory failure. However, Brg1 proteins were not completely deleted from cortical and hippocampal neurons at birth (Fig. 5, 1 DIV). We cultured mixed cortical/hippocampal neurons from P13 and P21 DG revealed that the most enriched group of genes encode extracellular matrix-associated proteins (P = 5.9 × 10⁻⁸) (Fig. 4D). High enrichment was also observed in genes encoding growth factor binding proteins, cell adhesion proteins, cytoskeleton regulators, plasma membrane proteins, and calcium signaling pathway components (Fig. 4D), which are all closely related to synapse development. The enrichment of the target genes in these synapse-associated pathways indicates that neuronal Brg1 and nBAF complexes specifically regulate genes involved in synapse formation, maturation, and plasticity. To further understand the molecular functions of Brg1 in synaptic gene regulation, we compared the Brg1-regulated genes in developing DG with known synaptic genes (52) and with human genes linked to autism as shown in the SFAI (Simons Foundation Autism Research Initiative) gene database (www.sfari.org). The P13 DEG data set was also used for comparison as P13 is a stage when synaptic and neurological defects are apparent in Brg1 mutant mice. There are significant overlaps between Brg1-regulated genes and synaptic autism genes (Fig. 4E). Thus, Brg1 specifically regulates synaptic genes in developing neurons; the abnormal expression of these genes may contribute to autism pathogenesis.

FIG 3 Syn1-Cre-mediated Brg1 deletion in neurons impairs synapse maturation in vivo. Individual DG granule neurons in fixed P21 Brg1+/F and Syn1-Cre Brg1+/F hippocampal slices were injected with Lucifer yellow to enable visualization. (A) Representative two-photon confocal microscopy images of dendritic spines. Scale bar, 3 μm. (B to D) Average spine densities (B), classification of spines (C), and average spine volumes from Brg1+/F (n = 18) and Syn1-Cre Brg1+/F (n = 20) neurons (D) are shown. Values in the graphs are averages plus standard errors. *, P < 0.05 (Student’s t test).

Brg1 regulates synaptic genes in developing hippocampus. The identification of many transcription factors and epigenetic regulators as autism risk genes suggests that the regulation of the synaptic gene network is a key step to control synaptogenesis in normal development and in diseases. To determine how Brg1 regulates synapse development, we performed RNA-seq to compare the gene expression profiles in control and Syn1-Cre Brg1+/F neurons (Fig. 4; see also Table S1 in the supplemental material). We analyzed P13 and P21 DG because in Syn1-Cre Brg1+/F hippocampus, Brg1 was not deleted in CA1 neurons but was completely deleted in DG granule neurons at this development stage (Fig. 2A). Analyses of two-photon confocal microscopy images of dendritic spines showed that although Brg1 mutant granule neurons displayed similar spine densities (Fig. 3A and B), there was a significant increase in thin spines in Brg1 mutant neurons (Fig. 3C), which indicates impaired synapse maturation. Similar to the individual Brg1-deleted CA1 neurons, these Brg1-deleted DG granule neurons also displayed significantly reduced dendritic spine volumes (Fig. 3D). Since spine volume correlates well with synapse maturation and activities, the synapse defects in both Brg1-deleted CA1 pyramidal neurons in hippocampal slice cultures and in DG granule neurons in vivo indicate that Brg1 is likely required for synapse development and maturation in general.

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January 2016 Volume 36 Number 1
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E18.5 control or BAF53b-Cre Brg1f/f mice. Using a Rosa-YFP Cre reporter (54), we observed a complete overlap between yellow fluorescent protein-positive (YFP+/H11001) cells and neuronal marker HuC/D staining in BAF53b-Cre Rosa-YFP cultures (Fig. 5, bottom panel), confirming that BAF53b-Cre is pan-neuron specific. In BAF53b-Cre Brg1f/f Rosa-YFP cultures, Brg1 proteins became undetectable in YFP+/H11001 neurons after 5 to 7 days in culture (Fig. 5, 7 DIV and 14 DIV). Brg1 was detected in all nonneuronal cells. Brg1 mutant (BAF53b-Cre Brg1f/f) and control neurons had similar viabilities and cell numbers, indicating that Brg1 is not required for neuron survival in general. We therefore used this culture system to study Brg1 function in neuronal activity-dependent gene regulation and synapse plasticity.

**Brg1 is required for MEF2-mediated gene activation.** To determine the function of Brg1 in neuronal activity-induced gene activation, we measured gene expression profiles in the cultured control and Brg1 mutant neurons under the basal and depolarized conditions. Cultured cortical neurons were used to identify Brg1-regulated neuronal activity-induced genes because these cultures have a high content of neurons (>80%) and are suitable for molecular and biochemical experiments. Many previous studies of activity-induced gene regulation were performed using similar culture conditions (19, 26, 53). The target genes and regulatory mechanisms identified here could be applied to many other experimental systems.

**FIG 4** Brg1 regulates synaptic genes in developing neurons. (A) Brg1-regulated genes in P13 and P21 DG identified with RNA-seq were compared. The Venn diagrams show the numbers of common genes activated or repressed by Brg1 in each developmental stage. (B) Examples of neuronal genes activated and repressed by Brg1 in both P13 and P21 DG. (C) Brg1 ChIP-qPCR experiments were performed using P13 and P21 DG. Enrichment of Brg1 binding to the regulatory regions of the indicated target genes compared to results with IgG ChIP was observed. A region in the CD4 gene was used as a Brg1 binding negative control. Values in the graph are shown as averages plus standard errors (n = 3). (D) List of the most enriched Brg1-regulated biological processes identified by DAVID gene ontology analysis of the RNA-seq data set obtained from both P13 and P21 DG. (E) Significant overlap between Brg1-regulated genes in developing DG and synaptic genes (52) and autism genes (SFARI gene database). Fisher’s exact test was used to calculate P values.
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YFP
that at 1 DIV, Brg1 was not completely deleted in YFP-labeled neurons in rons. Mixed cortical and hippocampal neurons cultured from E18.5 embryos BAF53b-Cre activity-induced genes (Fig. 6A). Of these 76 genes, 74 were not tion with the activity-induced genes yielded 76 Brg1-regulated
Brg1
Brg1
FIG 5 BAF53b-Cre-mediated Brg1 deletion in cultured cortical/hippocampal neurons. (A) BAF53b-Cre was used to mediate Brg1 deletion in cultured neurons. Mixed cortical and hippocampal neurons cultured from E18.5 embryos with the indicated genotypes at 1 DIV, 7 DIV, and 14 DIV were stained with antibodies against Brg1, YFP reporter, and the neuronal marker HuC/D. Note that at 1 DIV, Brg1 was not completely deleted in YFP-labeled neurons in Brg1 mutant culture, whereas at 7 DIV and 14 DIV, Brg1 was completely deleted in YFP−Brg1 mutant neurons. At 14 DIV, the YFP reporter completely costained with all HuC/D-positive cells, confirming the pan-neuron-specific feature of BAF53b-Cre transgene. DAPI, 4′,6′-diamidino-2-phenylindole.

determine the effect of Brg1 deletion on activity-induced gene transcription. By comparing transcription profiles under basal and depolarized conditions in control neurons, we identified 1,943 DEGs (fold change of >1.5, P value of <0.05), of which 1,254 were increased by neuron depolarization (see Table S2 in the supplemental material). By comparing the RNA signals from de- polarized control and Brg1 mutant neuron cultures, we found that 219 genes were downregulated after Brg1 deletion. The intersection with the activity-induced genes yielded 76 Brg1-regulated activity-induced genes (Fig. 6A). Of these 76 genes, 74 were not regulated by Brg1 under basal conditions but only at the activity-induced level. Interestingly, 15 of the 74 Brg1-regulated activity-induced genes are also target genes of the MEF2 family of transcription factors (Fig. 6A). Previously, MEF2 target genes were identified from neurons cultured under similar conditions (26). By comparing the MEF2 target genes with our activity-induced neuronal gene list, we obtained 57 activity-induced MEF2 targets; of these, 15 are also regulated by Brg1 (Fig. 6A). The overlap rate is significantly higher than the overlap between two groups of 74 and 57 genes randomly selected from the 1,254 activity-induced gene pool (P = 2.5 × 10−7). The common Brg1/MEF2 target genes include genes known to be important for synapse structure and plasticity such as BDNF, Kcnal, Homer1, Nrl4a1 (Nur77), and PCDH17 (24). We confirmed the impaired induction of several Brg1/MEF2 target genes by neuronal depolarization in Brg1 mu
t cortical neuron cultures (Fig. 6B). Junb, a gene that is not a MEF2 target, was used as a control. Brg1 deletion did not signifi
cantly change activity-induced Junb expression. Moreover, the fact that Brg1 regulated the activity-dependent expression of only 74 genes (<10% of all activity-induced genes) (Fig. 6A) indicates that Brg1 is required for neither activation of the upstream Ca2+ signaling nor activity-induced gene expression in general. Brg1 deletion in neurons did not impair the protein levels or activation of MEF2 proteins by dephosphorylation, as indicated by Western blotting of MEF2C and MEF2D (Fig. 6C). Thus, Brg1 is specifically required for gene activation mediated by certain transcription factors such as MEF2.

The MEF2 family of transcription factors contains four members that have high homology in their DNA binding domains. MEF2C is the major form expressed in the cortex and has been shown to play a predominant role in neuronal synapse development and function (55). A fusion of the MEF2C DNA-binding and dimerization domain with the VP16 activation domain serves as an activator of MEF2 target genes and bypasses the need for MEF2-specific coactivators (25). To determine whether Brg1 is required for MEF2-mediated transcription activation, we exam-
ined the effect of Brg1 deletion on the expression of an MEF2-
activated reporter gene. Although the reporters are different from endogenous genes, plasmids could incorporate nucleosomes and have been used successfully to test the transcription regulator functions of Brg1 (53). A luciferase reporter with three MEF2 response elements (MRE-Luc) (25) was cotransfected with plasmids for expression of MEF2C or MEF2-VP16 or a control plasmid into cultured BAF53b-Cre Brg1−/−FRP or control neurons. As expected, in control neurons, MRE-Luc was minimally expressed in the resting stage but was induced by either depolarization or co-expression of MEF2C or MEF-VP16 (Fig. 6D). In Brg1 mutant neurons, both depolarization-induced and MEF2C-induced reporter expression was significantly impaired. Interestingly MEF2-
VP16 activated MRE-Luc to the same levels in the presence and absence of Brg1 (Fig. 6D). The different requirements for Brg1 in activation of MRE-Luc by exogenous MEF2C and MEF2-VP16 suggest that Brg1 functions as a coactivator of MEF2C and that this requirement is bypassed by MEF2-VP16. Importantly, defective depolarization-induced expression of endogenous MEF2 tar-
gets such as Kcnal caused by Brg1 deletion could be rescued by expression of MEF2-VP16 (Fig. 6E). Therefore, Brg1 is required for MEF2-mediated gene activation.

MEF2C is required for the activity-dependent recruitment of Brg1 to target genes. MEF2 regulates activity-dependent target genes by exchanging cofactors from corepressors to coactivators upon neuron activation. Since Brg1 is a potential coactivator for MEF2-activated gene expression, we examined the dynamic bind-
ing of Brg1 to MEF2 targets. Cultured cortical neurons at 7 DIV were depolarized by a 1-h KCl treatment. Brg1 ChIP was per-
formed, and the signals in the regulatory regions of activity-de-
dependent MEF2 target genes in resting and depolarized neurons were compared. Depolarization of the neurons significantly in-
duced the binding of Brg1 to these genes (Fig. 7A). GAP43 is a neuronal Brg1 target gene that is not induced by neuronal activi-
ties and is not an MEF2 target; Brg1 was not further recruited to
FIG 6. Brg1 is required for MEF2-mediated gene activation. (A) Analyses of Brg1-regulated activity-dependent gene expression using RNA-seq data sets obtained from BAF53b-Cre Brg1<sup>F/F</sup> Brg1 mutant and control cultured cortical neurons at 7 DIV were treated with or without KCl for 6 h. The Venn diagrams show the intersections of Brg1-activated genes and activity-induced genes (left panel) and the significant overlap between Brg1-regulated and MEF2-regulated activity-induced genes (right panel). (B) Impaired expression of activity-induced MEF2 target genes in Brg1 mutant neuronal cultures as indicated by RT-qPCR analyses. Junb was used as a non-MEF2 target gene control. (C) Western blot showing MEF2 proteins in control and Brg1 mutant neuronal cultures as indicated by RT-qPCR measurement. Values in the graphs are shown as averages plus standard errors. Significance was determined using a test or analysis of variance with a post hoc test. ** P < 0.01 (n = 3).

The function of MEF2 in synapse remodeling and plasticity is most clearly demonstrated by data indicating that MEF2 activator overexpression in neurons reduces synapse and dendritic spine densities (25, 29). To determine whether Brg1 is required for MEF2-mediated dendritic spine/synapse elimination, we transfected plasmids for expression of MEF2C, MEF2-VP16, or a control Brg1 mutant neuronal cultures in response to KCl depolarization as indicated by RT-qPCR measurement. Values in the graphs are shown as averages plus standard errors. Significance was determined using a test or analysis of variance with a post hoc test. ** P < 0.01 (n = 3).

Brg1 is required for MEF2-mediated dendritic spine elimination. The function of MEF2 in synapse remodeling and plasticity.
In control neurons, MEF2C expression significantly reduced dendritic spine densities. In CA1 neurons where Brg1 was deleted by Cre induction, dendritic spine densities were significantly lower than those in control neurons; moreover, MEF2C failed to further reduce spine densities in the Brg1 mutant neurons (Fig. 8C and D). The expression of MEF2C did not significantly change the ratio between the thin and mushroom-shaped or stubby spines in either control or Brg1-deleted neurons (Fig. 8E). These experiments indicate that Brg1 is required for MEF2C-mediated dendritic spine/synapse elimination in hippocampal neurons in both dissociated and slice cultures.

**DISCUSSION**

In this study, using different Cre systems that are most suitable for the required studies, we found that the ASD-associated chromatin remodeler Brg1 regulates the expression of genes involved in synapse development and plasticity during development and in response to neuronal activities. During neuronal development, Brg1 is required for synapse formation and maturation in several types of neurons. In response to neuronal activities, Brg1 is recruited to MEF2 target genes to control synapse elimination (Fig. 8C and D). The expression of MEF2C did not significantly change the ratio between the thin and mushroom-shaped or stubby spines in either control or Brg1-deleted neurons (Fig. 8E). These experiments indicate that Brg1 is required for MEF2C-mediated dendritic spine/synapse elimination in hippocampal neurons in both dissociated and slice cultures.

**Brg1 is critical for synaptic gene regulation and synapse development.** Brg1 and BAF complexes regulate gene activation or repression by modulating chromatin structures either directly by ATP-dependent chromatin remodeling or indirectly by recruiting other epigenetic regulators (8–10). The recruitment of BAF complexes to target genes mostly requires sequence-specific transcription factors. Functional studies of BAF subunits in different developmental tissues at different stages revealed distinct cell-type-specific functions of BAF complexes. The diverse subunit compositions of BAF complex in different cell types may provide interactions with tissue-specific transcription factors to target the complex to loci that are required for specific developmental programs. Previous studies have shown that BAF complexes in embryonic stem cells, neural progenitors, and neurons have distinct functions in each cell type (19, 20, 56). There are few overlapping target loci of Brg1/BAF in different tissues (44, 57). In this study, we showed that synaptic genes are specific Brg1 targets in developing neurons. A large number of Brg1-regulated genes have known functions in neuronal and synapse development. We speculate that Brg1 supports a transcription program that coordinates synapse formation and maturation. However, it is not clear which transcription factors are involved in activating or repressing these genes. Our observation that Brg1 is a MEF2 coactivator enables us to speculate that MEF2 is one of the transcription factors that mediate Brg1 functions in synaptic gene regulation and in synapse maturation during development. Interestingly, we observed that Brg1 regulates both synapse formation and MEF2C-mediated synapse elimination. The seemingly paradoxical role of Brg1 in synapse development reflects the diverse functions of Brg1 in neuron development and the dynamic synapse developmental processes. At early postnatal stage, synapse/dendritic spines are actively formed and eliminated. Synapse forma-

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**FIG 7** MEF2 is required for the activity-induced recruitment of Brg1 to target genes. (A) Brg1 ChIP-qPCR experiments were performed using cultured MEF2CFlox/flox control and Emx1-Cre MEF2CFlox/flox cortical neurons treated with or without KCl for 1 h. Significant increases in Brg1 binding to the regulatory regions of all the activity-induced MEF2 target genes were observed. Gap43 was used as a non-activity-dependent gene control. Induction was significantly diminished in MEF2C mutant cortical cultures. **, P < 0.01 (analysis of variance with a post hoc t test). (B) Interactions between endogenous MEF2C and Brg1 were evaluated in cell lysates from cultured cortical neurons treated with or without KCl for 1 h. Samples were immunoprecipitated with Brg1 antibody or an IgG control. MEF2C and Brg1 were detected by Western blotting.
tion peaks around the second week postnatally in mouse brain, whereas synapse elimination peaks as neurons mature in the third week after birth, partially due to the increased activities of MEF2 family transcription factors (58–61). Therefore, proteins that play diverse functions during synapse development may appear to cause the opposite effects on synapse numbers, depending on the developmental stages when the genes are altered and on MEF2 activities at the specific time point. For example, it has been reported that the autism-associated fragile X mental retardation protein (FMRP) bidirectionally regulates synaptogenesis as a function of developmental age and MEF2 activity (60). Therefore, we propose that Brg1 is required for synapse formation and maturation by regulating synaptic structural genes. In addition, Brg1 also regulates MEF2C-mediated activity-induced gene expression and synapse elimination. The two scenarios described in the proposed model about nBAF functions during development and in

**FIG 8** Brg1 is required for MEF2C-induced dendritic spine elimination. (A and B) Brg1 is required for MEF2C-mediated dendritic spine elimination in cultured hippocampal neurons. BAF53b-Cre Brg1F/F mutant and control hippocampal neuron cultures were transfected with plasmids expressing GFP and MEF2C, MEF2-VP16, or control MEF2A-VP16 at 7 DIV. GFP-labeled neurons were imaged at 14 DIV (A), and dendritic spine densities were measured and compared (B). (C to E) Organotypic hippocampal slice cultures from P6 Brg1F/F mice were biolistically transfected with plasmids for expression of Cre, GFP, and MEF2C or a control at 1 DIV, GFP-labeled CA1 neurons were imaged after 5 days (C), and the dendritic spine densities (D) and classifications (E) were analyzed. Scale bar, 5 μm. Values in the graphs are averages ± standard errors. **, P < 0.01; *, P < 0.05 (analysis of variance with a post hoc t test). The numbers of neurons examined in each group are shown in the bar graph. (F) A model illustrating the function of Brg1 in synapse development and plasticity. During synaptic development, nBAF complexes are recruited by specific transcription factors to regulate expression of a significant number of genes required for synapse formation and maturation (left panel). In response to neuronal activity-triggered Ca2+ signaling, activated MEF2 proteins recruit nBAF complexes to MEF2 targets and regulate the activity-induced genes required for synapse elimination and plasticity (right panel). These two scenarios are not mutually exclusive and may reflect the different developmental stages of neurons.
activated neurons are not mutually exclusive and reflect diverse functions of Brg1 during different synapse developmental stages (Fig. 8F).

In addition to synaptic genes, Brg1 deletion in dentate gyrus also led to the increased expression of several growth factors such as Igf1 and Igf2 (data not shown), which may compensate for the impaired synapse formation. Therefore, the subtle defects in synaptic spine densities in Syn1-Cre Brg1^+/+ DG neurons in vivo (Fig. 3) relative to those in Cre-expressing individual CA1 neurons in hippocampal cultures (Fig. 1) could be due to the non-cell-autonomous compensatory mechanisms. Alternatively, the different defects could be caused by the different developmental stages of the neurons examined. However, we could not exclude the possibility that the different defects are due to the regional differences between dentate gyrus granule neurons and CA1 neurons. Nevertheless, our experiments clearly demonstrated that Brg1 is required for synapse maturation for both neuron types and that it is required cell autonomously for synapse formation in CA1 neurons.

**nBAF complexes function in activity-dependent gene activation.** The ability of neurons to convert transient stimuli into long-term changes in brain function underlies long-lasting neural plasticity; activity-dependent gene expression plays a central role in this process. Much evidence suggests that the cooperation between transcription factors and chromatin regulators controls the rapid response of neurons to stimuli as well as long-lasting changes in neuron function (24, 62, 63). Despite the understanding that chromatin regulation is important in neuronal plasticity, the biochemical and molecular mechanisms remain largely unclear.

Analyses of BAF53b knockout neurons demonstrated that nBAF complexes regulate activity-dependent dendrite growth, suggesting that signaling resulting from Ca^{2+} influx leads to chromatin regulation (19). In this study, by deleting Brg1 specifically in neurons and by comparing the Brg1-regulated genes under basal and depolarized conditions, we identified a specific group of genes that are regulated by Brg1 during activation of neurons. These genes significantly overlap activity-dependent MEF2 targets. We demonstrated the requirement of Brg1 for the expression of MEF2C targets and the requirement of MEF2C for Brg1 recruitment in response to neuronal depolarization. These results indicate that Brg1 functions as an essential coactivator of MEF2C-mediated transcription. This regulation is consistent with our observation that Brg1 is essential for MEF2C-mediated synapse elimination, which may also contribute to nBAF complex functions in neurodevelopmental diseases. However, in addition to MEF2, Brg1 may regulate additional transcription factors or pathways that are important for synapse development. Brg1 mutant mice display early defects in synapse formation and maturation, whereas MEF2C mutant neurons have defects in synapse elimination, which is a relatively late stage in synapse development. Deletions of Brg1 using inducible Cre lines in neurons at later developmental stages after synapse formation may reveal its requirement for synapse elimination in vivo and additional functions in mature neurons.

One remaining question is how nBAF complexes, including Brg1 and its 10 tightly associated subunits, are recruited to MEF2 targets in response to Ca^{2+} signaling activation. Several modifications to MEF2 lead to an exchange of corepressors for coactivators (30, 31). One possibility is that Ca^{2+} signaling induces MEF2 modification changes that facilitate the recruitment of Brg1. This is supported by our observation of an increase of MEF2C-Brg1 coimmunoprecipitation efficiency in neurons in response to KCl treatment. However, the interaction between MEF2C and Brg1 is moderate. Therefore, additional mechanisms may also contribute to the recruitment of Brg1 in response to neuronal activation. It is possible that changes in the local chromatin environment induced by MEF2 activation help recruit nBAF complexes through several histone modification binding domains in various BAF subunits (8).

In this study, we found that the core BAF subunit Brg1 is critical for synapse development, maturation, and plasticity. The close functional connections of Brg1 to the ASD-associated MEF2 proteins discovered here provide molecular and cellular mechanisms for the role of BAF complexes in neurodevelopmental diseases. In Brg1 mutant mice, the mutant phenotypes are most severe during development and become less severe in adults. It is possible that mice develop compensating mechanisms that enable recovery from the defects. Interestingly, in adult neurons, the Brg1 homolog Brm is highly expressed, and this might compensate for Brg1 deletion. Mutations in Brm, but not in Brg1, have been linked to schizophrenia (64). Thus, during different developmental stages, specific BAF complexes may be required for different aspects of neuronal development and function. The understanding of the functions and mechanisms of epigenetic regulators in ASD and other neurodevelopmental disorders may provide new treatment strategies for these diseases.

**ACKNOWLEDGMENTS**

We thank Qiu Wang for technical support and mouse colony maintenance. We are grateful to Andrew Yoo, Lei Chen, and Gerald Crabtree (Stanford University) for generating the BAF53b-Cre transgenic mouse. We thank Pierre Chambon (IGBMC, France), Luis Parada (UT Southwestern Medical Center), and Eric Olson (UT Southwestern Medical Center) for providing transgenic or knockout mice, and Kacey Rajkovich, Makoto Taniguchi, and Christopher Cowan for providing reagents. The behavior tests were performed in the Behavior Core Facility in UT Southwestern Medical Center. The RNA-seq experiments with the basic analyses were performed in the UT Southwestern Medical Center Sequencing Facility.

**FUNDING INFORMATION**

The American Cancer Society (American Cancer Society, Inc.) provided funding to Jiang Wu under grant number 627880. The March of Dimes Foundation (March of Dimes Births Defect Foundation) provided funding to Jiang Wu under grant number FY-12-434. HHS | NIH | National Institute of Mental Health (NIMH) provided funding to Jiang Wu under grant number R21MH102820.

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