Neurobiology of Disease 34 (2009) 71–77

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Evidence for both neuronal cell autonomous and nonautonomous effects of methyl-CpG-binding protein 2 in the cerebral cortex of female mice with *Mecp2* mutation

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ARTICLE INFO

Article history:
Received 6 October 2008
Revised 8 December 2008
Accepted 13 December 2008
Available online 8 January 2009

Keywords:
Rett syndrome
MeCP2
Mouse models
Brain
Motor cortex
Spines
Morphometry

ABSTRACT

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder caused by mutations in the gene *MECP2*, encoding methyl-CpG-binding protein 2 (MeCP2). Few studies have explored dendritic morphology phenotypes in mouse models of RTT and none have determined whether these phenotypes in affected females are cell autonomous or nonautonomous. Using confocal microscopy analysis we have examined the structure of dendrites and spines in the motor cortex of wild-type (WT) and Mecp2-mutant mice expressing green fluorescent protein (GFP). In Mecp2 GFP female mice age 6–7 months we found significant decreases in the density of spines, width of dendrites, size of spine heads, while increases were found in the length of spine necks, dendritic irregularities, spineless spots, and long spines. We show for the first time that a lower density of spines and smaller spine head area are phenotypes that distinguish MeCP2+ from MeCP2− dendrites in female Mecp2 GFP mice. In Mecp2 GFP male mice at three weeks of age, we found reduced spine density, thinner apical oblique dendrites and increased dendritic irregularities and long spines. Significantly, the changes affected both MeCP2− and MeCP2+ neurons, pointing to the ability of MeCP2− to impact the structure of MeCP2+ neurons. Our findings are evidence that MeCP2 deficiency results in both cell autonomous and nonautonomous changes.

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Introduction

Rett syndrome (RTT) results from the presence of mutations in the gene encoding methyl-CpG-binding protein 2 (MeCP2) on the X chromosome (Amir et al., 1999). The disorder affects primarily young girls with a prevalence of 1 in 10,000–15,000 (Hagberg, 1995; Hagberg, 1989). The brain is severely affected. Girls with RTT show developmental regression, diminished cognitive ability, loss of acquired speech skills, stereotypic hand movements, seizures, breathing irregularities and autonomic dysfunction (Hagberg, 1995; Hagberg, 1989; Nomura and Segawa, 2005; Percy, 2002; Rett, 1966). Studies performed on the brains of subjects with RTT and on mouse models of RTT show that the Mecp2 mutation has relatively little influence on neurogenesis and neuronal migration. The organization of cortical layers and the structure of the hippocampus are largely unaffected (Belichenko et al., 2008; Belichenko et al., 1997; Belichenko et al., 1994; Guy et al., 2001; Leontovich et al., 1999; see review in Armstrong, 2005). In contrast, striking changes in neuronal maturation are seen including: 1) significant reductions in the number and length of dendrites; 2) decreased dendritic spine number and regional loss of spines in the cortex; and 3) disorganization of afferent fibers to the spines of pyramidal neurons and in the shape of the axonal bundles in which they travel (Armstrong et al., 1995; Armstrong, 1992; Belichenko et al., 1994; Belichenko and Dahlström, 1995). The evidence is thus consistent that RTT exerts pathogenetic events that impact the maturation of neurons and the circuits in which they participate (Belichenko et al., 2008; Belichenko et al., 1994).

Because the *MECP2* gene is located on the X chromosome (Amir et al., 1999), random inactivation of this chromosome (see review in Payer and Lee, 2008) in RTT females renders them mosaic for neurons expressing normal MeCP2 activity (MeCP2+), and neurons expressing abnormal MeCP2 or neurons completely lacking MeCP2 (MeCP2−). An interesting question is whether disease manifestations are due to abnormalities only in neurons that fail to express normal MeCP2 or, alternatively are linked to changes in all neurons. Three possibilities related to cell autonomous and nonautonomous effects of the Mecp2-mutation can be envisioned: i) MeCP2− neurons are severely damaged and impact MeCP2+ neurons structure and function through their linkage in circuits; ii) MeCP2+ neurons substantially improve aspects of the abnormal phenotypes in MeCP2− neurons through linkage in circuits; and iii) MeCP2− neurons and MeCP2+ neurons are independent in their manifestations of disease pathology. In this case only MeCP2− neurons are responsible for neurological symptoms of RTT. In the present study we ask whether or not

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.nbd.2008.12.016

abnormal phenotypes affect only MeCP2− neurons or extend to MeCP2+ neurons. We chose to investigate whether or not the MeCP2 mutation displays cell autonomous or non-cell autonomous effects in female Mecp2 mutant mice. To carry out this work we crossed WT male mice expressing green fluorescent protein (GFP; GFP-M line; Feng et al., 2000) with female Mecp2-mutant mice (Dr. A. Bird strain, Mecp2B, Guy et al., 2001). Combining GFP with MeCP2 immunoreactivity (IR) and high resolution confocal microscopy, we examined dendrite and dendritic spine morphology in the motor cortex of heterozygous female Mecp2B GFP mice, defining these structures in MeCP2+ or MeCP2− neurons. To compare the severity of dendritic phenotypes in females with brains in which all neurons are MeCP2−, we examined the same phenotypes in male mice produced by the same mating. Comparing MeCP2− neurons in Mecp2B males to neurons in WT males, we discovered significant decreases in the density of spines, width of dendrites, and increases in dendritic irregularities (i.e. swellings or narrowings) and long spines. When we compared MeCP2− neurons in Mecp2B females to neurons in WT female mice, significant decreases in the density of spines, width of dendrites, size of spine heads, and increases in the length of spine necks, dendritic irregularities, long spines and spineless spots were found. The same phenotypes were evident comparing MeCP2+ neurons in Mecp2B females with neurons in WT females with the exception of dendritic irregularities. The changes in MeCP2− neurons in females were in general less severe than in neurons in Mecp2B males. Remarkably, changes in spine density and in the area of spine heads were phenotypes that clearly distinguish MeCP2+ from MeCP2− dendrites in female Mecp2B GFP mice. These data are evidence for both cell autonomous and nonautonomous changes in neurons due to MeCP2 deficiency.

Materials and methods

All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and with an approved animal protocol from the Stanford University Institutional Animal Care and Use Committee.

Creating Mecp2-mutant mice expressing green fluorescent protein

Details of maintenance and using Mecp2 mouse colony were described previously (Belichenko et al., 2008). To generate wild-type (WT) and Mecp2-mutant mice that express green fluorescent protein (GFP), female mice that were heterozygous for MeCP2 with an $Mecp2^{tm1.1Bird}$-mutation on a C57BL/6J background (Mecp2B; Guy et al., 2001) were crossed to male mice that carry GFP-M transgene (Feng et al., 2000) under a mix genetic background (C57BL/6J and B6EiC3). Male and female offspring of this cross were used in the present study (referred here as WT GFP and Mecp2B GFP). To distinguish WT GFP and Mecp2B GFP mice, we extracted genomic DNA from tail samples and genotyped them by polymerase chain reaction (PCR) for *Mecp2* and *Sry* (for sexing) by using protocols supplied by Jackson Laboratory and *Sry* primers previously described (Jordan et al., 2007). GFP-M genotype was identified by presence of fluorescence under fluorescence microscope and by PCR using tail DNA. The primers used were: forward primer, 5′AGCAAAGACCCCAACGAGAA3′ and reverse primer, 5′GGCGGCGGTCACGAA3′. Each mouse was genotyped twice. Immunofluorescence method with anti-MeCP2 antibody was used for additional confirmation of genotype in Mecp2B GFP female mice. We used male mice at 21 days old and female mice at 6–7 months old when RTT-related neurological phenotypes were evident.

Brain processing

WT and Mecp2B mice were deeply anesthetized with sodium pentobarbital (200 mg/kg i.p.) (Abbott Laboratories, North Chicago, IL), weighed, and transcardially perfused for 1 min with 0.9% sodium chloride (10 ml) and then for 10 min with ice cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 (100 ml). After perfusion the brain was immediately removed. The weight of the entire brain (including the olfactory bulbs, cortex, hippocampus, cerebellum, brain stem, and the cervical spinal cord through C1–C2) was recorded. The brain was sectioned coronally at 100-μm with a Vibratome (series 1000; TPI Inc., St. Louis, MO), and sections were placed in 0.1 M PBS. For MeCP2 immunoreactivity (IR), free-floating sections from WT GFP and Mecp2B GFP female were preincubated with 5% nonfat milk in PBS for 1 h and then were incubated with a rabbit antibody to MeCP2 (Cat. # 07-013, lot 27908, Upstate, Lake Placid, NY) at a dilution 1:100 overnight at 4 °C. Sections were rinsed in PBS (20 min, three changes) and incubated for 1 h at room temperature with biotinylated donkey anti-rabbit IgG (1:200; Jackson Immunoresearch, West Grove, PA). After being rinsed with PBS (20 min, three changes), sections were incubated with streptavidin Quantum dot 655 conjugate (QD655; 1:500; Cat. # SA306, lot 0601019035, Chemicon International, Temecula, CA) for 1 h at room temperature. After again rinsing in PBS, sections were either mounted onto glass slides and coverslipped using 90% glycerol in water or counterstained with the 5 μM fluorescent dye acridine orange (AO; Cat. # A-6014, lot 57H3627, Sigma Chemical Co., St. Louis, MO) in PBS for 30 s and rinsed in PBS as described (Belichenko and Dahlstrom, 1995) and than mounted. Sections from WT GFP and Mecp2B GFP male were mounted onto glass slides and coverslipped using 90% glycerol in water and imaged with confocal microscope.

Confocal microscopy

To study dendritic and spine morphology in male and female WT and Mecp2-mutant mice we used confocal microscopy imaging of GFP expressing neurons in layers V–VI in motor cortex. Dual-channel confocal microscopy was used for imaging colocalization of GFP and MeCP2 IR to distinguish MeCP2+ and MeCP2− neurons in Mecp2B female mice or for imaging colocalization of AO staining and MeCP2 IR to count number of MeCP2+ neurons within population of all neurons in WT and Mecp2B female mice. Single-channel confocal microscopy was used for imaging GFP expressing neurons to study dendritic morphology in male and female mice. Confocal microscopy was performed as described (Belichenko et al., 2004). In brief, slices with GFP expressing neurons were examined and scanned in a Radiance 2000 confocal microscope (BioRad, Hertfordshire, UK) attached to a Nikon Eclipse E800 fluorescence microscope. The laser was an argon/krypton mixed gas laser with exciting wavelengths for GFP, QD655, or AO (488λ). The emission was registered with HQ515/30 “green” filter for GFP or AO and with 660LP “far-red” filter for QD655. LaserSharp software (BioRad) was used to establish optimal conditions for collecting images.

Sections with QD655 staining of MeCP2 IR were studied under the following optimal conditions: the lens was a ×60 objective (Nikon; Plan Apo ×60/1.40 oil); laser power was 30% for both GFP and for QD655, single optical sections was scanned with a sequential mode; the zoom factor was 2; scan speed was 500 lines per second (lps); each optical section was the result of three scans followed by Kalman filtering; the size of the image was 512×512 pixels (i.e. 87×87 μm).

The morphology of GFP-expressed neurons and their spines was studied under the following optimal conditions: the lens was a ×60 objective (Nikon; Plan Apo 60×/1.40 oil); laser power was 30%, optical sections were scanned at increments of 0.5 μm; the zoom factor was 10; scanning was at 500 lps; each optical section was the result of three scans followed by Kalman filtering; pixel size was 0.039 μm; the size of the image was 512×512 pixels. Each image was saved as a stack of individual optical sections and as a z-projection. Four slices per mouse were used: 20–40 cells for layers V–VI motor cortex per mouse