Neuroscience 168 (2010) 624–632

DIMINISHED PREVALENCE BUT PRESERVED SYNAPTIC DISTRIBUTION OF N-METHYL-D-ASPARTATE RECEPTOR SUBUNITS IN THE METHYL CpG BINDING PROTEIN 2(MeCP2)-NULL MOUSE BRAIN

E. MALISZEWSKA-CYNA,[a,b] D. BAWA[a] AND J. H. EUBANKS[a,b,c,d]*

[a]*Division of Genetics and Development, Toronto Western Research Institute, University Health Network, 399 Bathurst Street, Toronto, ON M5T 2S8, Canada*

[b]*Department of Physiology, University of Toronto, Toronto, ON M5S 1A8, Canada*

[c]*Department of Surgery (Neurosurgery), University of Toronto, Toronto, ON M5S 1A8, Canada*

[d]*Institute of Medical Science, University of Toronto, Toronto, ON M5S 1A8, Canada*

Abstract—In this study, we examined the prevalence and distribution of NMDA receptor subunits within crude synaptic membranes derived from the brains of mice lacking methyl CpG binding protein 2 (MeCP2). Our results show that the distribution of NMDA receptor subunits within the detergent soluble, detergent resistant, and postsynaptic density microdomains is preserved at MeCP2-null synapses. However, analysis of the NMDA receptor subunit expression revealed a decrease in the prevalence of the GluN1 and GluN2A subunits in MeCP2-null tissue. Collectively, these results indicate that synaptic membrane microdomains at synapses of the MeCP2-null brain develop normally, and that NMDA receptor subunits are properly targeted and distributed within them. The under-representation of the GluN1 and GluN2A subunits suggests that MeCP2-null synapses contain fewer mature NMDA receptor complexes, and raises the possibility that impaired NMDA receptor ontogeny could contribute to Rett syndrome pathophysiology.

Key words: brain, autism–spectrum disorder, methyl DNA-binding factor, lipid raft, post-synaptic density.

Rett syndrome (Rett, 1966; MIM 312750) is a neurological disorder caused primarily by mutations in the X-linked gene encoding methyl CpG binding protein 2 (*MECP2*) (Amir et al., 1999). Cardinal neural features of clinical Rett syndrome include diminished brain mass (Jellinger and Seitelberger, 1986), attenuated neuronal complexity (Armstrong et al., 1998; Leontovich et al., 1999), and increased neuronal packing density in different brain regions (Armstrong, 2002). Mice deficient in MeCP2 have been generated (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002), and these mice recapitulate many Rett-like phenotypes (Kishi and Macklis, 2004; Fukuda et al., 2005; Stearns et al., 2007; Belichenko et al., 2009). Collectively, these observations have spawned the hypothesis that Rett syndrome is a condition in which brain development stalls, and synapses fail to mature (Johnston et al., 2001; Percy, 2002; Glaze, 2004). Despite knowing the causal gene, however, it remains largely unknown why synaptic maturation is impaired in the MeCP2-deficient brain.

Several candidate systems have been postulated to contribute to Rett syndrome pathophysiology. One system, recognized for its role in synaptic development, is the N-methyl-D-aspartate (NMDA) receptor system. The majority of NMDA receptors are heteromeric combinations of a GluN1 subunit (formerly designated NR1) and different GluN2 (GluN2A–GluN2D) subunits (formerly designated NR2A–NR2D, Collingridge et al., 2009) (Cull-Candy et al., 2001). However, while GluN2B is expressed throughout the brain from embryonic development, GluN2A displays a more restricted expression pattern, predominantly being expressed in forebrain regions of the perinatal and adult brain (Watanabe et al., 1992; Wenzel et al., 1997; Monyer et al., 1994). Evidence suggests that GluN2A-containing receptors can replace GluN2B-containing receptors at many postsynaptic densities as they functionally mature (Bellone and Nicoll, 2007; Sans et al., 2000; Petralia et al., 2005), and that this GluN2B to GluN2A switch is required for the proper synaptic maturation of many excitatory synapses (Townsend et al., 2003; Fu et al., 2005).

While the function of NMDA receptors remains to be evaluated in MeCP2-deficient neurons, convergent data suggest that alterations in NMDA receptor expression and/or function could play a role in the etiology of Rett syndrome. For example, MeCP2 regulates the activity-dependent expression of the GluN2B subunit in normal rat neurons (Lee et al., 2008), suggesting that misregulation of GluN2B could be evident in MeCP2-deficient neurons. Further, radioligand binding studies conducted on post-mortem Rett syndrome brain have revealed significant decreases in NMDA receptor binding density in cortical and basal ganglia regions (Blue et al., 1999a,b), and microarray profiling studies have reported altered expression of NMDA receptor subunits in both patient and MeCP2-null

*Correspondence to: J. H. Eubanks, Room Mac 13-423, Toronto Western Hospital, 399 Bathurst Street, Toronto, ON, M5T 2S8, Canada. Tel: +1-416-603-5800 ext. 2933; fax: +1-416-603-5745.
E-mail address: jeubanks@uhnres.utoronto.ca (J. H. Eubanks).

Abbreviations: BDNF, brain derived neurotrophic factor; GluN1, glutamate receptor NMDA type subunit 1; GluN2A, glutamate receptor NMDA type subunit 2A; GluN2B, glutamate receptor NMDA type subunit 2B; GluN2D, glutamate receptor NMDA type subunit 2D; GP50, glycoprotein of 50 kilodaltons; HRP, horse radish peroxidise; IGF-1, insulin-like growth factor 1; MeCP2, methyl CpG binding protein 2; NMDA, N-methyl-D-aspartate; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; PSD-95, postsynaptic density protein of 95 kilodaltons; ROD, relative optical density; RPM, revolutions per minute; SDS-PAGE, sodium dodecyl sulfate polyacrilamide gel electrophoresis; TBST, tris–buffered saline with tween 20.

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2010.03.065

mouse brain regions (Colantuoni et al., 2001; Chahrour et al., 2008). Finally, a previous study from our group showed decreased GluN2A subunit expression in the hippocampus of symptomatic MeCP2-null mice, which occurred coincident with impairments in synaptic plasticity (Asaka et al., 2006). Here, we extend from these studies by examining the prevalence and microdomain localization of NMDA receptor subunits within crude synaptic preparations from the MeCP2-null mouse brain. Our results show that specific NMDA receptor subunit expression is altered at MeCP2-null synapses in a manner consistent with attenuated synaptic maturation, and supports the possible involvement of the NMDA receptor system in Rett syndrome pathophysiology.

EXPERIMENTAL PROCEDURES

Animal subjects

The MeCP2-deficient mouse model generated by Guy et al. (2001) (Mecp2$^{tm1\text{-}Bird}$) (obtained from Jackson Laboratories, Bar Harbor, ME, USA) was used for this study. Genotyping was determined by the polymerase chain reaction (PCR) using the following primers to detect the wild-type *Mecp2* allele: 5′-GGTAAAGACCCATGTGACCC-3′ and 5′-GGCTTGCCACATGACAA-3′, and the following primers to detect the mutant *Mecp2* allele: 5′-GGTAAAGACCCATGTGACCC-3′ and 5′-TCCACCTAGCCTGCCTGTAC-3′. PCR amplification was conducted on an MJ Research Thermocycler using a program of 35 cycles of denaturing at 94 °C for 45 s, annealing at 60 °C for 60 s, and extending at 72 °C for 50 s. Products were visualized on Ethidium Bromide-stained agarose gels. Mice were sacrificed by cervical dislocation between 70 and 90 days of age when they displayed hind-limb clasp impairments when elevated (Guy et al., 2001). Whole brains were extracted and stored in liquid nitrogen until use. All mutant mice were males and wild-type mice were matched by sex and age. All procedures were conducted in accordance with the guidelines established by the Canadian Council on Animal Care, and all animal experimentation was reviewed and approved by local Animal Care Committees prior to the onset of the study.

Preparation of synaptic membranes

Crude synaptic membranes were isolated as described previously (Besshoh et al., 2005, 2007) with minor modifications. In brief, frozen whole brains were slowly thawed to 4 °C, and homogenized with a Potter bench-type homogenizer (model number AA 2R629) in ice cold solution A (0.32 M sucrose, 0.5 mM $CaCl_2$, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM NaF, 2 mM sodium orthovanadate, 20 mM p-nitrophenyl phosphate, and 20 mM glycerophosphate) supplemented with a mixture of proteinase inhibitors (5 μg/mL each Antipain, Aprotinin, Leupeptin, Calpain Inhibitor III, and PMSF), at a ratio of 10 ml solution A per gram wet weight tissue. The homogenizer speed was set to 70 RPM, and 15–18 strokes of 30–35 s total duration were used for each tissue. The Pellet (P1) was isolated by centrifugation at 700×*g* for 5 min at 4 °C, and re-suspended in 5 ml of solution A and centrifuged again as above. The supernatants from both spins (S1) were pooled, transferred to ultra spin centrifuge tubes, and centrifuged at 15,000×*g*, for 13 min at 4 °C. The subsequent pellet was collected, re-suspended in solution A, and centrifuged a second time as above. The supernatant (S2) was collected and stored. The resulting pellet (P2) was then re-suspended in 200 μl of 40% (w/v) sucrose in solution B (1 mM NaF, 2 mM sodium orthovanadate, 20 mM glycerophosphate, and 5 μg/mL each of Antipain, Aprotinin, Leupeptin, and Calpain Inhibitor III). Protein concentrations were determined using the Bradford assay. Unless specified, all solutions were made with de-ionized distilled water (specific resistance 18.2 MΩ/cm, Milli-Q system), and chemicals and inhibitors were purchased from Sigma-Aldrich, Canada (Mississauga, ON, Canada).

Solubilization and sucrose density gradient fractionation

Solubilization was done by incubating 4 mg of re-suspended P2 homogenate protein with TritonX-100 (made in water and supplemented with all 5 μg/mL each of Antipain, Aprotinin, Leupeptin and Calpain Inhibitor III) to give a final detergent concentration of 0.5% (w/v) and protein-to-detergent ratio of 1:1 (w/w). After gentle rocking for 30 min at 4 °C, the detergent–protein suspension was placed below a discontinuous sucrose gradient (6 ml of 5% and 5 ml of 30% sucrose in solution B) and centrifuged at 100,000×*g* for 18 h at 4 °C. After centrifugation, 1 ml aliquots were collected from the top of gradient, and the pellet was re-suspended in 200 μl of solution A.

Gel electrophoresis and immunoblotting

Equal volumes from each fraction (12.5 μl of fractions 1–12 and 2.5 μl of the re-suspended postsynaptic density fraction) from the sucrose gradients were heated in SDS sample buffer, and resolved on 5% stacking/10% resolving acrylamide gels in Tris–glycine Laemmli running buffer. The proteins were transferred at 4 °C overnight in standard transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS) to a nitrocellulose membrane, and pre-hybridized for 2 h at room temperature in blocking solution (Tris–buffered saline containing 0.05% Tween-20 (v/v) (TBST) and 5% (w/v) non-fat dry milk). Blots were then incubated overnight at 4 °C with primary antibody in blocking solution. The antibodies used were directed against: Flotillin-1 (1/3000; Biosciences, Cat # 610820), GP-50 (1/5000), PSD-95 (1/1000; BD Biosciences, Cat # 610495), GluN1 (1/1000; BD Biosciences, Cat # 556308), GluN2A (1/750; AbD Serotec, Cat # 6870-1059), and GluN2B (1/1000; AbD Serotec, Cat # 6870-1109). After washing in TBST (3×20 min per wash), HRP-linked secondary antibodies (1/5000 dilution, Jackson ImmunoResearch, West Grove, PA, USA) were applied, and incubated for 2 h at room temperature. After extensive washing in TBST, specific immunoreactivity was visualized by enhanced chemiluminescence. If required, blots were stripped by incubating at 55 °C for 30 min in stripping buffer (10% SDS, 1 M Tris pH 6.7, 2-mercaptoethanol), followed by washing in TBST and blocking for 1 h at room temperature in blocking solution. The blots were then re-probed with primary antibody as described above.

Immunoblot analysis and statistics

The relative optical densities (RODs) were measured for each fraction from individual immunoblots (MCID 6.0, St. Catherine's ON, Canada). Blots from wild-type and mutant mice were processed together, and exposed to a common film. The percentage change in ROD was then determined and plotted by normalizing the experimental values to control, and expressing these as percent changes from control. The resulting mutant and wild-type values for all experiments were compared using non-paired, two-tailed Student's *t*-test, and significance levels were set at $P<0.05$. A Bonferroni post-hoc correction for multiple comparisons was applied where applicable.

RESULTS

Brain mass and protein yield differ between MeCP2-null and age-matched wild-type control mice

Whole brains from MeCP2 deficient ($n=7$, male) and age-matched controls ($n=7$, male) were used in this study.