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Increased dendritic complexity and axonal length in cultured mouse cortical neurons overexpressing methyl-CpG-binding protein MeCP2

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Rett syndrome is caused by loss-of-function mutations in the gene encoding the methyl DNA-binding factor MeCP2. As brain mass and neuronal complexity tend to be diminished in Rett patients, we tested whether MeCP2 directly influences the morphological complexity of developing neurons. Our results show that cultured mouse neurons overexpressing MeCP2β (MECP2A) develop more complex morphologies, having longer axonal and dendritic processes, and an increased number of axonal and dendritic terminal endings. We then tested whether overexpressing a mutant form of MeCP2β lacking its carboxyl terminus would elicit the same effects. Interestingly, while neurons overexpressing this mutant failed to enhance axonal and dendritic process elongation, the complexity of their axonal and dendritic processes remained significantly elevated. Taken together, these data support the hypothesis that MeCP2 directly regulates neuronal maturation and/or synaptogenesis, and provides evidence that MeCP2 may influence neuritic elongation and process branching through different mechanisms.

Keywords: Rett syndrome; Methyl DNA-binding factor; Neuronal culture; Transient overexpression; Axon; Dendrite; Morphology

Introduction

Rett syndrome (Rett, 1966) is a neurological disorder affecting primarily young girls in which the brain apparently fails to mature properly (Kerr and Engerstrom, 2001; Kozinetz et al., 1993). Although there is no demonstrable decrease in neuronal number, the Rett brain is both undersized and underweight when compared to age-matched nonaffected children (Jellinger and Seitelberger, 1986). Symptomatic onset of Rett syndrome is generally not observed until stages of postnatal development when normal cortical neuron maturation is occurring (Johnston et al., 2003; Percy, 2002). Detailed postmortem morphological analysis of cortical neurons from Rett individuals reveals the degree of dendritic branching, and the length of dendritic processes, to be significantly attenuated from age-matched non-Rett cortical neurons (Armstrong, 1992; Armstrong et al., 1998; Leontovich et al., 1999). These observations have led to the hypothesis that Rett syndrome is a disorder that results from an impairment of normal synaptic development or maturation (Johnston et al., 2001).

Mutations in the gene encoding the methyl DNA-binding factor MeCP2 have been identified as the cause for the majority of clinical Rett syndrome cases (Amir et al., 1999). MeCP2 is the prototypical member of a family of related methyl DNA-binding proteins that function by recruiting specific histone deacetylase containing co-repressor complexes to methylated chromatin locales (Jones et al., 1998; Kokura et al., 2001; Nan et al., 1998). As the most common mutations in Rett children occur either within the methyl DNA-binding domain or the transcriptional repressor domain of MeCP2 (see database at http://www.mecp2.org.uk), these mutations would likely affect the ability of MeCP2 to bind methylated DNA templates, or affect its ability to associate with histone deacetylases. Indeed, mutant mice that lack MeCP2 develop many of the hallmarks of clinical Rett syndrome, confirming that abrogated MeCP2 function promotes the neurological phenotype (Chen et al., 2001; Guy et al., 2001). Consistent with the neuropathological observations, the brain mass and the size of specific forebrain neurons is smaller in mice lacking MeCP2 than in wild-type controls (Chen et al., 2001). Taken together with the attenuated morphological complexity observed in

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cortical neurons from Rett patients, these data are consistent with MeCP2 playing a critical role in the maturation of neurons from an immature to a phenotypically mature state (Johnston et al., 2001; Kerr and Engerstrom, 2001).

Recently, in studies attempting to correct the Rett-like disorder in animals lacking functional MeCP2, Collins et al. (2003) and Luikenhuis et al. (2004) demonstrated that the neuronal overexpression of MeCP2 above a certain threshold caused a delayed, but progressive, neurological condition. The condition in these overexpressing mice was distinct from that of mice lacking MeCP2, suggesting that a critical window of MeCP2 expression level must be maintained to allow normal neuronal maturation and function. However, it remains unclear how the overexpression of MeCP2 affects the morphological and functional properties of neurons. To begin addressing how MeCP2 regulates these properties, we tested whether the transient overexpression of MeCP2 would alter the normal viability or the morphological complexity of cultured mouse embryonic cortical neurons. In this study, we show that the overexpression of wild-type MeCP2 markedly increases the morphological complexity of cultured cortical neurons, while the overexpression of a clinically relevant mutant form of MeCP2 fails to promote the same morphological effects.

Materials and methods

Plasmids and DNA constructs

A plasmid containing the full-length mouse MeCP2 cDNA was obtained from Research Genetics (I.M.A.G.E. clone 1395411) and used as template DNA for the polymerase chain reaction (PCR). The entire coding region of wild-type mouse MeCP2 was isolated using the polymerase chain reaction with the following oligonucleotide primers: sense 5′-GCCACCATGTATCCATATGACTGCCCAGACTATGCTATGGTAGCTGGGATGTTA-3′ (which introduces a consensus Kozak initiation sequence (Kozak, 1987) linked to a hemagglutinin (HA) sequence tag followed by the first 18 nucleotides of the mouse MeCP2 open reading frame); and antisense 5′-TTTCTCGAGAAGTCAGCTAACTCTCTCGGT-3′ (ACGT Inc, Toronto Ontario). The mutant form of MeCP2 ($MeCP2^{293}$) was isolated using the HA-tagged wild-type cDNA clone as a template for the polymerase chain reaction with the following primers: sense 5′-AAAGGTACCGCCACCATGTATCCATATGAC-3′ and antisense 5′-AAAGATATCTTATATGGAAGACTCCTTCACGGC-3′. This "sense" primer targets the Kozak consensus and proximal region of the HA tag present in the wild-type MeCP2 expression cassette generated above. The amplified products were subcloned into the pCRII plasmid vector (GIBCO Invitrogen, Grand Island, NY), and then shuttled into a modified pTRACER-CMV2 expression vector in which the cytomegalovirus promoter drives transgene expression (GIBCO Invitrogen). This modified vector lacks the EF-1 promoter and the GFP reporter gene of the original vector. To serve as a control, a construct was also generated in which the short form of methyl CpG binding domain-containing factor 3 (MBD3b) was similarly tagged at its amino terminus with the HA sequence. This HA-tagged MBD3b expression plasmid (HA-MBD3) was constructed using the polymerase chain reaction with the following primers: sense 5′-GCCACCATGGGCTATCCATATGACGTCCCAGACTATGCGGGCGCAATGGAGCGGAAGAGCCCC-3′ and antisense: 5′-TTTGGAAAGGAGGTGGAC-3′. For this reaction, a mouse MBD3 cDNA clone that we had previously isolated (Jung et al., 2003b) was used as the template. The amplified products were then subcloned into the pNN265-CMV expression vector (a gift of Dr. Eric Kandel, Columbia University), in which the CMV promoter also drives transgene expression. To serve as an additional control, a β-galactosidase cDNA (a gift of Dr. R. Bremner, Toronto Western Research Institute) was introduced into the same vector. The reporter gene construct that was used for each co-transfection was generated from the original pTRACER-CMV plasmid, except the GFP cDNA of the original was replaced with an enhanced GFP (eGFP) cDNA. The fidelity of each construct was confirmed by DNA sequencing (ACGT Inc).

Primary cortical and N1E 115 cultures

Animal experimentation was conducted only after full review and approval of the proposed experiments by local animal care committees, in accordance with guidelines established by the Canadian Council on Animal Care. Embryonic mice (day E15) were isolated from timed pregnant dams (CD-1; Charles River, St Constant, QC) by Cesarean section. Briefly, neocortical tissue was isolated by microdissection, digested with papain for 30 min at 37°C according to manufacturer's recommendation (Worthington Biochemical Corp, Lakewood, NJ), separated from debris by density gradient centrifugation, and cell number and viability quantified by hemocytomer assessment and trypan blue exclusion. The cells were then plated upon poly-L-ornithine (MW > 300,000, Sigma-Aldrich, St. Louis, MO) coated 12-mm-diameter glass cover slips (Bellco Glass, Vineland, NJ), in minimal essential medium and 10% heat-inactivated fetal bovine serum at a density of 5×10^5 cells per cover slip. After allowing 3 h for the cultures to settle, total medium was replaced with Neurobasal Growth Medium [Neurobasal medium, supplemented with B27, Glutamax (GIBCO Invitrogen), penicillin (10 μg/ml), and streptomycin (10 u/μl)], as described previously (Brewer, 1995). Cultures to be used for test and control conditions were equivalently seeded, and randomized prior to use. In our hands, these condition yield cultures comprised of approximately 90% neurons (identified by MAP2 immunoreactivity), 7% astrocytes (identified by GFAP immunoreactivity), and less than 0.1% microglia (identified by Mac1 immunoreactivity), at in vitro culture days 5–6. This frequency of representation did not significantly differ from culture to culture. The identity of the remaining cells has yet to be determined. N1E 115 cells were obtained from ATCC (Manassas, VA), and were cultured in Dulbecco's modified eagle medium with 10% fetal bovine serum, penicillin (10 μg/ml), and streptomycin (10 u/μl) (GIBCO Invitrogen). Cells were maintained at 37°C, in a 5% CO_2 humidified incubator. Medium was replaced every 3–5 days for all cultures. N1E 115 cells were used exclusively to test the expression fidelity of the expression constructs. All viability and morphological examinations were performed exclusively in primary cortical cultures.

Transfection of primary neurons and N1E 115 cells

Transient neuronal transfections were performed by lipofection (Ohki et al., 2001). Test neurons were co-transfected with equimolar amounts (1:1) of eGFP reporter and HA-MeCP2 plasmids. For control neurons, similar equimolar amounts of eGFP reporter and β-galactosidase plasmids were used. Briefly, embryonic mouse cortical cells were prepared as discussed above (designated day 0