MicroRNAs downregulated in neuropathic pain regulate MeCP2 and BDNF related to pain sensitivity

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ARTICLE INFO

Article history:
Received 25 May 2015
Revised 11 August 2015
Accepted 16 August 2015

Keywords:
MeCP2
miRNA
Neuropathic pain
BDNF

ABSTRACT

Nerve injury induces chronic pain and dysregulation of microRNAs in dorsal root ganglia (DRG). Several downregulated microRNAs are predicted to target MeCP2. MECP2 mutations cause Rett syndrome and these patients report decreased pain perception. We confirmed MeCP2 upregulation in DRG following nerve injury and repression of MeCP2 by miRNAs in vitro. MeCP2 regulates brain-derived neurotrophic factor (BDNF) and downregulation of MeCP2 by microRNAs decreased BDNF in vitro. MeCP2 T158A mice exhibited reduced mechanical sensitivity and MeCP2 T158A null mice have decreased Bdnf in DRG. MeCP2-mediated regulation of Bdnf in the DRG could contribute to altered pain sensitivity.

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1. Introduction

Chronic pain is a prevalent, disabling health condition affecting more than 100 million people in the United States [1]. Neuropathic pain caused by injury or dysfunction of the nervous system can result in peripheral and central sensitization, a state of hyperexcitability due to reduction in threshold and an amplification in the responsiveness of nociceptors [2]. Elucidation of signaling pathways underlying pain hypersensitivity is crucial in improving our understanding of molecular mechanisms that drive plasticity in the nervous system promoting the development and maintenance of chronic pain states [3]. Animal models of neuropathic pain have been pivotal in the exploration of molecular mechanisms of pain underlying nerve injury [4]. Our miRNA profiling study in the dorsal root ganglion (DRG) from a nerve injury model identified 63 miRNAs differentially regulated compared to sham controls [5]. Bioinformatic prediction indicates 15 downregulated miRNAs are predicted to target the 3′ untranslated region (3′UTR) of Mecp2. Translational repression of MeCP2 mediated by binding of miRNAs to the 3′UTR has been reported [6–10].

MeCP2 binds methylated DNA, and together with co-repressors or transcriptional activators, mediates downstream changes in gene expression either directly or by altering chromatin structure [11,12]. Mutations in the X-linked methyl-CpG-binding protein 2 (MECP2) gene cause Rett syndrome (RTT) [13,14]. Decreased pain perception is commonly reported in children with RTT [15,16]. The abnormal sensitivity can be as high as 75%, with a potential relationship between hyposensitivity and specific mutations in the MECP2 gene [15]. The observations from RTT patients indicate that decreased functional MeCP2 contributes either directly or indirectly to reduced pain sensitivity. Since reduced pain sensitivity observed in RTT patients results from a decrease in functional MeCP2 protein, we postulated that a decrease in miRNAs that bind and repress translation of MeCP2 will cause an increase in MeCP2 levels and thus contribute to hypersensitivity.

MeCP2 can mediate downstream transcriptional changes of a large number of genes and depending on its interacting protein partners and target genes, MeCP2 can act either as an activator or repressor [12,17]. MeCP2 is a known regulator of Bdnf [18], a neurotrophin important in both neuronal development and peripheral pain mechanisms [19]. BDNF levels in the hypothalamus correlated with MeCP2 levels, with lower levels in...
Mecp2-null mice and higher levels in MECPT overexpressing mice compared to controls [12]. The role of BDNF in both inflammatory and neuropathic pain is well established [20–23]. Here we investigated if miRNAs downregulated in a rodent model of neuropathic pain that modulate MeCP2 expression, can induce changes in Bdnf levels in Neuro-2a cells.

Several mouse models have been generated for investigating MeCP2 function [24] including alteration of the endogenous Mecp2 gene, or introduction of the human MECPT gene with RTT-associated mutations. One of the most common mutations observed in RTT is in amino acid T158, located at the C terminus of the methyl-CpG binding domain of MeCP2. It has been reported that 70.6% of patients with this mutation have decreased pain sensitivity [15]. The phenotype of MeCP2 T158A knockin mice that express 50% of the wild-type level of MeCP2 had a slower reaction to a conductive heat stimulus [26,27]. We sought to assess the pain sensitivity of MeCP2 T158A mice as well as expression of Bdnf to test our hypothesis that MeCP2 plays a role in mediating pain sensitivity and confirm the functional implication of a mutation in the methyl binding domain.

Thus, here we sought to determine whether miRNAs downregulated after nerve injury regulate MeCP2 and hence modulate Bdnf expression, contributing to hypersensitivity. We used MeCP2 T158A mice to determine if a point mutation in the methyl binding domain can attenuate pain sensitivity, and we measured expression of Bdnf in the DRG from MeCP2 T158A mice and MeCP2-null mice to determine the regulatory role of MeCP2 on Bdnf in the DRG.

2. Materials and methods

2.1. Cell culture, transfection and luciferase reporter assay

HEK293 and Neuro-2a cells obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO2. For the reporter assay HEK293 cells were co-transfected with precursor miRNA or anti-miRNA plasmid (GeneCopoeia) and luciferase reporter plasmid containing the target sequence of interest (1–2210 bp or 6360–8591 bp) was cloned downstream of the luciferase reporter gene as 4 constructs of ~2 kb fragment each (GeneCopoeia). The fragment with the miRNA target sequence of interest (1–2210 bp or 6360–8591 bp) was co-transfected with corresponding miRNA. The Luc-Pair Duo-Luciferase assay (GeneCopoeia) was used to measure firefly and renilla luciferase according to the manufacturer’s instructions. Firefly luciferase measurements normalized to renilla luciferase according to the manufacturer's instructions.

2.2. Western blot

Protein from Neuro-2a cells or DRG was isolated using radioimmunoprecipitation assay buffer (Thermo Scientific). For western blotting, 20 μg protein lysates were resolved by a 4–12% SDS–PAGE gel, transferred to the nitrocellulose membrane. The membranes were probed with MeCP2 antibody [25] at 1:3000 dilution overnight. Chemiluminescence was detected using FluoroChem M System (Protein Simple). The membrane was also probed with goat anti-rabbit GAPDH-HRP (1:2000 dilution, Santa Cruz) as a loading control. Quantification was determined using UN-SCAN-IT software, MeCP2 expression was normalized to GAPDH.

2.3. Immunocytochemistry

Neuro-2a cells grown on 12mm glass coverslips were transfected with miRNA precursor plasmids with GFP using X-tremeGENE HP DNA transfection reagent for 48 h. Cells were fixed in 4% formaldehyde and blocked in 10% normal goat serum followed by a 3 h incubation in 1:200 anti-MeCP2 antibody (mentioned above). Anti-Rabbit-IgG Atto 647N secondary antibody (Sigma) was used for detection of MeCP2. Coverslips were mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were acquired using the 60x objective on the Olympus 1 x 81 confocal microscope and Fluoview FV10-ASW software. Image J was used for quantification. MeCP2 levels were normalized to DAPI staining of the nucleus. Transfected (GFP positive) and untransfected (GFP negative) cells were then compared.

2.4. Quantitative RT-PCR

RNA was purified from Neuro-2a cells, DRG collected from spared nerve injury (SNI) model, sham control, Mecp2-null, Mecp2 T158A and the corresponding wild-type littermate mice using the mirVana RNA isolation kit (Life technologies). cDNA synthesis and qRT-PCR were performed as previously described [28]. The Assay ID for the Taqman primer probes used were Mm01193535_M1 (Mecp2) and Mm04230607_s1 (Bdnf). Gapdh was used as the normalizer and a t-test was used to perform statistical analysis.

2.5. Animal model of neuropathic pain

The care and use of all mice were approved by the Institutional Animal Care & Use Committee of Drexel University College of Medicine. The SNI model was generated using 8-week old C57BL/6 male mice (Taconic) as previously described [29,30]. Briefly, mice were anesthetized with isoflurane during surgery. The common peroneal and tibial nerves of the left paw were ligated, 2–4 mm of the nerve was sectioned and removed distal to ligation. Sham mice underwent the same surgical procedures as the SNI group without ligation and sectioning. Development of mechanical hypersensitivity was assessed using von Frey filaments [30] and the Mann–Whitney U test was used to calculate significance. L4 and L5 DRG on the ipsilateral side of surgery were collected 4 weeks post SNI surgery at 12 weeks of age.

2.6. Behavior studies using MeCP2 T158A mice

Male MeCP2 T158A mice and wild-type littermates were purchased from Jackson laboratories (Bar Harbor, Maine). Thermal and mechanical sensitivity were determined using the Hargreaves’ method [31] and von Frey filaments respectively. Behavior testing and tissue collection was conducted at 12 weeks of age. Mann–Whitney U test was used for statistical analysis.

3. Results

3.1. Chronic neuropathic pain induced MeCP2 upregulation in DRG

MeCP2 has been predicted to play a role in pain and modulation of MeCP2 has been observed in various inflammatory and neuropathic pain models [32,33]. To investigate whether MeCP2 expression is altered in a neuropathic pain state, we generated an SNI
model. We confirmed increased mechanical sensitivity and data 4 weeks post-surgery on 12 week old mice is shown in Fig. 1a. Though there was a trend, the increase in Mecp2 mRNA in DRG after nerve injury was not significant (Fig. 1b). However, there was a significant increase in MeCP2 protein in the SNI model (Fig. 1c). This increased expression of MeCP2 in a neuropathic pain state could in part be mediated by the reduced expression of miRNAs predicted to target Mecp2 in the DRG [5]. We therefore sought to determine the regulatory role of a selected subset of miRNAs on MeCP2 expression.

3.2. Confirmation of miRNAs predicted to target Mecp2 3’UTR

To experimentally validate the bioinformatics prediction of miRNAs targeting the Mecp2 3’UTR, a luciferase reporter assay was performed. We tested a subset of miRNAs that were decreased in the DRG from a nerve injury model based on seed sequence complementarity, varying positions throughout the 3’UTR, the presence of multiple binding sites, and those not predicted to target Bdnf mRNA. Reduction in luciferase expression and therefore, miRNA binding to the Mecp2 3’UTR was validated for miR-19a, miR-301 and miR-132 (Fig. 2), while miR-17 and miR-181 did not reduce luciferase activity (data not shown).

3.3. MeCP2 expression is modulated by miRNAs

To further evaluate regulation of MeCP2 expression from miRNA binding, Neuro-2a cells were transfected with precursor miRNAs or antagonirs that are inhibitors of endogenous miRNAs. Our data indicate that transfection of Neuro-2a cells with miR-19a, miR-301 and miR-132 did not decrease Mecp2 mRNA (Fig. 3a) but reduced MeCP2 protein as shown in western blot (Fig. 3b) and immunocytochemistry of endogenous MeCP2 in cells transfected with individual miRNAs (Fig. 3d–f). The antagonirs of miR-301 and miR-132 increased MeCP2 expression (Fig. 3c). We did not observe an increase in MeCP2 protein after the transfection of miR-19a inhibitor which could be due to a low endogenous level of miR-19a in Neuro-2a cells, the inhibition of which would not lead to the modulation of MeCP2 expression. Since we did not observe significant changes in Mecp2 mRNA after over expressing miRNAs, we conclude that Mecp2 regulation by miRNAs tested is mediated by translational repression and not through RNA degradation.

3.4. miRNA-mediated decrease of MeCP2 lead to concomitant reduction in Bdnf

To determine if miRNA mediated regulation of MeCP2 affects expression of the MeCP2 target genes, we measured Bdnf mRNA after transfecting Neuro-2a cells with miR-19a, miR-301 and miR-132. Fig. 4a shows that there was reduction in Bdnf transcripts after overexpressing miRNAs. Additionally, inhibiting endogenous miR-132 and miR-301 resulted in increased Bdnf transcript (Fig. 4b). The miRNAs used in this study were confirmed to target the Mecp2 3’UTR, but are not predicted to bind the Bdnf 3’UTR. Thus, the reduced expression of Bdnf can be a direct consequence of miRNA mediated regulation of MeCP2.

3.5. MeCP2 T158A mice have reduced mechanical sensitivity

Mice expressing 50% of the wild-type level of MeCP2 and female Mecp2+/− mice have reduced thermal sensitivity [26,34]. We wanted to further investigate the effect of MeCP2 mutations on pain behavior by assessing nociception in MeCP2 T158A mice. We observed that MeCP2 T158A mice have reduced mechanical sensitivity compared to wild-type littermate control mice (Fig. 5a). We found no significant changes in thermal sensitivity as determined by Hargreaves’ method between MeCP2 T158A and wild-type littermate control mice (data not shown). These results indicate a role for MeCP2 in mediating mechanical sensitivity.

3.6. Bdnf is downregulated in the DRG of Mecp2-null mice and MeCP2 T158A mice

Several studies using mouse models have shown a direct correlation between MeCP2 and Bdnf expression in the brain indicating...
Regulation of MeCP2 by miRNAs. (A) Taqman analysis of MeCP2 in Neuro-2a cells 48 h after miRNA transfection showed no significant reduction in mRNA ($n=3$). (B) Western blot analysis after transfection of Neuro-2a cells with miR-301, miR-132 and miR-19a significantly reduced MeCP2 protein ($n=3$). (C) MeCP2 expression increased when cells were transfected with anti-miR (miRNA inhibitors) for miR-301 and miR-132; ($n=3$, representative blot shown). (D and E) Immunocytochemistry and quantification for MeCP2 in Neuro-2a cells transfected with miR-132. (F and G) Immunocytochemistry and quantification for MeCP2 in Neuro-2a cells transfected with miR-19a. (H and I) Immunocytochemistry and quantification for MeCP2 in Neuro-2a cells transfected with miR-301. MeCP2 levels were normalized to DAPI staining of the nucleus and the graph represents MeCP2 expression relative to untransfected cells. Representative images are shown, $n=3$. Significance was determined using Student $t$-test, $p$ value $^{*}<0.05$, $^{**}<0.001$, $^{***}<0.001$. 
that MeCP2 regulates Bdnf [18]. We examined Bdnf expression in DRG from MeCP2 T158A mice. Fig. 5b shows a reduction in Bdnf mRNA levels in MeCP2 T158A mice compared to control. Thus MeCP2 mediated regulation of Bdnf expression in DRG appears to be similar to that observed in brain. We then investigated Bdnf levels in DRG from MeCP2 T158A mice to determine if a loss of function mutation would yield a similar result (Fig. 5c). We observed a decrease in Bdnf mRNA in the DRG of MeCP2 T158A mice as was previously reported in the brain [25]. These results indicate that in DRG, the role of MeCP2 as an activator is important for precise regulation of Bdnf and alteration of Bdnf in DRG may be a contributing factor leading to aberrant pain sensitivity in RTT.

3.7. Bdnf is decreased in MeCP2 T158A SNI model compared to wild-type SNI model mice

To determine how changes in MeCP2 in the DRG affect pain behavior, we generated an SNI model using MeCP2 T158A mice and wild-type littermate controls. Unfortunately, conducting mechanical and thermal sensitivity assessment post-SNI surgery...
was not possible in the MeCP2 T158A mouse; post-surgical over grooming behavior resulted in injuries compared to wild-type littermates. We collected the DRG from MeCP2 T158A mice 4-weeks post SNI and from wild-type SNI mice, and measured Bdnf transcripts. After nerve injury, MeCP2 T158A mice have decreased Bdnf compared to wild-type littermate control mice (Fig. 5d). Bdnf levels did not differ significantly between SNI and sham MeCP2 T158A mice (data not shown). This suggests that in the absence of fully functional MeCP2, the upregulation of Bdnf is impaired even after nerve injury, again suggesting the regulatory role of MeCP2 on Bdnf.

4. Discussion

The role of MeCP2 in the development and function of the central nervous system is well established and neuronal dysfunction contributes to symptoms associated with RTT [14,35]. Previous studies investigating the role of MeCP2 in pain have shown differences in expression pattern of MeCP2 in DRG and spinal cord from various rodent models of pain. An increase in MeCP2 has been associated with the development of neuropathic pain in the chronic constriction injury model in rats 14 days after injury [36]. MeCP2 is known to play an important role in signaling in the superficial dorsal horn upon induction of peripheral inflammation by injecting complete Freund’s adjuvant (CFA) in rat ankle joint. This study showed that phosphorylation of MeCP2 led to the release of DNA-bound MeCP2, relieving the repression on a few MeCP2 target genes in lamina I neurons. These genes were upregulated rapidly in this model of inflammatory pain [37]. MeCP2 expression was increased in the superficial dorsal horn 7 days following CFA injection in the ankle joint but decreased 7 days following SNI [38]. This study also showed that MeCP2 levels were decreased in activating transcription factor 3 (ATF3)-positive neurons in DRG after SNI [38]. Another report showed an increase in MeCP2 transcripts in the spinal cord 2 h after peripheral injection of formalin [9]. Upregulation of MeCP2 in the central nucleus of the amygdala was reported in the CFA model [39]. Differences in models, tissues, and time points can influence gene expression and so we performed qPCR and western blot analysis for MeCP2 using DRG from a mouse SNI model of neuropathic pain 4 weeks after surgery. We observed a significant increase of MeCP2 protein in DRG.

MeCP2 can regulate microRNAs (miRNAs) [40] and dysregulation of miRNAs was observed in the cerebella of MeCP2-null mice [41]. miRNA-mediated alteration of MeCP2 was reported to alter pain threshold in wild-type mice. miR-124a was downregulated in spinal cord after peripheral noxious stimulation with formalin and this was associated with an increase in MeCP2 mRNA. Intravenous administration of a miRNA-124a inhibitor enhanced the nociceptive behavior and miRNA-124a mimic significantly reduced formalin-induced inflammatory pain. In addition to confirming that MeCP2 is a target for miR-124a, this study showed administration of miR-124a mimic induced a decrease in MeCP2 mRNA and miR-124a inhibitor enhanced MeCP2 levels in the spinal cord [9]. In another study, intrathecal miR-124 treatment reversed persistent hyperalgesia induced by carrageenan and prevented the development of mechanical allodynia in the SNI model of chronic neuropathic pain [42]. Thus future studies investigating the role of miRNAs validated to target MeCP2 will be beneficial in confirming their role in pain.

Our previous study investigating miRNA changes in DRG identified several miRNAs predicted to target MeCP2 3’UTR. The miRNAs were downregulated after nerve injury and because of the expected inverse correlation in expression between miRNAs and genes they regulate, we selected a subset of 5 miRNAs for further studies. Of these, miR-19a, miR-301 and miR-132 were confirmed to bind and regulate MeCP2 by translational repression. miR-132 is a previously validated miRNA for MeCP2 [6] and we included it as a positive control. This reduction of MeCP2 protein resulted in decreased Bdnf, indicating that activation by MeCP2 is an important mechanism of regulation for normal expression of BDNF. Our findings that inhibition of miRNA-mediated repression increased MeCP2 and Bdnf is similar to what was reported for miR-132 in cultured rat neurons [6].

Alterations in BDNF expression is a common phenomenon in both RTT and pain states, albeit in reverse direction. BDNF is an important modulator of sensory neurotransmission in nociceptive pathways both at spinal and supraspinal levels and has an important role in the development of central sensitization that underlies many forms of hyperalgesia [19]. Many of the biological effects of BDNF are mediated by the high affinity postsynaptic receptor TrkB. TrkB signaling contributes to both the induction and maintenance of injury induced persistent pain and Trk inhibitors have been developed for the treatment of pain [43]. In addition to correlation of BDNF levels in the hypothalamus with MeCP2 levels, with lower levels in MeCP2-null mice and higher levels in MeCP2 overexpressing mice compared to controls [12], disruption of total BDNF levels and secretion in MeCP2-null mice indicate that functional MeCP2 is important for BDNF expression [44]. BDNF-related therapies have shown a reversal of cardiorespiratory deficits in MeCP2-null mice and is being pursued for the amelioration of RTT-like neurological symptoms in mouse models [18]. Our qPCR studies showed that Bdnf levels in the DRG were downregulated in both MeCP2-null and MeCP2 T158A mice. Thus the reduction in endogenous Bdnf could be one of the contributing factors leading to decreased pain sensitivity. After SNI surgery, lower levels of Bdnf in MeCP2 T158A mice compared to wild-type mice further indicates that increased Bdnf in the DRG after nerve injury is in part mediated by MeCP2. A recent study investigating the role of MeCP2 in pain regulation and morphine reward showed that MeCP2 and histone dimethyltransferase C9a induce a transcriptional de-repression of Bdnf in central nucleus of the amygdala, promoting pain behavior through BDNF upregulation [39]. Thus in addition to direct activation of Bdnf, MeCP2 in its role as a transcriptional repressor of C9a, further enhance BDNF levels in chronic pain.

Our studies showed that mice with the loss of function mutation, T158, have reduced mechanical sensitivity compared to wild-type littermates. MeCP2 T158A mice expressing 50% of the wild-type level of MeCP2 and female MeCP2 T158A mice have reduced thermal sensitivity in a hot plate test [26,34] but there are no reports to date investigating their mechanical sensitivity. Our assessment of thermally induced nociception using the Hargreaves’ method showed no differences in MeCP2 T158A mice and wild-type littermates. Differences in the thermal sensitivity could be due to the specific mutation of MeCP2 or the method of measuring thermal sensitivity. Since BDNF release in dorsal horn in neuropathic pain model is associated with thermal hyperalgesia [45,46], we postulate that other modes of action may be playing a role in mediating mechanical sensitivity in T158A mice. Studies investigating function of DRG neurons in naïve animals have shown that delivering BDNF directly to DRGs induced mechanical allodynia. Conversely, after nerve injury direct administration of anti-BDNF antibody to injured DRG attenuated mechanical allodynia [47]. MeCP2 and BDNF are expressed in microglia and astrocytes in addition to neurons and additional studies are needed to determine the contribution of these different cell types in modulating aberrant pain sensation in MeCP2 T158A mice. A recent study investigated the role of miR-183, a miRNA that was significantly downregulated in the ipsilateral L5 DRG after SNL [48]. Intrathecal administration of miR-183 attenuated mechanical allodynia and this was associated with the downregulation of BDNF transcripts.
in DRG [48]. This study demonstrated that downregulation of BDNF in DRG was correlated with attenuated mechanical allodynia in SNL model of neuropathic pain. Our data shows that mutations in Mecp2 can reduce mechanical pain sensitivity. Since MeCP2 can mediate downstream transcriptional changes of a large number of genes, we cannot rule out the contribution of other dysregulated genes (due to T158A mutation in methyl binding domain of MeCP2) to aberrant pain sensitivity in MeCP2 T158A mice.

In conclusion, our results indicate that miRNA-mediated regulation of MeCP2 can contribute to the mechanical hypersensitivity observed in neuropathic pain models by altering BDNF in the DRG. Neuropathic pain induces MeCP2 and BDNF expression in the DRG; conversely, RTT mouse models lacking or expressing reduced levels of MeCP2 have decreased Bdnf that can contribute to mechanical hyposensitivity. Further investigations including in vivo delivery of miRNAs targeting Mecp2 followed by characterization of pain behavior and investigation of MeCP2 expression and downstream targets in naïve mice, RTT and pain models can provide insights on therapeutic options for both pain and RTT.

Author contributions

S.A. conceived and supervised the study; M.M. and S.A. designed experiments; M.M. and Y.T. performed experiments; M.M. analyzed the data; Z.Z. provided reagents and critical input regarding the study design; M.M. and S.A. wrote the manuscript; Z.Z. made manuscript revisions.

Acknowledgments

This work was supported by grants from Commonwealth Universal Research Enhancement (CURE), Pennsylvania Department of Health and Rita Allen Foundation to Seena Ajit. The funding agency had no role in study design; M.M. and S.A. wrote the manuscript; Z.Z. made manuscript revisions.

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