

Differential Expression and Regulation of Brain-Derived Neurotrophic Factor (*BDNF*) mRNA Isoforms in Brain Cells from *Mecp2*^{308/y} Mouse Model

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Abstract Rett syndrome (RTT) is a severe neurodevelopmental disease caused by mutations in methyl-CpG-binding protein 2 (*MECP2*), which encodes a transcriptional modulator of many genes including *BDNF*. *BDNF* comprises nine distinct promoter regions, each triggering the expression of a specific transcript. The role of this diversity of transcripts remains unknown. MeCP2 being highly expressed in neurons, RTT was initially considered as a neuronal disease. However, recent studies have shown that MeCP2 was also expressed in astrocytes. Though several studies explored *Bdnf* *IV* expression in *Mecp2*-deficient mice, the differential expression of *Bdnf* isoforms in *Mecp2*-deficient neurons and astrocytes was never studied. By using TaqMan technology and a mouse model expressing a truncated *Mecp2* (*Mecp2*^{308/y}), we firstly showed in neurons that *Bdnf* transcripts containing exon I, IIb, IIc, IV, and VI are prominently expressed, whereas in astrocytes, *Bdnf* transcript containing exon VI is preferentially expressed, suggesting a specific regulation of *Bdnf* expression at the cellular level. Secondly, we confirmed the repressive role of

Mecp2 only on the expression of *Bdnf* VI in neurons. Our data suggested that the truncated *Mecp2* protein maintains its function on *Bdnf* expression regulation in neurons and in astrocytes. Interestingly, we observed that *Bdnf* transcripts (I and IXA), regulated by neural activity induced by bicuculline in *Mecp2*^{308/y} neurons, were not affected by histone deacetylase inhibition. In contrast, *Bdnf* transcripts (IIb, IIc, and VI), regulated by histone deacetylation, were not affected by bicuculline treatment in wild-type and *Mecp2*^{308/y} neurons. All these results reflect the complexity of regulation of *Bdnf* gene.

Keywords Rett syndrome · *Mecp2* · *Mecp2*^{308/y} · BDNF · BDNF isoforms · Neurons · Astrocytes

Introduction

Rett syndrome (RTT, MIM 312750) is an X-linked neurodevelopmental disorder representing one of the most frequent causes of severe intellectual deficiency in females. RTT infants develop normally until 6 to 18 months of age but then show progressive loss of neurodevelopmental milestones (Bienvenu and Chelly 2006). Clinical features include deceleration of brain growth, loss of motor skills including stereotypic hand movements with loss of purposeful hand use, absence of speech, seizures, autistic behaviors, and respiratory irregularity (Hagberg et al. 1983). De novo mutations in X-linked *MECP2* encoding methyl-CpG-binding protein 2 (MeCP2) are responsible for most cases of RTT (Amir et al. 1999). MeCP2 levels in the brain are low during embryonic stages and increase steadily during the first few days after birth (Balmer et al. 2003; Kishi and Macklis 2004; Shahbazian et al.

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2002), a postnatal period of intense synapse formation and maturation.

MeCP2 belongs to the family of DNA-binding proteins and was originally hypothesized to silence gene transcription by binding to methylated CpG dinucleotides, usually within gene promoter regions (Jones et al. 1998). This model predicts that MeCP2 deficiency should result in the transcription of normally repressed genes. However, the observation that the majority of modulated genes are activated in *Mecp2*-overexpressing mice and downregulated in *Mecp2* knockout mice (Chahrouh et al. 2008) suggested that MeCP2 is also an activator of gene transcription.

As the symptoms of RTT are primarily neurological, MeCP2 function was originally hypothesized to be restricted to neurons (Guy et al. 2001; Chen et al. 2001). It appears that MeCP2 binding to DNA and subsequent transcriptional modulation is a dynamic process and dependent on external signals such as Ca^{2+} (Chen et al. 2003; Zhou et al. 2006). For example, neuronal membrane depolarization triggers calcium-dependent phosphorylation at serine 421 and release of MeCP2 from the *Bdnf* promoter IV, thereby facilitating transcription (Zhou et al. 2006). These results indicate that MeCP2 plays a key role in the transcription of neuronal activity-dependent gene regulation, such as the brain-derived neurotrophic factor Bdnf.

Although MeCP2 is important for neuronal function, many studies suggest that the function of other cell types, particularly astrocytes, is impaired by MeCP2 defects. Although MeCP2 levels are roughly 5-fold lower in astrocytes than in neurons (Maezawa et al. 2009), recent studies suggest that loss of MeCP2 in astrocytes contributes to RTT-like symptoms and restoration of MeCP2 can rescue some of these defects (Lioy et al. 2011).

Bdnf is a unique activity-dependent neurotrophic factor widely expressed in the brain and has long-term effects on neuronal survival, development, and synaptic plasticity. It modulates the strength of existing synaptic connections and acts in the formation of new synaptic contacts (Thoenen 1995). Interestingly, the structure of the *BDNF* gene in humans and rodents is unusual in that it contains several 5' non-coding exons, each possessing a unique promoter region driving the transcription of a common 3' exon that contains the coding region of the mature Bdnf protein (Metsis et al. 1993; Timmusk et al. 1993; Timmusk et al. 1995). In mouse and human, exon I transcripts contain an inframe AUG that can serve as an alternative translation initiation codon, extending the coding region by eight amino acids (Timmusk et al. 1993). The 3' untranslated region of the coding exon also contains two polyadenylation sites. This gene structure gives rise to several different *Bdnf* mRNA transcripts, each possessing one of the non-coding exons followed by the common *Bdnf* coding exon (exon IX, formerly exon V). Originally, four non-coding exons were believed to exist, designated *Bdnf* exons I, II, III, and IV,

with the coding exon designated as exon V. In 2007, four new non-coding exons have been discovered that are interspersed among the originally characterized exons (Aid et al. 2007), and a new nomenclature for the *Bdnf* gene and associated transcripts has been proposed. The new non-coding *Bdnf* exon designations are exon I and exon II (unchanged from previous characterizations), exon III (new), exon IV (formerly characterized as exon III), exon V (new), exon VI (formerly characterized as exon IV), exon VII (new), exon VIII (new), and exon IXA (new). For clarity, we use the newer designation in this report (Aid et al. 2007).

Though several reports studied *Bdnf IV* transcript in *Mecp2*-deficient mice, differential expression of *Bdnf* mRNA isoforms in *Mecp2*-deficient neurons and astrocytes was unknown. Moreover, because RTT is primarily due to missense and nonsense mutations in the *MECP2* gene, we focused on a male mouse model expressing a targeted truncated *Mecp2* (*Mecp2*^{308/y}). It appears that the *Mecp2*^{308/y} model might mimic the behavioral symptoms of patients with RTT closer than mouse models carrying null mutations (Chen et al. 2001; Guy et al. 2001). These male transgenic mice develop progressive neurological alterations beginning at around 6 weeks that include tremor (pronounced stereotypic forelimb motions and claspings), motor impairment, hypoactivity, increased anxiety by 8 months, and seizures (Shahbazian and Zoghbi 2002).

Material and Methods

Animals

*Mecp2*³⁰⁸ mice developed by Shahbazian et al. (2002) were obtained from the Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME, USA). Females heterozygous for the mutation were mated with C57BL/6J wild-type males (Jackson Laboratory). Pups were genotyped using genomic DNA isolated from tail snips to determine *Mecp2* allele type according to the protocol designed by the original investigators (Shahbazian et al. 2002). Gender was determined using primers for the *Sry* gene on Y chromosome, which were 5'-CTCATCGGAGGGCTAAAGTG-3' and 5'-AAGCTTTGCTGGTTTTTGGA-3'. The University of Paris Descartes Institutional Animal Care and Use Committee approved all animal protocols. To avoid bias due to variations in the rate of inactivation of the X chromosome in females, all experiments were carried out from male *Mecp2*^{308/y} newborn and their wild-type male siblings.

Mecp2-Deficient and Control Astrocyte Cultures

P0 newborns from *Mecp2*^{308/+} heterozygous mice were used for the preparation of dissociated hippocampal and cortical

cell cultures. Primary astrocytes cultures were prepared from the male mouse *Mecp2*^{308/y} hippocampus and cortex according to previously described methods. Briefly, brains were dissected, hippocampal and cortical tissue were isolated by microdissection. Tissues were mechanically dissociated following incubation at 37 °C for 30 min in 0.05 % trypsin. Dissociated cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) with 10 % FBS (Gibco). After 10 days in vitro, wild-type and mutant confluent astrocytes cultures were used for total RNA extraction with the Qiagen RNeasy kit (Qiagen, Courtaboeuf, France) as described by the manufacturer.

Primary Cultures of Neurons

Embryonic mice (day E15) were isolated from timed pregnant *Mecp2*^{308/+} heterozygous females. Cortical and hippocampal tissues were isolated by microdissection, digested with trypsin for 30 min at 37 °C. The cells were then plated upon polylysine-coated 12-mm diameter glass cover slips treated by laminin (20 mg/ml). After incubation of 3 h in minimal essential medium and 10 % heat-inactivated fetal bovine serum, total medium was replaced with neurobasal growth medium (Gibco) supplemented with 2 % B27 (Invitrogen, Carlsbad, CA, USA), 1 % glutamax (Gibco), and 0.2 % of mycozap (Lonza, Basel, Switzerland). In our hands, these conditions yield cultures comprising of approximately 95 % neurons (identified by MAP2 immunoreactivity).

RNA Extraction and Quantitative Real-Time PCR

Cells were treated with a final concentration of 40 μM bicuculline methobromide (Tocris Bioscience, Minneapolis, MN, USA) for 1 h at 37 °C, or with 100 nM Trichostatin A (Sigma Aldrich, Saint Louis, MO, USA) for 19 h at 37 °C, or with solvent (DMSO). Briefly, total RNA was isolated from cells with RNeasy kit (Qiagen). To remove residual DNA, total RNA was treated with DNaseI (Qiagen, Frankfurt, Germany) according to manufacturer's instructions. Total RNA was then converted to cDNA using the Maxima First Strand cDNA kit (Thermo Scientific, Carlsbad, CA) under conditions recommended by the manufacturer. Quantitation was performed by spectrophotometry (Nanodrop, ThermoScientific). *Bdnf* transcript-specific primers (Table 1) were designed to cross an intron or span intron/exon boundaries to further limit the effect of potential genomic DNA contamination using Integrated DNA Technologies Design software (<http://eu.idtdna.com/scitools/Applications/RealTimePCR/>). Primer pairs designed to quantify the murine *Gad1* and *Arc* transcripts in neurons were as follows: *Gad1* forward sequence, 5'-ACTCAGCGGC ATAGAAAGGG-3'; *Gad1* reverse sequence, 5'-GAAG AGGTAGCCTGCACACA-3' and *Arc* forward sequence, 5'-ATCCTGGCACCTGGCCC-3'; and *Arc* reverse sequence, 5'-TCCGCCTGCCATGGCTGAGT-3'. Specificity of each primer was checked by genomic

Table 1 Primers and TaqMan probes to study *Bdnf* transcripts (I to IXA)

<i>Bdnf</i> transcript	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')	TaqMan probe (5'-3')	Reporter	Quencher	PCR product size (bp)
I	TTACCTTCCTGCATCTGT TGG	GTCATCACTCTTCT CACCTGG	AACATTGTGGCTTTGC TGCCTGG	FAM	NFQ	97
Ila	GAGAGCAGAGTCCATTTCAGC	CCTTCATGCAACCG AAGTATG	CCTTGGACAGAGCCAG CGGATTT	FAM	NFQ	143
Ilb	AGAGTCAGATTTGGAGC GG	GTCATCACTCTTCT CACCTGG	AGACTCTTGGCAAGCT CCGGTTC	FAM	NFQ	145
Ilc	GGCTGGAATAGACTCTTGGC	GTCATCACTCTTCT CACCTGG	CGGGTTGGTATACTGGGT TAACTTTGGG	FAM	NFQ	138
III	GGGCTCCTGCTTCTCAAG	ATGCAACCGAAGTA TGAAATAACC	CCTGGTGGAACTGGGC TCAAGAA	FAM	NFQ	149
IV	AGCTGCCTTGATGTTTAC TTTG	CGTTTACTTCTTTC ATGGGCG	AGGATGGTCATCACTCTT CTCACCTGG	FAM	NFQ	139
V	AACCATAACCCCGCACAC	ATGCAACCGAAGTATG AAATAACC	AAACCTTCCCACCT TCCCG	FAM	NFQ	151
VI	GGACCAGAAGCGTGAC AAC	ATGCAACCGAAGTATG AAATAACC	ACCAGGTGAGAAGAGTGA TGACCATCC	FAM	NFQ	136
VII	TGAAAGGGTCTGCGGA AC	GTCATCACTCTTCTCA CCTGG	AGGATGGACAGAGGGT CGGATACAG	FAM	NFQ	138
VIII	CTGATTGCTGAAAATGGT GTCTG	AGTTGCTTG TCCGTGG	TCATACTCGTTGCATG AAGGCGG	FAM	NFQ	142
IXA	AGTTCTAACCTGTTCTGT GTCTG	CGTTTACTTCTTCAT GGGCG	ACAGTTCCACCAGTTC CACCAGG	FAM	NFQ	149

alignment (www.ncbi.nlm.nih.gov/blast/blast.cgi). Each PCR contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems), and 5 µl of the diluted cDNA in a final volume of 25 µl. Final quantitation was done using the comparative CT method and is reported as a relative measure of the expression of each *Bdnf* transcript, which were all normalized to *beta-actine* transcript as calibrator gene. The identity and expected size of the single PCR product were confirmed by 2 % agarose gel electrophoresis and by Sanger sequencing.

Immunostaining

Cells (neurons and astrocytes) were washed twice with PBS and fixed with 4 % paraformaldehyde at 4 °C during 10 min, incubated with appropriated antibodies (anti-γ-aminobutyric acid (Sigma-Aldrich), anti-GFAP, sc-6170 Santa Cruz; anti-MAP2, MAB364 Merck Millipore, Billerica, MA, USA) and then mounted with Fluoromount mounting medium with DAPI (Interchim, Montluçon, France) and analyzed with a Leica DMRA2 fluorescence microscope DMRA2 (Leica microsystems, Leitz, Germany) using MetaMorph software (Molecular Devices LLC, Sunnyvale, CA, USA).

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) or fold change relative to control. Type-II ANOVA with post hoc Tukey’s test was used for analysis using the GraphPad Prism (GraphPad Software, San Diego, CA). *p*<0.05 is considered significant. Student’s *t* test was used as appropriate.

Results

Bdnf Expression Profiling in Neurons and Astrocytes

Expression of various *Bdnf* transcripts was initially described by Aid et al. (Aid et al. 2007) when the authors firstly established the complete murine genomic organization of the *Bdnf* gene. However, they focused on whole brain and/or whole brain structures, known to be heterogeneous at the cellular level. In a first step, we investigated relative expression levels of the eleven *Bdnf* transcripts (I to IXA) known to be under the control of separate promoters in primary cultures of astrocytes and neurons from the cortex and hippocampus from *Mecp2*^{+/-y} male mice (Fig. 1).

In primary cortical and hippocampal neurons from E15 embryonic mice, *Bdnf* transcripts containing exon I was prominently expressed. *Bdnf* transcripts containing exons IIb, IIc, IV, and VI were moderately expressed, and *Bdnf* IXA was weakly expressed (Fig. 1a). In primary cortical and hippocampal astrocytes from P0 newborn, *Bdnf* transcripts containing exon VI was prominently expressed, and *Bdnf* I, IIc, IV, and IXA were moderately expressed, and *Bdnf* IIb was slightly expressed. In both cell types, no expression of *Bdnf* IIa, III, V, VII, and VIII was detected (Fig. 1b).

In the hippocampus, *Bdnf* transcripts I, IIb, IIc, and IV were more expressed in neurons than in astrocytes with 10- to 74-fold variations. In the cortex, those *Bdnf* transcripts I, IIb, IIc, and IV were more expressed in neurons than in astrocytes with 4- to 18-fold variations (Fig. 1). However, in both cerebral structures, *Bdnf* transcript VI was 2-fold more expressed in astrocytes than in neurons. Taking into account our results, *Bdnf* transcripts for which no significant expression was detected were not further studied.

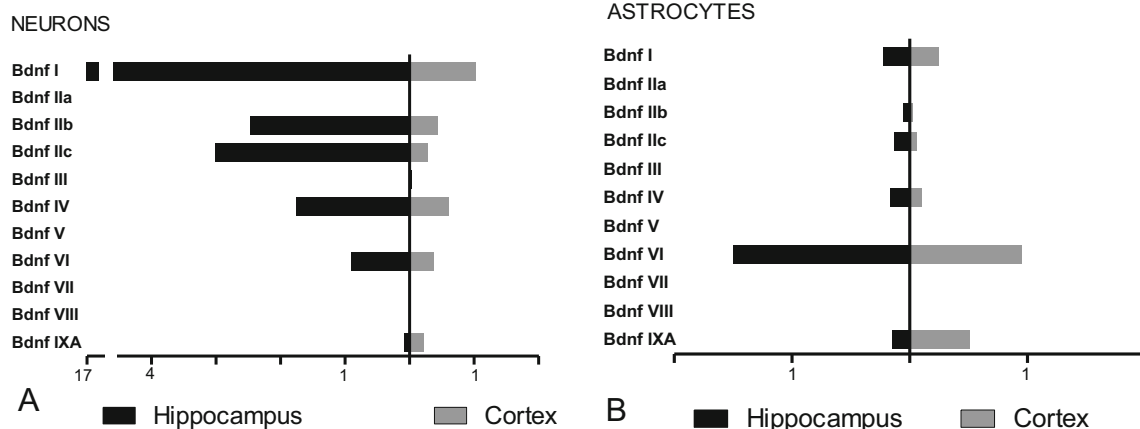


Fig. 1 *Bdnf* expression profiling in neurons (a) and astrocytes (b) from the hippocampus and cortex of wild-type mice: relative expression of each *Bdnf* transcripts. RNA isolated from neurons or astrocytes were

subjected to real time RT-quantitative PCR with TaqMan probes against each specific *Bdnf* transcript. The horizontal axis represents the relative quantification of each *Bdnf* transcript

Mecp2 Deficiency on Bdnf Transcription in the Cortex and Hippocampus

To study the influence of *Mecp2* on *Bdnf* expression pattern, we studied by RT-quantitative PCR (qPCR) the relative expression level of *Bdnf* mRNA isoforms (transcripts I, IIb, IIc, III, IV, VI, and IXA) in primary cultures of neurons and astrocytes at different stages of maturation and in various brain structures (cortex and hippocampus) from wild-type and *Mecp2*-deficient male mice (*Mecp2*^{308/y}). We firstly analyzed *Bdnf* expression in neurons obtained from cortex taken from E15 embryos brain from five litters. After 9 days of in vitro culture (DIV), a critical period for dendritic elongation and branching (Wayman et al. 2006), *Mecp2* deficiency results only in a significant overexpression of *Bdnf* transcript VI, one of the highest expressed *Bdnf* transcript ($p < 0.05$) (Fig. 2a). Similarly, expression of *Bdnf* transcript IIc appears to increase in *Mecp2*-deficient neurons ($p = 0.07$) (Fig. 2a). Interestingly, we did not observe significant changes in *Bdnf* expression levels in hippocampal neurons at 20 DIV from E15 embryos brain from two litters comparing *Mecp2*^{308/y} to wild-type neurons (Fig. 2b).

When we applied the same quantitative approach in primary astrocyte cultures from hippocampal structures from P0 *Mecp2*^{308/y} male newborns, levels of all *Bdnf* transcripts were

similar in *Mecp2*-deficient and WT hippocampal astrocytes at 10 DIV (Fig. 2c).

Effect of Neuronal Depolarization by Bicuculline on Bdnf Transcription in Wild-Type and *Mecp2*-Deficient Neurons

To study *Bdnf* transcripts whose expression is regulated by neuronal activity, we studied effect of membrane depolarization induced by bicuculline, an inhibitor of GABAergic receptors. Experiments were performed on wild-type and *Mecp2*^{308/y} neurons, treated with a final concentration of 40 μ M bicuculline methobromide or vehicle (DMSO). At this concentration, bicuculline induced overexpression of the *Arc* gene, required for long-term plasticity, more importantly in cortical neurons compared to hippocampal neurons (Fig. 1S). Moreover, RT-PCR of the *Gad1* gene and immunofluorescence analysis for GABA showed the presence of GABAergic neurons in neuronal cultures from the cortex and hippocampus (Fig. 2S, Fig. 3S). In wild-type cortical and hippocampal neurons, bicuculline treatment had no significant effect on the expression level of all *Bdnf* transcripts (Figs. 3 and 4; Fig. 4S). However, in *Mecp2*-deficient hippocampal neurons at 20 DIV, a late developmental stage reflecting synapse formation (Kato-Negishi et al. 2004), bicuculline treatment increased significantly expression level

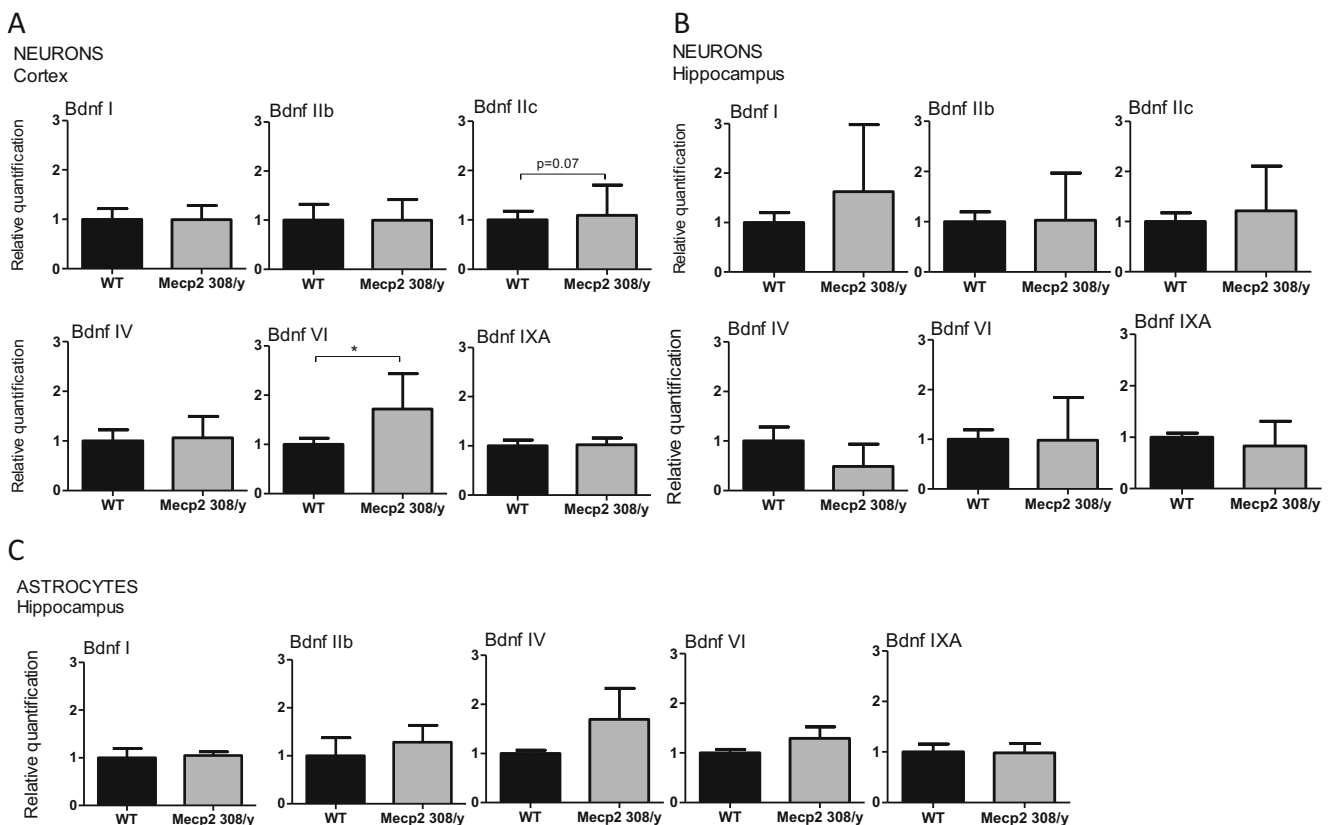
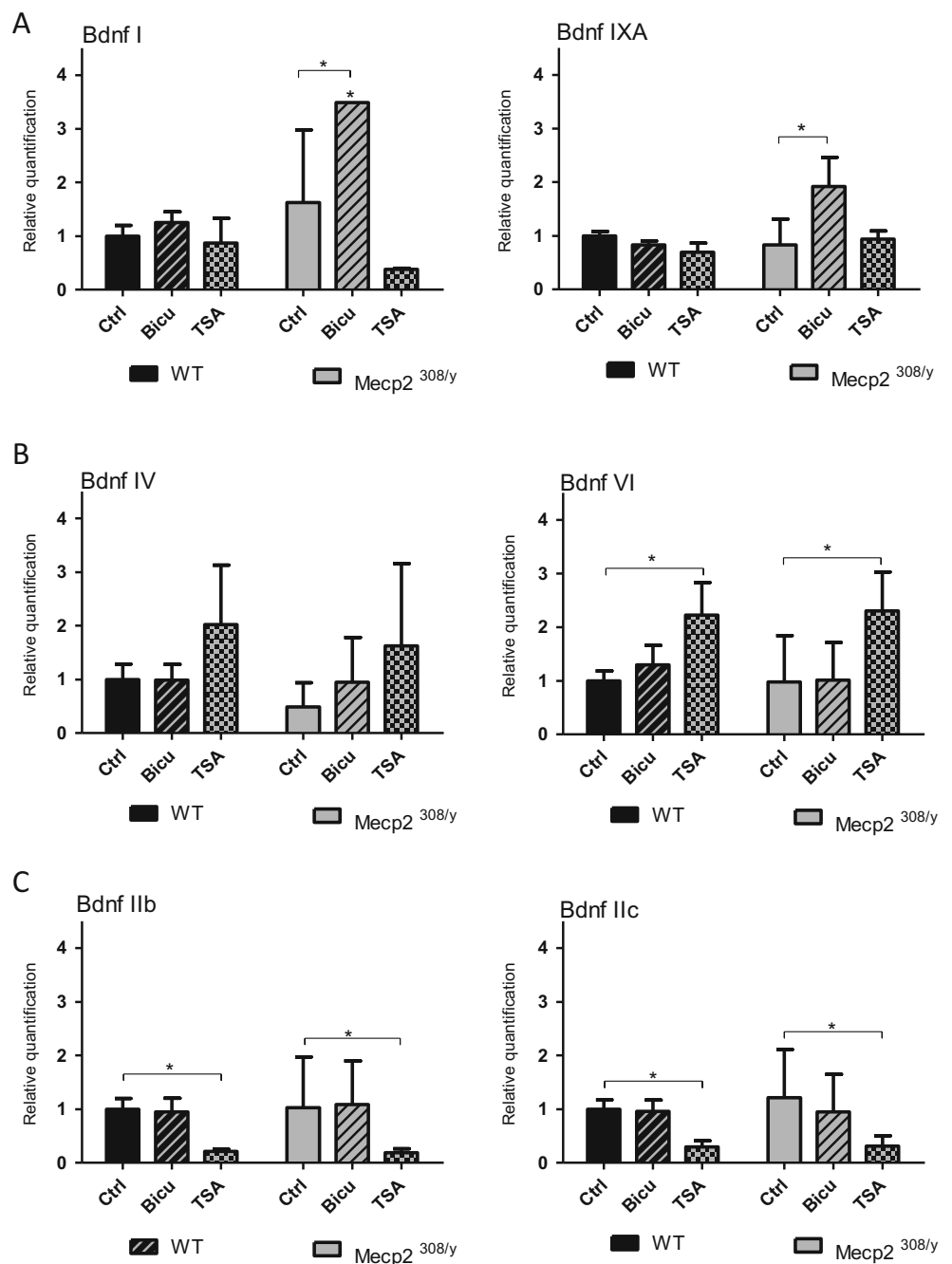


Fig. 2 Relative expression level of *Bdnf* transcripts I, IIb, IIc, IV, VI, and IXA from *Mecp2*^{308/y} and wild-type (*WT*) mice in the cortical neurons (a), hippocampal neurons (b), and hippocampal astrocytes (c)

Fig. 3 Effect of bicuculline (*bicu*) and trichostatin A (*TSA*) on *Bdnf* transcription in wild-type (*WT*) and *Mecp2*^{308/y} hippocampal neurons: real-time RT-quantitative PCR analysis showing the relative changes of *Bdnf* transcripts I, IXA (a), IV, VI (b), IIb, and IIc (c) after 1 and 19-h exposure to bicuculline and trichostatin A, respectively. *Ctrl* control as untreated cells. *Bars* represent means \pm SEM. Asterisk indicates $p < 0.05$



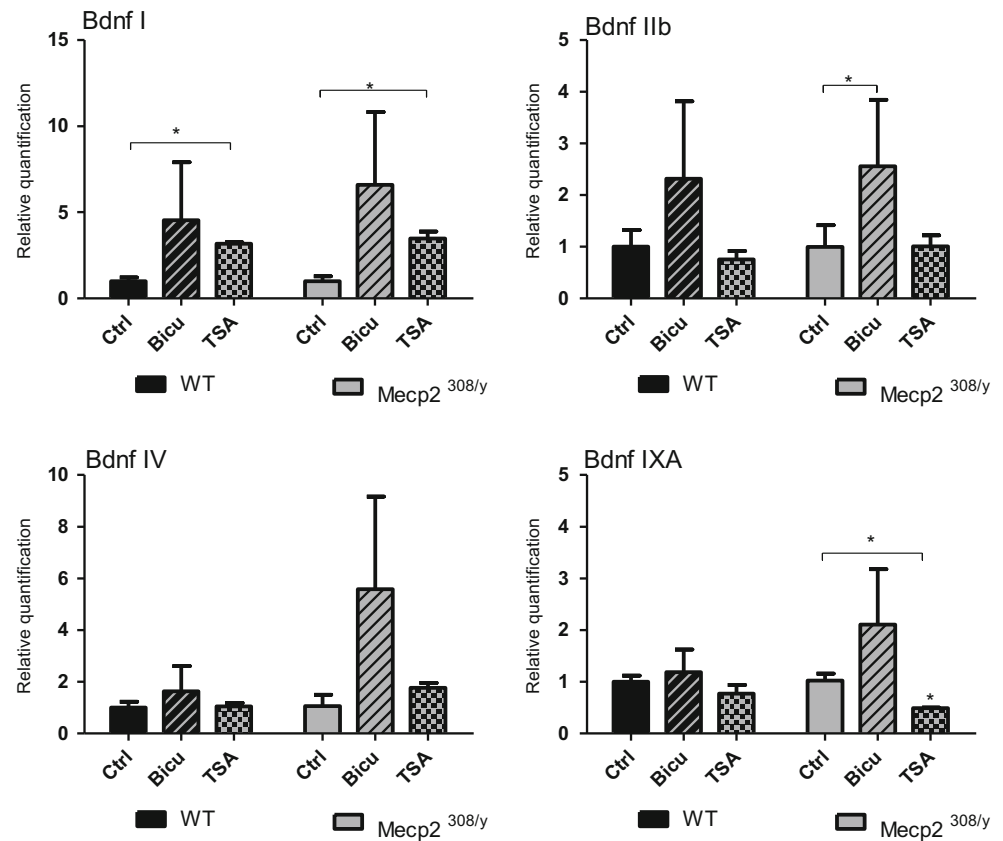
of *Bdnf* transcripts I and IXA ($p < 0.05$) (Fig. 3a). Moreover, in neurons at 9 DIV from *Mecp2*^{308/y} cortex, *Bdnf* transcripts I, IIb, and IV tend or were significantly overexpressed by bicuculline treatment ($p = 0.07$, $p < 0.01$, and $p = 0.07$, respectively) (Fig. 4).

Effect of Chromatin Remodeling Induced by Histone Deacetylase Inhibition on *Bdnf* Expression Pattern

Previous reports have shown that histone deacetylases (HDACs) participate in gene silencing via deacetylation of

histones H3 and H4 (Nan et al. 1998). After HDAC dissociation, histone acetylation becomes more evident within *Bdnf* exon IV, which facilitates its transcription (Martinowich et al. 2003). At 20 DIV, trichostatin A (TSA), an inhibitor of histone deacetylases, significantly increased level of *Bdnf* transcript VI in wild-type and *Mecp2*-deficient hippocampal neurons ($p < 0.05$), but significantly reduced expression levels of *Bdnf* transcripts IIb and IIc ($p < 0.05$) (Fig. 4). However, TSA treatment did not affect expression levels of *Bdnf* transcripts IIb, IIc, IV, and VI in wild-type and *Mecp2*^{308/y} cortical neurons at 9 DIV (IIb, IV, Fig. 4; IIc, VI, Fig. 3S), and in contrast

Fig. 4 Effect of bicuculline (*bicu*) and trichostatin A (*TSA*) on *Bdnf* transcription in wild-type (*WT*) and *Mecp2*^{308/y} cortical neurons: real-time RT-quantitative PCR analysis showing the relative changes of *Bdnf* transcripts I, IIb and IV and IXA after 1 and 19-h exposure to bicuculline and trichostatin A, respectively. *Ctrl* control as untreated cells. *Bars* represent means \pm SEM. Statistical analysis was determined by analysis of variance (*ANOVA*). Asterisk indicates $p < 0.05$



significantly increased level of *Bdnf* transcripts I compared to untreated wild-type or *Mecp2*^{308/y} neurons (Fig. 4). We also noted that in *Mecp2*^{308/y} cortical neurons, TSA treatment led to a significant decrease in expression levels of *Bdnf* transcript IXA ($p = 0.05$) (Fig. 4).

Interestingly, we observed that expression levels of *Bdnf* transcripts I and IXA, regulated by bicuculline in *Mecp2*^{308/y} hippocampal neurons, were not affected by histone deacetylase inhibition (Fig. 3a). In contrast, *Bdnf* transcripts (IIb, IIc, and VI), regulated by histone deacetylation, were not affected by bicuculline treatment in wild-type and *Mecp2*^{308/y} hippocampal neurons (Fig. 3b, c).

Discussion

It has long been believed that the *Mecp2* protein was expressed exclusively in neurons and was not expressed in glial cells. Rett syndrome was thus regarded as a purely neuronal pathology (Shahbazian and Zoghbi 2002). Recently, several groups have also demonstrated the expression of *Mecp2* in glial cells, particularly in astrocytes. It was also shown that co-culture of *Mecp2*-deficient astrocytes with wild-type neurons resulted in a disruption of dendritic arborization, suggesting a non-cell autonomous effect and the

astrocytes involvement in the pathophysiology of Rett syndrome (Ballas et al. 2009; Maezawa et al. 2009). *Mecp2* is currently considered as a modulator of gene transcription and the *Bdnf* gene is one of the few genes to have been described as a target gene in a reproducible manner (Maezawa et al. 2009; Chang et al. 2006). Although the regulation of *Bdnf* expression has been explored through several studies, the regulation of this gene in glial cells was not studied with enthusiasm. Only few studies have investigated the consequences of MeCP2 deficiency on the complexity of the *Bdnf* gene transcription in both neurons and astrocytes. One recent work showed a higher *Bdnf* mRNA level in the hippocampus of *Mecp2*^{308/y} mice as compared to wild-type littermates (De Filippis et al. 2010). However, Zoghbi and collaborators previously reported that quantitative analysis of *Bdnf* expression in the hippocampus of wild-type and *Mecp2*^{308/y} mice did not reveal significant abnormalities (Moretti et al. 2006).

In this study, we show for the first time the *Bdnf* expression pattern in both neurons and astrocytes from wild-type and *Mecp2*^{308/y} mice, and the effect of bicuculline, and TSA-induced chromatin remodeling on this expression pattern.

Firstly, we studied the expression of *Bdnf* isoforms and observed that the major transcripts are *Bdnf* transcripts I, IIb, IIc, and IV in neurons. However, *Bdnf* expression pattern varied depending on the brain structure (cortex and hippocampus) and the type of the neural cell (neurons and astrocytes). In

hippocampal astrocytes, *Bdnf* transcript VI is the most expressed isoform. Our results are thus in agreement with the previous study of Aid and colleagues showing a tissue-specific and developmental regulation of the expression of the different *Bdnf* transcripts (Aid et al. 2007). The *Bdnf* expression pattern we identified from P0 astrocyte culture is similar to the one obtained from P1 mouse brains with a predominance of *Bdnf* transcripts IIc, IV, VI, and IXA (Aid et al. 2007).

Secondly, we studied *Bdnf* isoforms expression in the model of MeCP2-deficient mice (*Mecp2*^{308/y}) characterized by the production of a nonfunctional truncated MeCP2 protein. Previously, De Filippis and colleagues showed an overexpression in total *Bdnf* transcript in the hippocampus of *Mecp2*^{308/y} mice (De Filippis et al. 2013), but the differential expression of *Bdnf* isoforms had never been previously studied in this model. In basal conditions, our results show a significant increase only in the expression of *Bdnf* transcript VI in *Mecp2*-deficient cortical neurons compared to wild-type cells. Our results are thus in agreement with the model of MeCP2 as a transcriptional repressor initially described by Nan et al. (Nan et al. 1998; Li and Pozzo-Miller 2014) specifically for *Bdnf* transcript VI. Because we did not find significant changes in the expression of the *Bdnf* transcript IV, we can suggest that in the absence of membrane depolarization, the MeCP2 truncated protein (*Mecp2*³⁰⁸) may be able to bind to the *Bdnf* IV promoter and prevent its expression (Ballas et al. 2009; Chen et al. 2003).

The mechanisms regulating *Bdnf* expression by MeCP2 have been the subject of a vast literature and two complementary models have been described (Zheng et al. 2012; Li and Pozzo-Miller 2014). The first model involves a neuronal activation induced by ionic Ca²⁺, triggering MeCP2 phosphorylation at position 421 (Ser421) and its release from *Bdnf* promoter IV, and subsequent binding of the phosphorylated cAMP response element-binding protein (CREB) at Ser133, required for *Bdnf* expression (Chen et al. 2003). The second model suggests that neuronal depolarization decreased methylation of CpG sites within *Bdnf* promoter IV (Martinowich et al. 2003). Such demethylation triggers the dissociation of MeCP2 and its corepressors (e.g., Sin3A and HDAC1) from *Bdnf* promoter IV, and CREB's affinity for this *Bdnf* regulatory region is then correspondingly increased, reflecting another gating mechanism. However, experimental data obtained by using pharmacological inhibitor of DNA methyltransferases showed varying effects depending on transcripts and cell type (Aid et al. 2007; Lubin et al. 2008; Nelson et al. 2008).

To study the effect of cell activation on the *Bdnf* expression pattern, we treated cortical and hippocampal neurons by the pharmacological agent bicuculline, able to inhibit the GABA A receptors. This treatment induced a significant increase of *Bdnf* transcript I and IXA in *Mecp2*^{308/y} hippocampal neurons, and *Bdnf* transcripts I, IIb, and IV only in *Mecp2*^{308/y} cortical neurons. These results suggest that the regulation of

the expression of few *Bdnf* transcripts is dependent of neuronal activity by mechanisms dependent and independent of MeCP2.

Finally, given the involvement of histone deacetylases in gene silencing via deacetylation of histones H3 and H4 (Jones et al. 1998; Nan et al. 1998; Martinowich et al. 2003), we studied the effect of the inhibitor of HDACs activity trichostatin A (TSA), on the *Bdnf* expression pattern in wild-type and mutated cells. Our results showed a significant overexpression of *Bdnf* transcript I in wild-type and mutated cortical neurons after treatment and *Bdnf* transcript VI in wild-type and mutated hippocampal neurons. In contrast, in wild-type and mutated hippocampal neurons, inhibition of HDACs resulted in a surprisingly decreased expression of *Bdnf* transcripts IIb and IIc. Our results may suggest a histone acetylation-dependent regulation of the promoter regions of *Bdnf* transcripts I and VI. Consistent with this view, a pharmacological DNMT inhibitor differentially activated *Bdnf* regulatory regions: exons I and III in cultured neuro2A neuroblastoma cells (Aid et al. 2007), exons I, IV, and VI in rat hippocampus (Lubin et al. 2008). The mechanism must take place in the presence or absence of functional MeCP2 protein.

Moreover, previous reports showed that the inhibitor of histone deacetylase activity TSA has differential effects on *Bdnf* promoters. Levels of *Bdnf* transcript containing exon I increased in a dose-dependent manner, producing up to a 20-fold change at 250 nM for 24 h over untreated rat hippocampal neurons. However, levels of *Bdnf* exon IV increased only 2-fold at the same concentration (or no effect at other concentrations 100 or 500 nM) suggesting that the regulatory regions for exons I and IV respond differently to an HDAC inhibitor, and that different mechanisms are responsible for altering chromatin structure for each of these promoters in response to NMDA (Tian et al. 2010). Taken together, these data confirm that the chromatin conformation modulated by histone acetylation state could regulate the expression of *Bdnf* transcripts differently depending on the cell type and brain structure. Interestingly, in hippocampal neurons, when neuronal activity regulates the expression of specific *Bdnf* transcripts (I and IXA), chromatin conformation modulated by histone acetylation state has no influence.

In conclusion, many studies have shown that altered regulation of the expression of *Bdnf* dependent particularly from neuronal activity contributes to neuronal phenotype observed in Rett syndrome such as abnormalities of dendritic arborization (Chen et al. 2001; Kishi and Macklis 2004) and synaptic plasticity (Zhou et al. 2006; Moretti et al. 2006; Larimore et al. 2009). In the absence of MeCP2, *Bdnf* transcript expression is thus increased. However, the level of the Bdnf protein is reduced in the brain of *Mecp2*-deficient mice and in patients with Rett syndrome, and overexpression of Bdnf corrects cell phenotype in *Mecp2*-deficient neurons (Chang et al. 2006; Larimore et al. 2009). The complexity of the transcriptional

regulation of *Bdnf* could participate in this apparent discrepancy. While the vast majority of studies published to date have focused on the *Bdnf* transcript IV, other minor and major *Bdnf* transcripts such as *Bdnf*I and VI could play a leading role. For example, in mature neurons, the *Bdnf* transcript I promoter is the target of the repressor RE1-silencing transcription factor (REST). In absence of *Mecp2*, the *REST* gene is overexpressed and blocks the induction of transcription linked to neuronal activation (Abuhatzira et al. 2007). This *Bdnf* transcript I, regulated by activity and chromatin conformation, could play an important role in neuronal phenotype observed in patients with Rett syndrome. Moreover, given that more recent research suggests that the subcellular localization of a *Bdnf* transcript is likely based on its specific upstream non-coding exon, it has been found that *Bdnf* transcripts containing non-coding exons II and VI appear to be localized in both the soma and the proximal dendrites of several classes of rat cortical and hippocampal neurons, while *Bdnf* transcripts containing non-coding exons I and IV remain restricted to the soma only (Aliaga et al. 2009; Chiaruttini et al. 2009; Pattabiraman et al. 2005). Our data suggest that in *Mecp2*^{308/y} neurons, neuronal activity causes an increase of *Bdnf* transcripts containing either exons I, IIb, and IXA, and may affect specifically the distribution of *Bdnf* mRNA in cellular compartments.

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