

# A Role for Diminished GABA Transporter Activity in the Cortical Discharge Phenotype of MeCP2-Deficient Mice

Liang Zhang<sup>1,2,3</sup>, Robert G Wither<sup>4,5</sup>, Min Lang<sup>2,4,5</sup>, Chiping Wu<sup>1,4</sup>, Elena Sidorova-Darmos<sup>4,5</sup>, Hristo Netchev<sup>4</sup>, Catherine B Matolcsy<sup>4</sup>, Orlando Carter Snead<sup>2,3</sup> and James H Eubanks<sup>\*,2,4,5,6</sup>

<sup>1</sup>Division of Fundamental Neurobiology, Toronto Western Research Institute, University Health Network, Toronto, ON, Canada; <sup>2</sup>University of Toronto Epilepsy Research Program, University of Toronto, Toronto, ON, Canada; <sup>3</sup>Department of Medicine (Neurology), University of Toronto, Toronto, ON, Canada; <sup>4</sup>Division of Genetics and Development, Toronto Western Research Institute, University Health Network, Toronto, ON, Canada; <sup>5</sup>Department of Physiology, University of Toronto, Toronto, ON, Canada; <sup>6</sup>Department of Surgery (Neurosurgery), University of Toronto, Toronto, ON, Canada

Cortical network hyper-excitability is a common phenotype in mouse models lacking the transcriptional regulator methyl-CPG-binding protein 2 (MeCP2). Here, we implicate enhanced GABA<sub>B</sub> receptor activity stemming from diminished cortical expression of the GABA transporter GAT-1 in the genesis of this network hyper-excitability. We found that administering the activity-dependent GABA<sub>B</sub> receptor allosteric modulator GS-39783 to female *Mecp2*<sup>+/-</sup> mice at doses producing no effect in wild-type mice strongly potentiated their basal rates of spontaneous cortical discharge activity. Consistently, administering the GABA<sub>B</sub> receptor antagonist CGP-35348 significantly decreased basal discharge activity in these mice. Expression analysis revealed that while GABA<sub>B</sub> or extra-synaptic GABA<sub>A</sub> receptor prevalence is preserved in the MeCP2-deficient cortex, the expression of GAT-1 is significantly reduced from wild-type levels. This decrease in GAT-1 expression is consequential, as low doses of the GAT-1 inhibitor NO-711 that had no effects in wild-type mice strongly exacerbated cortical discharge activity in female *Mecp2*<sup>+/-</sup> mice. Taken together, these data indicate that the absence of MeCP2 leads to decreased cortical levels of the GAT-1 GABA transporter, which facilitates cortical network hyper-excitability in MeCP2-deficient mice by increasing the activity of cortical GABA<sub>B</sub> receptors.

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## INTRODUCTION

Methyl-CPG-binding protein 2 (*MECP2*) is a ubiquitously expressed epigenetic factor that regulates normal transcriptional activity. Loss-of-function mutations of *MECP2* can cause Rett syndrome, which is one of the leading genetic causes of severe intellectual disability in females (Amir *et al*, 1999). In addition to Rett syndrome, alterations in normal MeCP2 prevalence or function have also been implicated in a host of neurodevelopmental conditions (Neul, 2012; Van Esch *et al*, 2005). Given the commonality of involvement of altered MeCP2 function in this spectrum of neural disorders, delineating the mechanisms through which MeCP2 dysfunction leads to the development of specific neurological phenotypes may shed light upon pathogenesis cascades common to a number of genetic and non-genetic conditions that share overlapping phenotypes.

To facilitate such investigations, several mouse models have been developed that either lack MeCP2 or express a clinically-relevant mutant form of MeCP2 (Katz *et al*, 2012). These mice display a constellation of behavioral and neurological deficits; many of which mirror symptoms also seen in patients with different types of *MECP2* mutations (Katz *et al*, 2012). One feature commonly associated with the absence of MeCP2 is neural network hyper-excitability (Zhang *et al*, 2008; Calfa *et al*, 2011; Ward *et al*, 2011; McLeod *et al*, 2013). We have previously reported rhythmic cortical spike and wave EEG discharges in both male and female mice expressing a null *Mecp2* allele (D’Cruz *et al*, 2010; Wither *et al*, 2012; Lang *et al*, 2014). Shahbazian *et al* (2002) also reported myoclonic jerks coupled with high-amplitude bilateral cortical spike and wave discharges in the *Mecp2*<sup>308/y</sup> mouse, and Goffin *et al* (2011) reported evidence for cortical network hyper-excitability in the *Mecp2*<sup>T158A/y</sup> mouse model. There are also circuitry-specific components to the genesis of these discharges. Ward *et al* (2011) showed cortical network hyper-excitability in mice globally lacking MeCP2, but not in mice selectively lacking MeCP2 from HOX-B1 hindbrain neurons, whereas Goffin *et al* (2014) showed network hyper-excitability in mice lacking MeCP2 only in forebrain inhibitory neurons, but not

\*Correspondence: Dr J Eubanks, Toronto Western Hospital, 8KD-417, 60 Leonard Avenue, Toronto, ON M5T 2S8, Canada, Tel: +1 416 603 5800, ext. 2933, Fax: +1 416 603 5745, E-mail: jeubanks@uhnres.utoronto.ca

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in mice selectively lacking MeCP2 in excitatory forebrain neurons. These latter data suggest that GABAergic circuits may be instrumental for defining network hyper-excitability in the MeCP2-deficient brain, but to date, the underlying mechanisms for how hyper-excitability neural networks arise in these models remain poorly understood. Using cortical discharge activity as an index of network hyper-excitability, our present study investigates how specific GABAergic signaling mechanisms influence the genesis and regulation of cortical network hyper-excitability in MeCP2-deficient mice. Our results indicate that enhanced GABA<sub>B</sub> receptor activity stemming at least in part from decreased cortical expression of the GABA transporter GAT-1 have a key role in the genesis of MeCP2-deficient cortical network hyper-excitability.

## MATERIALS AND METHODS

### Ethics Statement

All animal experimentation was conducted in accordance with the guidelines of the Canadian Council of Animal Care, and the work was thoroughly reviewed and approved by local animal care committees before initiation (UHN Animal Use Protocol 1321.7).

### Animal Subjects

Female and male *Mecp2*<sup>tm1.1Bird</sup> mice (Guy *et al*, 2001) were used in this study. These animals were obtained from Jackson Laboratories (Bar Harbor, ME, USA) or bred in-house, and maintained on a pure C57Bl/6J background. Male and female mice were subjected to the experimental procedures described below when mice displayed clear Rett-like behavioral phenotypes (Katz *et al*, 2012); male *Mecp2*<sup>tm1.1Bird</sup> mice were studied between 60 and 80 days, and female *Mecp2*<sup>tm1.1Bird</sup> mice were studied between 14 and 18 months of age. Gender and age-matched wild-type mice on a pure C57Bl/6J background served as controls. Genotyping of these mice was done via PCR as described previously (Guy *et al*, 2001; Jugloff *et al*, 2008). All animals were housed in a vivarium that was maintained between 22 and 23 °C, and employed a standard 12-h light on/off cycle commencing at 0600 h.

### Implantation Surgery

Experimental and control animals were implanted with polyimide-insulated stainless steel microelectrodes (125 μm) in somatosensory cortex (Bregma, -0.8 mm; lateral, 1.8 mm; depth, 1.5 mm) following the procedures outlined previously (Wu *et al*, 2008; D'Cruz *et al*, 2010; Lang *et al*, 2013). The reference electrode was implanted superficially into the frontal brain area (Bregma, +2.8 mm; lateral, 1.8 mm; depth, 0.5 mm). Following implantation, animals were allowed at least 7 days of post-surgical recovery before further experimentation.

### EEG Recording and Analysis

EEG recordings were collected as described previously (D'Cruz *et al*, 2010; Lang *et al*, 2013), and data analyzed using the pCLAMP software (Molecular Devices, Sunnyvale,

CA, USA). All EEG recordings were made from freely moving mice for different periods of time. For female *Mecp2*<sup>+/−</sup> mice, the recording sessions were 2 h long on consecutive days. For male *Mecp2*<sup>−/−</sup> mice, discharge profiles were assessed for a minimum of 1 h per day, with at least 1 week between sessions. For pharmacological characterization, baseline EEG activities were collected for ~30 min before drug administration, and the drug effects evaluated for 1 h following administration. A washout period of at least 2 days was employed before a subject was administered any other pharmacological compound.

### Characterization of Epileptiform Discharge Events

Two independent investigators blinded to experimental conditions visually examined EEG traces, and their individual counts were averaged. Concordance rates between the two counters were greater than 90%. Excitatory discharge events were defined as rhythmic spike waveforms, having amplitudes of at least 1.5-fold background, durations of at least 0.4 s, and frequencies between 6 and 10 Hz (D'Cruz *et al*, 2010; Wither *et al*, 2012, 2013; Lang *et al*, 2013, 2014). Long discharge events were defined similarly to typical discharge events, but required a minimum duration of 5 s and an average frequency of between 4 and 6 Hz. The frequency of discharges was determined by generating spectral plots using the Fast Fourier Transformation (FFT) function in pCLAMP (Molecular Devices) with rectangular binning, 50% window overlap, and a spectral resolution of 0.5 Hz.

### Drugs and Chemicals

Unless specified otherwise, all general reagents and buffers were obtained from Sigma-Aldrich (Mississauga, ON, Canada). The drugs and dosages used in this study were CGP-35348 (Tocris, Bristol, UK) at 100 mg/kg; GS-39783 (Tocris) at 2.5 and 5 mg/kg; 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (THIP) (Sigma-Aldrich) at 5 mg/kg; and NO-711 (Sigma-Aldrich) at 1 and 2.5 mg/kg. All drugs were diluted in saline and administered intraperitoneally to the animals.

### Immunoblotting

Mice were killed by isoflurane overdose, and their brains isolated rapidly on ice. Cerebral cortices were isolated and frozen on dry ice before being partially thawed, and homogenized by sequential passage through 20, 23, and 25½ gauge syringe needles in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a mixture of protease inhibitors (PMSF 40 ng/ml, Antipain 2 ng/ml, Pepstatin A 2 ng/ml, Leupeptin 20 ng/ml, Aprotinin 20 ng/ml, and MDL28170 20 ng/ml). The crude homogenate was centrifuged at 12 000 *g* for 5 min to remove insoluble material, and aliquots of the remaining supernatant collected and stored at -80 °C until use. Protein concentrations were determined using the Folin method (Bio-Rad, Hercules, CA, USA; Cat #500-0116). SDS-PAGE and western blot procedures were conducted as described previously (Asaka *et al*, 2006; Wither *et al*, 2013; Sidorova-Darmos *et al*,

2014) using the following antibodies and dilutions: anti-GAT-1 (1:1000; Abcam, Cambridge, MA, USA; Cat #ab426), anti-GABA<sub>A</sub> receptor delta subunit (1:1000, Phosphosolutions, Aurora, CO, USA; Cat #868-GDN), anti-GABA<sub>B</sub> receptor R2 subunit antibodies (1:1000; Millipore, Temecula, CA, USA; Cat #AB2255; and 1:200; Alomone Labs, Jerusalem, Israel; Cat #AGB-002), and anti-GAPDH (1:15 000; Chemicon, Billerica, MA, USA; Cat #MAB374). The HRP-linked secondary antibodies employed were goat-anti-rabbit (1:5000; GE Healthcare, Buckinghamshire, UK; Cat #NA934), goat-anti-mouse (1:5000; GE Healthcare; Cat #NA931) or donkey-anti-guinea-pig (1:5000; Jackson ImmunoResearch, West Grove, PA, USA; Cat #AP193P). Immunoreactivity was visualized by enhanced chemiluminescence (GE Healthcare, Cat #RPN2106).

### Immunoblot Quantification and Analysis

Immunoreactive protein levels were determined using densitometric analysis as described previously (Asaka *et al*, 2006; Sidorova-Darmos *et al*, 2014). In brief, blots were scanned and visualized using Fluor-S MultiImager (Bio-Rad), and the density of the bands was determined using the rectangular density function in the Quantity One (Bio-Rad) analysis program. Film background was subtracted from the calculated specific immunoreactive product to obtain the overall protein density values. Specific target protein levels were then normalized to their corresponding GAPDH protein levels to account for any minor protein loading discrepancies.

### Statistical Analysis

Student's *t*-tests were used for direct comparisons between two normally distributed groups. For comparisons between multiple groups, one-way ANOVA with Bonferroni *post hoc* correction for multiple comparisons was used. Significance was set at  $p < 0.05$ . Mean and standard error of the mean are presented throughout the text and figures.

## RESULTS

### Spontaneous Excitatory Discharge Activity Differs Between Male and Female MeCP2-Deficient Mice

Cortical EEG recordings were used to identify spontaneous discharge activity in male and female MeCP2-deficient mice. Recordings from 14- to 18-month female *Mecp2*<sup>+/-</sup> mice revealed the presence of one predominant form of excitable discharge activity in all of the mice examined ( $n = 24$ ). These discharges had an average incidence rate of  $95.3 \pm 7.7$  events per hour, an average duration of  $1.1 \pm 0.1$  s, and an average frequency of  $7.7 \pm 0.3$  Hz (Figure 1 and Table 1). In contrast to the single discharge population seen in female *Mecp2*<sup>+/-</sup> mice, however, *Mecp2*<sup>-/-</sup> male mice of 50–60 days age ( $n = 8$ ) displayed two distinct discharge populations: a population of short duration discharges similar to those of female *Mecp2*<sup>+/-</sup> mice, and a distinct population of longer duration discharges not observed in any of the female *Mecp2*<sup>+/-</sup> mice (Figure 1 and Table 1). The short-duration discharge population had an average incidence rate of

$41.2 \pm 8.8$  per hour, an average duration of  $0.8 \pm 0.1$  s, and an average frequency of  $6.1 \pm 0.3$  Hz. The novel 'long-duration' discharges were present in seven of eight *Mecp2*<sup>-/-</sup> male mice, and had an average duration of  $72.8 \pm 14.0$  s ( $p < 0.0001$  relative to 'short duration' discharges in female *Mecp2*<sup>+/-</sup> mice, Table 1). The incidence rate of the 'long-duration' discharge events was  $2.5 \pm 0.7$  events per hour, which is significantly less frequent than that of the 'short' events seen in the same mice ( $p = 0.0012$ , Table 1). The average frequency of the 'long' discharge waveforms was also significantly slower than that of the 'short' discharges ( $5.5 \pm 0.3$  Hz;  $p = 0.047$ , Table 1). These discharge activities were specific to MeCP2-deficient mice, as no cortical spike and wave discharge activity having at least 0.5 s duration was observed in any of the 10 male or 22 female wild-type mice examined (Table 1).

### Enhancing GABA<sub>B</sub> Receptor Activity Increases Cortical Epileptiform Discharges in *Mecp2*<sup>+/-</sup> Mice

Given the network hyper-excitability phenotype observed in mice selectively lacking MeCP2 in forebrain GABAergic neurons (Goffin *et al*, 2014), we examined how altering specific GABA-related signaling components would affect cortical excitatory discharge activity. We first tested whether stimulating GABA<sub>B</sub> receptors would affect network hyper-excitability using the GABA<sub>B</sub> receptor-positive allosteric modulator GS-39783, whose modulatory actions only affect GABA<sub>B</sub> receptors upon activation by endogenously released GABA (Pin and Prézeau, 2007; Froestl, 2010). At 2.5 mg/kg, GS-39783 failed to induce cortical discharge activity in 14 of the 15 female wild-type mice examined (Figure 1). This same dose, however, elevated the discharge incidence rate of female *Mecp2*<sup>+/-</sup> mice from a baseline of  $88.5 \pm 10.8$  to  $143.9 \pm 14.8$  discharge events per hour ( $p = 0.0014$ ;  $n = 14$ , Figure 4), but did not significantly alter the duration or waveform frequency of the discharges (Figure 4). The administration of a higher dose of GS-39783 (5 mg/kg) similarly increased the cortical discharge incidence rate in *Mecp2*<sup>+/-</sup> mice from a baseline of  $68.2 \pm 7.5$  to  $124.4 \pm 15.4$  discharges per hour ( $p = 0.0002$ ,  $n = 14$ , Figure 4), and also lengthened the average discharge duration (to  $1.6 \pm 0.1$  s post-GS-39783 from  $1.2 \pm 0.1$  s baseline,  $p = 0.002$ , Figure 4), and slowed discharge waveform frequency (from  $8.1 \pm 0.2$  Hz to  $7.7 \pm 0.2$  Hz,  $p = 0.03$ , Figure 4). Interestingly, this same 5 mg/kg dose of GS-39783 was sufficient to induce short discharge-like events in all female wild-type mice tested ( $n = 8$ , Figure 1) that had discharge incidence rates ( $42.8 \pm 11.0$  per hour), durations ( $1.1 \pm 0.1$  s), and frequencies ( $6.0 \pm 0.4$  Hz) that were similar to those seen in female *Mecp2*<sup>+/-</sup> mice under baseline conditions (Figure 1 and Table 1).

### Enhancing Extra-Synaptic GABA<sub>A</sub> Receptor Activity Decreases Cortical Excitatory Discharge Patterns in Female *Mecp2*<sup>+/-</sup> Mice

The enhancement of discharge activity with GS-39783, which requires GABA<sub>B</sub> receptors to be bound by GABA for its potentiating effects, suggests that GABA levels may be elevated in the *Mecp2*<sup>+/-</sup> mouse cortex. This is intriguing, as both the excessive stimulation of GABA<sub>B</sub> receptors

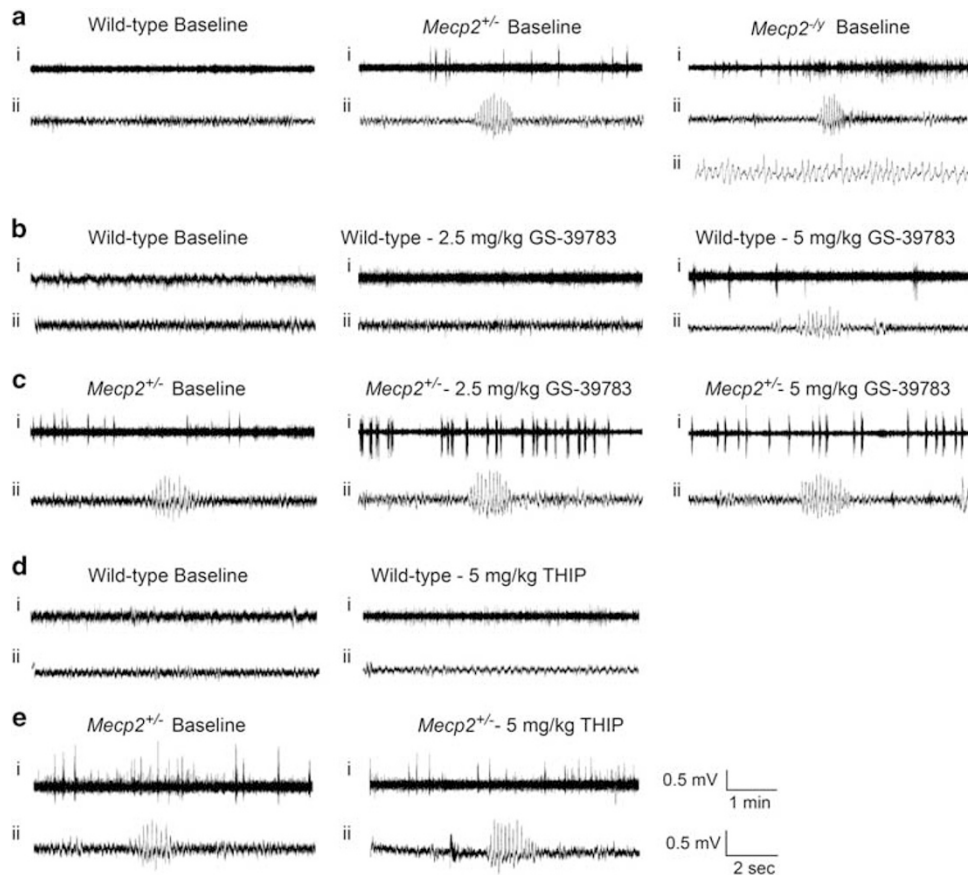
(Snead, 1992) and the overstimulation of tonic extra-synaptic GABA<sub>A</sub> receptors can each induce cortical discharge activity in a variety of rodent seizure models (Cope *et al*, 2009). To test whether selective stimulation of extra-synaptic GABA<sub>A</sub>

receptors would similarly exacerbate discharge activity, we administered THIP, which is a selective agonist for the GABA<sub>A</sub> receptor  $\delta$ -subunit found exclusively at extra-synaptic locations (Nusser *et al*, 1998; Brown *et al*, 2002).

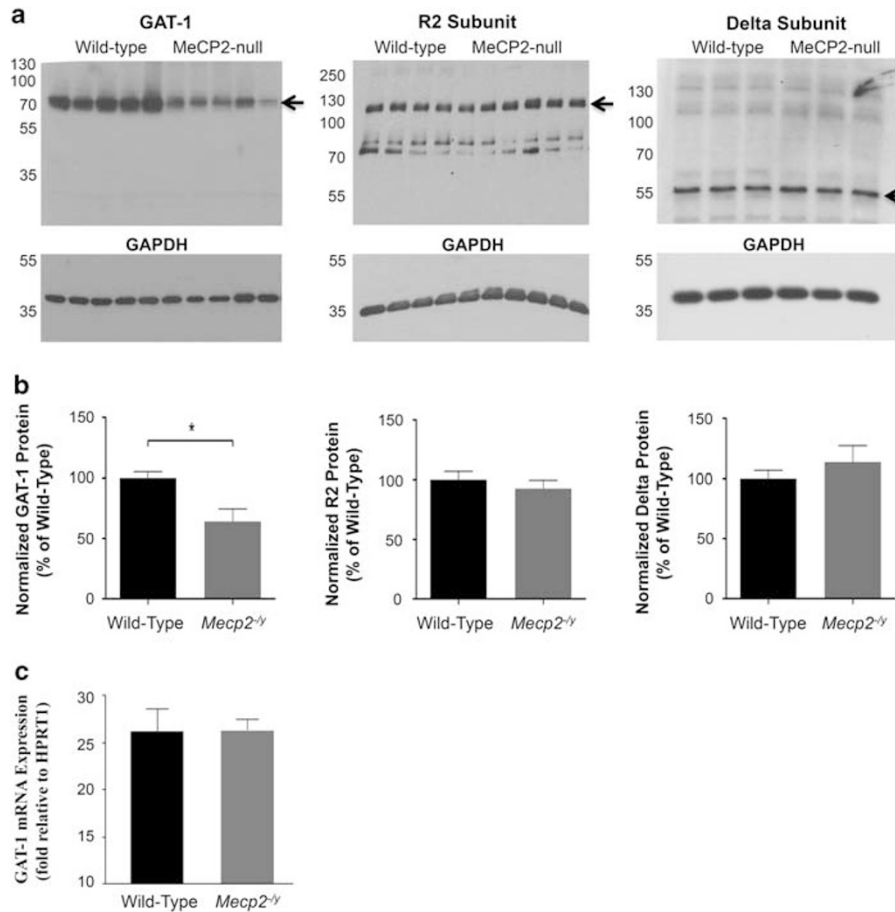
**Table 1** Epileptiform Discharge Properties Differ Between Male *Mecp2*<sup>-/-</sup> and Female *Mecp2*<sup>+/-</sup> Mice

Genotype	Discharge type	Discharges per hour	Discharge duration (s)	Discharge frequency (Hz)
14- to 18-Month-old female wild type (n=22)	None	0	NA	NA
14- to 18-Month-old female <i>Mecp2</i> <sup>+/-</sup> (n=24)	Short	95.3 ± 7.7	1.1 ± 0.1	7.6 ± 0.3
60- to 80-Day-old male wild type (n=10)	None	0	NA	NA
60- to 80-Day-old male <i>Mecp2</i> <sup>-/-</sup> (n=8)	Short	41.2 ± 8.8*	0.8 ± 0.1	6.1 ± 0.3*
60- to 80-Day-old male <i>Mecp2</i> <sup>-/-</sup> (n=7)	Long	2.5 ± 0.7	72.8 ± 14.0	5.5 ± 0.3

Summary table of baseline incidences, durations, and frequency components of epileptiform discharge events observed in wild-type male, wild-type female, *Mecp2*<sup>-/-</sup> male, and *Mecp2*<sup>+/-</sup> female mice. The incidence rate for 'short duration' discharge activity, and its average discharge frequency, differs significantly between male and female mutants (denoted by asterisk,  $p = 0.0003$  and  $p = 0.0099$ , respectively; unpaired *t*-test). In contrast, the average duration of the short discharge events did not significantly differ between male and female mutants ( $p = 0.989$ ). The parameters for the 'long duration' discharge events seen exclusively in male *Mecp2*<sup>-/-</sup> mice differed significantly from either the 'short duration' events present in the same male *Mecp2*<sup>-/-</sup> mice or the 'short duration' events present in 14- to 18-month-old female *Mecp2*<sup>+/-</sup> mice. No spike and wave discharges were observed in any of the male or female wild-type mice examined.



**Figure 1** The GABA<sub>B</sub> receptor-positive allosteric modulator GS-39783 exacerbates epileptiform discharge activity in female *Mecp2*<sup>+/-</sup> mice, whereas the extra-synaptic GABA<sub>A</sub> receptor agonist THIP attenuates discharge activity. (a) (i) Representative 5 min cortical EEG traces, (ii) expanded 10 s trace segments from a 14-month-old female wild-type mouse (left); a 14-month-old heterozygous female MeCP2-deficient mouse depicting typical short-duration discharge events (middle); and a 65-day-old male MeCP2-null mouse depicting both short-duration (ii) and long-duration (iii) discharge events (right). (b, c) Representative 5 min (i) and 10 s (ii) cortical EEG traces from 14- to 18-month-old female wild-type and *Mecp2*<sup>+/-</sup> mice under basal conditions (left panels) and following a 2.5-mg/kg dose (middle panels) or a 5.0-mg/kg dose (right panels) of the GABA<sub>B</sub> receptor-positive allosteric modulator GS-39783. Note the 2.5-mg/kg GS-39783 dose exaggerated discharge activity in female *Mecp2*<sup>+/-</sup> mice without affecting wild-type mice, while at 5.0 mg/kg GS-39783 induced short duration discharge events in wild-type mice. (d, e) Representative EEG traces from wild-type and *Mecp2*<sup>+/-</sup> mice following administration of 5 mg/kg of the extra-synaptic GABA<sub>A</sub> receptor  $\delta$ -subunit agonist THIP. This concentration of THIP had no effect on EEG activity in wild-type mice, and attenuated discharge activity in female *Mecp2*<sup>+/-</sup> mice.



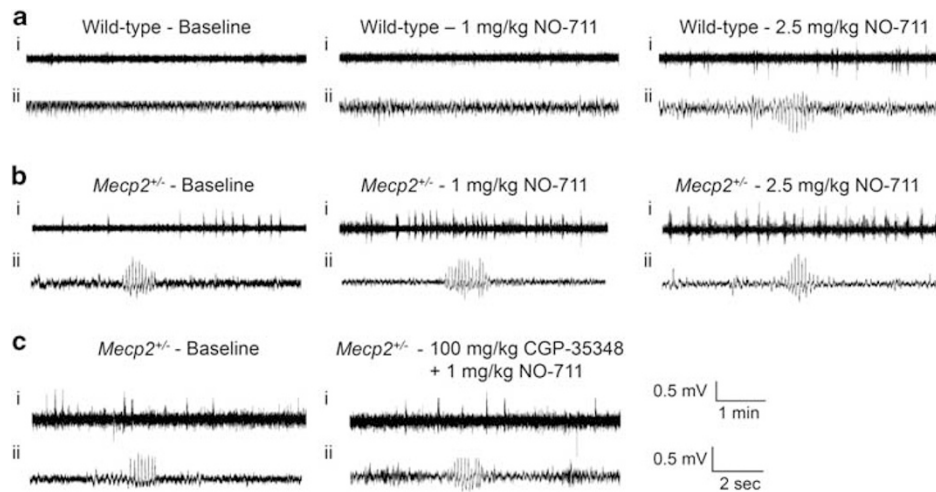
**Figure 2** GAT-1 protein levels, but not GABA<sub>B</sub> receptor R2 subunit or GABA<sub>A</sub> receptor delta subunit levels, are diminished in the *Mecp2*<sup>-/-</sup> mouse cortex. (a) Representative western blots illustrating the relative immunoreactive levels of GAT-1 (left blot), the R2 subunit of GABA<sub>B</sub> receptors (middle blot), and the delta subunit of extra-synaptic GABA<sub>A</sub> receptors (right blot) in cortical tissue extracted from 60- to 80-day-old male wild-type and *Mecp2*<sup>-/-</sup> mice. Arrows denote the product of the expected mass for each respective antibody. The level of GAPDH protein for each sample is shown as a protein loading control. (b) Histograms presenting the cumulative mean ± SEM of the densitometric intensity data for examining GAT-1 (*n* = 13 wild-type and *n* = 15 *Mecp2*<sup>-/-</sup>; 3 independent blots); the GABA<sub>B</sub> receptor R2 subunit (*n* = 12 wild-type and *n* = 15 *Mecp2*<sup>-/-</sup>; 3 independent blots), and the delta subunit of extra-synaptic GABA<sub>A</sub> receptors (*n* = 10 wild-type and *n* = 10 *Mecp2*<sup>-/-</sup>; 2 independent blots) normalized to their corresponding GAPDH levels. In all histograms, the wild-type values have been set to 100%. Asterisk denotes statistical significance (*p* < 0.05, rank-sum test). (c) Histogram presenting the mean ± SEM for GAT-1 mRNA levels as determined by real-time PCR. The values represent fold differences in GAT-1 expression in wild-type (*n* = 3) and male *Mecp2*<sup>-/-</sup> (*n* = 3) cortical samples relative to the expression of the internal reference *HPRT1* gene.

In female wild-type mice (*n* = 15), the administration of 5 mg/kg THIP failed to promote any discernible discharge activity (Figure 1) and had no overt sedative effects. In 14- to 18-month-old female *Mecp2*<sup>+/-</sup> mice (*n* = 12), however, this same dose of THIP significantly decreased the cortical discharge incidence rate from a baseline of 71.8 ± 12.2 to 25.8 ± 6.4 discharges per hour (*p* = 0.0002; Figures 1 and 4), without significantly affecting either the discharge duration or the waveform frequency (Figures 1 and 4).

#### GAT-1 Protein Levels, but not GABA<sub>B</sub> or Extra-Synaptic GABA<sub>A</sub> Receptor Subunits, are Diminished in the MeCP2-Null Cortex

The above results suggest that GABA<sub>B</sub> receptor tone is elevated in the MeCP2-deficient cortex. Such an effect could arise from increases in GABA<sub>B</sub> receptor prevalence, and/or from decreases in the expression or activity of GABA transport systems. Using western blotting, we compared the

expression levels of the GABA<sub>B</sub> receptor subunit R2, which is the requisite subunit for functional GABA<sub>B</sub> receptors (Bowery and Enna, 2000), as well as the expression of the extra-synaptic GABA<sub>A</sub> receptor subunit delta, and the GABA Transporter type 1 (GAT-1)—which is the primary transporter responsible for clearing synaptic GABA in the cortex (Conti *et al*, 2004)—between the wild-type and MeCP2-null cortex. These assessments focused on male mutants, as they display the more severe cortical hyper-excitable network phenotype, and allow us to avoid the expression variability seen in female mutants due to their mosaic MeCP2 expression patterns (Wither *et al*, 2013). No significant changes in expression were found for either the GABA<sub>B</sub> receptor R2 subunit or the GABA<sub>A</sub> receptor delta subunit (Figure 2). In contrast, the prevalence of GAT-1 in the same *Mecp2*<sup>-/-</sup> mouse cortical samples was decreased by 34.4 ± 10.7% from wild-type levels (*p* = 0.007, Figure 2). This decrease in GAT-1 protein was not paralleled by decreases in cortical GAT-1 mRNA expression; however, although



**Figure 3** The GAT-1 inhibitor NO-711 intensifies the epileptiform discharge phenotype of female *Mecp2*<sup>+/-</sup> mice. Representative 5 min (i) and 10 s (ii) traces showing cortical EEG activity patterns seen in 14- to 18-month-old wild-type mice (a) or 14- to 18-month-old female *Mecp2*<sup>+/-</sup> mice (b) under baseline conditions, and following the administration of the GAT-1 inhibitor NO-711 at 1.0 or 2.5 mg/kg. Although no effects were observed in wild-type mice with the 1.0-mg/kg dose, a strong enhancement of discharge activity was observed in *Mecp2*<sup>+/-</sup> mice. A similar increase in discharge activity was observed in *Mecp2*<sup>+/-</sup> mice following the administration of a 2.5-mg/kg dose of NO-711. However, unlike the 1.0-mg/kg dose, the administration of 2.5 mg/kg dose of NO-711 to female wild-type mice induced short duration discharge events similar to those seen in female *Mecp2*<sup>+/-</sup> mouse under basal conditions. (c) Representative traces stemming from *Mecp2*<sup>+/-</sup> mice treated first with 100 mg/kg of the GABA<sub>B</sub> receptor antagonist CGP-35348, followed 15 min later with 1.0 mg/kg of the GAT-1 inhibitor NO-711 are shown. Note the increase in discharge activity seen in (b) for the same dose of NO-711 is abolished by pre-treatment with the GABA<sub>B</sub> receptor antagonist.

chromatin immunoprecipitation assays confirmed that MeCP2 binds to the promoter region of the mouse *GAT-1* gene in mouse cortical tissue (Supplementary Figure 1), no significant differences between *GAT-1* mRNA were seen between cortical tissues from *Mecp2*<sup>-/-</sup> and wild type by quantitative real-time PCR analysis (Figure 2). These data suggest the decreases in *GAT-1* protein observed in the MeCP2-null cortex likely arise from post-transcriptional rather than transcriptional mechanisms. The decrease in *GAT-1* protein is intriguing, as impaired *GAT-1* enzymatic activity has been previously linked to the genesis of EEG discharge activity in cortical circuitries (Cope *et al*, 2009).

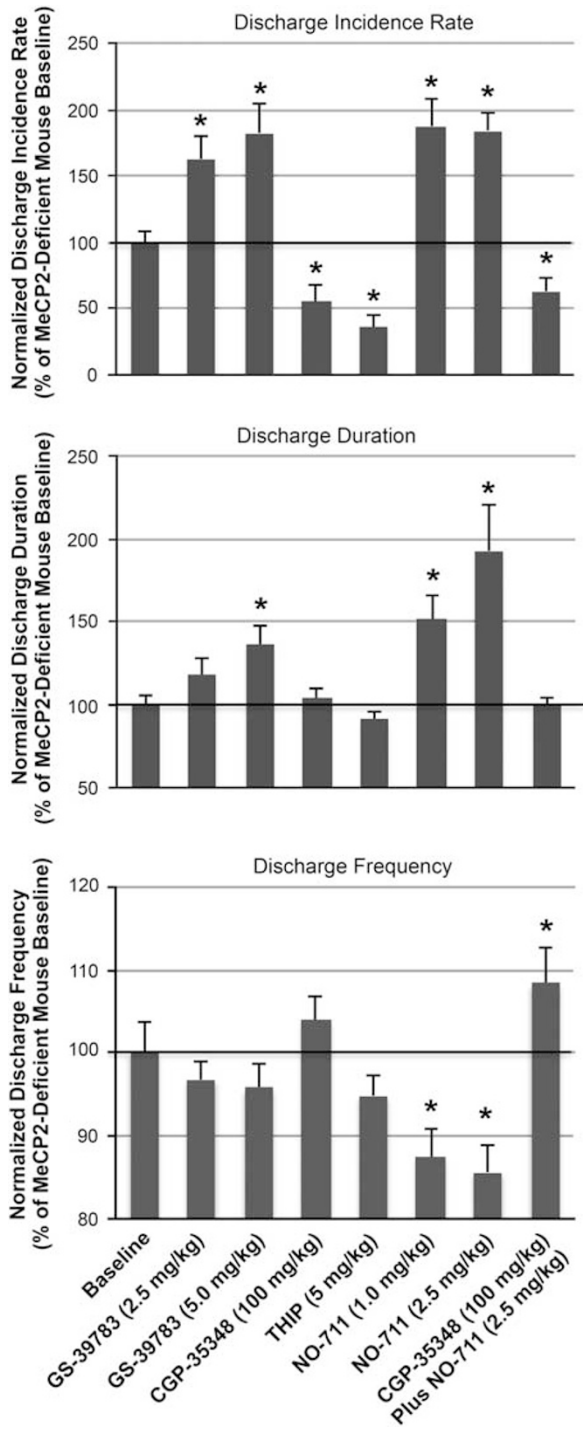
### GAT-1 Inhibition Increases the Severity of Discharge Events in Female *Mecp2*<sup>+/-</sup> Mice

The decreased prevalence of *GAT-1* protein provides a mechanism through which overstimulation of GABA<sub>B</sub> receptors could occur in the MeCP2-null cortex. If causal for network hyper-excitability, then further inhibition of the remaining *GAT-1* in MeCP2-deficient mice should further exacerbate their discharge phenotype. To test this, we administered different doses of the selective *GAT-1* inhibitor NO-711 (Borden, 1996) to both female *Mecp2*<sup>+/-</sup> mice and female wild-type mice. The administration of 1.0 mg/kg NO-711 failed to induce discharge activity in 12 of 14 wild-type mice examined (Figure 3), and only a single short discharge was seen in each of the other two mice (not shown). These results indicate that under normal conditions this level of *GAT-1* inhibition is primarily inconsequential for wild-type mice. In female *Mecp2*<sup>+/-</sup> mice ( $n=12$ ), however, this same 1.0 mg/kg dose of NO-711 significantly increased the discharge incidence rate from  $108.4 \pm 13.9$  to  $203.1 \pm 23.0$  events per hour ( $p=0.0002$ , Figures 3 and 4),

lengthened discharge duration from  $1.2 \pm 0.1$  s to  $1.8 \pm 0.2$  s ( $p=0.011$ , Figure 4), and slowed the discharge waveform frequency from  $7.7 \pm 0.2$  Hz to  $6.7 \pm 0.3$  Hz ( $p=0.0014$ , Figure 4). The administration of a dose of 2.5 mg/kg NO-711 produced a similar effect in female *Mecp2*<sup>+/-</sup> mice ( $n=12$ ) increasing discharge incidence rate from  $86.9 \pm 9.7$  to  $160.2 \pm 12.0$  events per hour ( $p=0.0001$ , Figures 3 and 4), lengthening discharge duration from  $1.1 \pm 0.1$  s to  $2.1 \pm 0.3$  s ( $p=0.006$ , Figure 4), and slowing the discharge waveform frequency from  $7.4 \pm 0.2$  Hz to  $6.3 \pm 0.2$  Hz ( $p=0.0001$ , Figure 4). Interestingly, administering the 2.5-mg/kg dose of NO-711 was sufficient to induce 'short' duration discharge events in 10 of 10 female wild-type mice (Figure 3) that had properties similar to the short duration discharges of female *Mecp2*<sup>+/-</sup> mice under basal conditions with incidence rates of  $25.3 \pm 13.4$  discharges per hour, discharge durations of  $1.0 \pm 0.1$  s, and discharge frequencies of  $6.7 \pm 0.6$  Hz. Collectively, these results not only illustrate the critical role of *GAT-1* in regulating cortical network hyper-excitability in female *Mecp2*<sup>+/-</sup> mice, but also show that the progressive inhibition of *GAT-1* can induce a discharge phenotype in wild-type mice that resembles that of female *Mecp2*<sup>+/-</sup> mice under basal conditions.

### Attenuating GABA<sub>B</sub> Receptor Activity Decreases Cortical Epileptiform Discharges in *Mecp2*<sup>+/-</sup> Mice

Given the positive effects on discharge activity seen with the GABA<sub>B</sub> receptor allosteric modulator GS-39783 and the elevated discharge activity seen with the *GAT-1* inhibitor NO-711, we next tested whether blocking GABA<sub>B</sub> receptors would decrease the discharge activity of female *Mecp2*<sup>+/-</sup> mice. Indeed, the administration of the selective GABA<sub>B</sub> receptor antagonist CGP-35348 (100 mg/kg; Snead, 1992)



**Figure 4** Cumulative effects of GABA modulating drugs on discharge properties in MeCP2-deficient mice. Histograms presenting the normalized mean and SEM for the different GABA modulating drugs tested on  $n = 12$ – $15$  female MeCP2-deficient mice. In each graph, the baseline discharge incidence rate (top panel), discharge duration (middle panel), and frequency (bottom panel) for each of drug and concentration as indicated. Baseline values before drug administration were set at 100%, and the data presented indicate the percentage change from baseline for each condition. Asterisks denote statistical significance relative to MeCP2-deficient baseline ( $p < 0.05$ ; one-way ANOVA with Bonferroni *post hoc* correction).

decreased the discharge incidence rate from  $90.6 \pm 15.6$  to  $50.0 \pm 11.4$  events per hour ( $n = 12$ ;  $p = 0.0016$ , Figure 4), but did not significantly affect average discharge duration or discharge frequency (Figure 4). Further, the pre-administration of 100 mg/kg of the GABA<sub>B</sub> receptor blocker CGP-35348 15 min before the administration of 1.0 mg/kg NO-711 completely prevented the increase in discharge activity seen in female *Mecp2*<sup>+/-</sup> mice treated with the same dose of only NO-711 ( $n = 12$ ; Figures 3 and 4). In fact, discharge rates remained below baseline (from  $106.7 \pm 12.4$  to  $67.2 \pm 7.5$  discharges per hour;  $p = 0.0014$ ), and the average frequency of the remaining discharges was also significantly increased (from  $7.2 \pm 0.2$  Hz to  $7.8 \pm 0.2$  Hz;  $p < 0.001$ ; Figure 4). Collectively, these results strongly implicate enhanced GABA<sub>B</sub> receptor activity stemming from diminished GAT-1 clearance of synaptic GABA as a key driving force for the cortical network hyper-excitability phenotype of female *Mecp2*<sup>+/-</sup> mice.

## DISCUSSION

Although neural network hyper-excitability is a major co-morbidity of Rett syndrome patients (Huppke *et al*, 2007), and also present in other conditions displaying errant MeCP2 expression (Gonzales and LaSalle, 2010), how impaired MeCP2 function leads to hyper-excitability neural circuits remains poorly understood. Using MeCP2-deficient mice as model systems and cortical epileptiform discharges as an index of network hyper-excitability, our results implicate enhanced GABAergic signaling as a contributing factor for the cortical discharge phenotype of MeCP2-deficient mice. Five principle findings emerge from our study: (1) cortical discharge patterns differ between male and female MeCP2-deficient mice, with male MeCP2<sup>-ly</sup> mice displaying population of long duration discharges not seen in female *Mecp2*<sup>+/-</sup> mice; (2) cortical discharge patterns in female *Mecp2*<sup>+/-</sup> mice are exacerbated by enhancing GABA<sub>B</sub> receptor activity and attenuated by GABA<sub>B</sub> receptor blockers; (3) the expression levels of GAT-1 are diminished in the MeCP2-deficient cortex; (4) the pattern of cortical discharges in female *Mecp2*<sup>+/-</sup> mice is exacerbated by inhibiting GAT-1 activity, and discharge activity can be induced in wild-type mice by more progressive GAT-1 inhibition; and (5) blocking GABA<sub>B</sub> receptors in female *Mecp2*<sup>+/-</sup> mice ameliorates the discharge activity increase induced by GAT-1 inhibition.

The overstimulation of GABA<sub>B</sub> receptors represents a recognized mechanism through which cortical discharge activity can be induced (Snead, 1992). Extra-synaptic levels of GABA are strongly regulated by GAT-1 activity (Conti *et al*, 2004), and decreasing the prevalence of activity of GAT-1 leads to increases in extracellular GABA levels and a heightening of both pre and postsynaptic GABA<sub>B</sub> receptor activity. Our data showing discharge activity is exacerbated in female *Mecp2*<sup>+/-</sup> mice by the GABA<sub>B</sub> receptor-positive allosteric modulator GS-39783—whose modulatory actions require GABA<sub>B</sub> receptors be activated by GABA (Pin and Prézeau, 2007; Froestl, 2010)—which are consistent with constitutively elevated GABA<sub>B</sub> receptor activation in the MeCP2-deficient cortex. The increased excitatory discharge activity induced by doses of the GAT-1 inhibitor NO-711

that had minimal or no effect on wild-type mice further supports this contention, as does the decreased discharge activity seen following the administration of the GABA<sub>B</sub> receptor antagonist CGP35348. These data therefore suggest that the decrease in GAT-1 prevalence in the MeCP2-deficient mouse cortex is rate-limiting for the establishment of a hyper-excitable cortical network, and that the discharge activity itself manifests through the enhanced activation of GABA<sub>B</sub> receptors. The observation that cortical discharges resembling those of *Mecp2*<sup>+/-</sup> mice can be induced in wild-type mice by increasing degrees of GAT-1 inhibition also supports this model.

Cortical GABAergic interneuronal networks are known to be involved in the initiation of cortical network hyperexcitability and seizures (Shiri *et al*, 2015). Recently, Goffin *et al* (2014) demonstrated that the large-scale loss of MeCP2 from forebrain GABAergic interneurons led to cortical hyperexcitability and behavioral seizures, whereas the restoration of MeCP2 in specific classes of interneurons in MeCP2-deficient mice ameliorated these deficits. However, the loss of MeCP2 from only excitatory neurons did not induce discharge activity. These results imply that the key MeCP2 target(s) responsible for the discharge phenotype are associated with cortical GABAergic interneurons. Our data indicate that decreased GAT-1 transporter expression in cortex regulates the discharge phenotype of MeCP2-deficient mice. It is therefore noteworthy that while some GAT-1 is expressed in astrocytes (Conti *et al*, 1998, 2004), the highest density of GAT-1 expression is found within GABAergic neurons (Chiu *et al*, 2002; Jensen *et al*, 2003; Conti *et al*, 1998), with inhibitory axon terminals in the neocortex displaying particularly high levels of GAT-1 expression (Chiu *et al*, 2002). Although we did not specifically assess the types of cells expressing GAT-1 in the MeCP2-deficient cortex, these collective results suggest that the decreases in GAT-1 expression we detect by immunoblotting, and those recently reported by Kang *et al* (2014) using immunohistochemistry, occur within the same forebrain inhibitory interneurons shown to be critical for epileptogenesis by Goffin *et al* (2014). These results therefore independently support the role of the GABAergic system in the manifestation of excitatory discharge activity in the MeCP2-deficient brain.

An additional interesting question that arises from our observations is whether or how decreases in GAT-1 activity might affect phasic synaptic GABA<sub>A</sub> receptor activity. Indeed, phasic GABA signaling through synaptic GABA<sub>A</sub> receptors is altered in different regions of the MeCP2-deficient brain (Chao *et al*, 2010; Zhang *et al*, 2010; Jin *et al*, 2013), and has been proposed to be an underlying cause of local synaptic hyperexcitability (Chao *et al*, 2010). Although heightened extra-synaptic GABAergic tone may initially seem inconsistent with attenuated phasic GABAergic synaptic activity, the two are not mutually exclusive. In fact, GAT-1 knockout mice display precisely this phenotype. In GAT-1 knockout mice, enhanced tonic GABAergic tone arises from the diminished ability of GABA to be removed from synaptic areas (Jensen *et al*, 2003). However, these mice also display decreased phasic GABA<sub>A</sub> receptor activity due to the diminished frequency of spontaneously released GABA from presynaptic inhibitory terminals (Jensen *et al*, 2003). Thus, the enhanced activity of

GABA<sub>B</sub> receptors we show here with GS-39783, and the decreased levels of phasic inhibition shown previously at MeCP2-deficient synapses (Chao *et al*, 2010; Jin *et al*, 2013), can co-exist in systems with decreased GAT-1 transporter activity. It is also noteworthy that many of the behavioral deficits and general phenotypes of GAT-1 knockout mice (Chiu *et al*, 2005; Liu *et al*, 2007) display considerable overlap with those of MeCP2-deficient mice (Katz *et al*, 2012). These include an impaired hind limb clasp reflex, pronounced tremor, decreased ambulatory and rearing activity, impaired balance, and altered anxiety responsiveness. Although these behavioral impairments are not unique to these two specific mutant mice, and additional work is required to establish whether or not MeCP2 and *GAT-1* are functionally connected, the phenotypic overlap between the two lines is intriguing and suggestive of common or convergent network origins.

Finally, although our study focused on mechanisms of cortical network hyperexcitability in MeCP2-deficient mice, the observations we present raise the possibility that altered MeCP2 expression in other chronic or acute neurological conditions could secondarily cause decreases in GAT-1 expression in these conditions, and thereby further negatively affect their neural circuitry homeostasis. In this regard, relatively subtle decreases in MeCP2 expression are sufficient to induce behavioral impairments in mice (Samaco *et al*, 2008; Kerr *et al*, 2008), and decreases in brain MeCP2 levels have been reported in neurological conditions such as Angelman syndrome, attention deficit hyperactivity disorder, and general autism (Nagarajan *et al*, 2006; Neul, 2012; Gonzales and LaSalle, 2010). Thus, the neural link we illustrate here between MeCP2 and GAT-1 expression may be worthy of assessment in other conditions in which imbalances of excitatory and inhibitory tone are believed to have a role in pathogenesis.

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The authors declare no conflict of interest.

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