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EXPLORING GABA FUNCTION IN RETT SYNDROME

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Abstract

Dendritic spines are critical elements in establishing excitatory synapses in cortical circuits. The alteration of dendritic spines in shape and number is believed to be critical for the refinement of neural circuits and the processing and storage of information within the brain. Neuropathological studies have demonstrated that a number of disease states, ranging from schizophrenia to autism spectrum disorders, display abnormal dendritic spine morphology or distribution. Rett syndrome, a severe form of autism spectrum disorder, is mainly caused by MeCP2 *de novo* mutations and its mechanism remains to be elucidated. Here we demonstrate that GABA functional deficits may be involved in the aberrant spine plasticity that, in part, underlie the pathophysiology of Rett syndrome. In order to study the mechanistic action of MeCP2 in controlling dendritic spine morphogenesis, the use of cultured mouse cortical neurons was employed. Our data suggests that GABA function may serve as an alternative therapeutic approach for the treatment of Rett syndrome.

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Chapter 1

Introduction

1.1 Rett Syndrome

Rett syndrome (RS) is a severe form of autism spectrum disorder (ASD) caused mainly by loss-of-function mutations in the X-linked methyl-CpG-binding protein 2, MeCP2 (Chahrour and Zoghbi, 2007). Linked to more than 95% of classical Rett syndrome cases, MeCP2 *de novo* mutations are key players in one of the leading causes of mental retardation and autistic behavior in females (Cohen and Greenberg, 2008). A transcriptional regulatory protein, MeCP2 is known to mediate a multitude of genes that contribute to neuronal survival and development and maintain the balance between synaptic excitation and inhibition (Abuhatzira et al., 2007).

Patients affected with RS experience normal development for 6-18 months, at which time they enter a period of motor regression and developmental stagnation. Rett patients generally suffer from a loss of social, cognitive, and language skills, a gain in repetitive stereotyped behavior, and seizures (Weaving et al., 2005; Chahrour and Zoghbi, 2007). In addition to the defects within the central nervous system, weight loss, cardiac and breathing abnormalities, and high mortality rate have also been reported in Rett syndrome patients (Erlandson and Hagberg, 2005).

The neuronal defects exhibited in Rett patients may stem from disrupted synaptic transmission (Dani and Nelson, 2009), disturbed excitation-inhibition ratio (Dani et al., 2005), and abnormal spine morphology and plasticity (Landi et al., 2012; Zoghbi and Bear, 2012), which have been described in animal models for RS. Signaling molecules such as BDNF (Chang et al., 2006; Zhou et al., 2006), mTOR (Ricciardi et al., 2011; Li et al., 2013), and insulin-like growth factor -1 (IGF-1) (Marchetto et al., 2010) have been documented as players in the molecular mechanism of Rett syndrome. However, the ultimate signaling molecule downstream to MeCP2 that directly regulates synaptic function has yet to be identified.

Since the molecular culprit for Rett syndrome is MeCP2, the investigation of different MeCP2 *de novo* mutations and correlation to phenotype will provide further insight on how the MeCP2 signaling pathway regulates neuronal development and function.

1.2 The Role of MeCP2 in Rett Syndrome

Reported in individuals with infantile autism, severe neonatal encephalopathy, bipolar disorder, and schizophrenia, the phenotypic spectrum of MeCP2 coding mutations extends far beyond Rett syndrome (Jian et al., 2006). MeCP2 expression is spatially and developmentally regulated and is widespread with some differences between brain regions. Relatively low during the embryo stage of development, MeCP2 expression is highest in mature postnatal neurons (Shahbazian et al., 2002; Cohen et al., 2003; Kishi and Macklis, 2004), but is also detected in glia and microglia cells (Maezawa and Jin, 2010; Derecki et al., 2012).

MeCP2 binds to methylated CpG pairs throughout the entire genome (Skene et al., 2010; Cohen et al., 2011) in order to mediate methylation-dependent repression by coupling DNA methylation to silencing transcriptional machinery (Jones et al., 1998; Jaenisch and Bird, 2003). This MeCP2 repression model has been validated for the regulation of various genes such as neurotrophic factor BDNF. Upon phosphorylation, MeCP2 is released from the BDNF promoter and elevates BDNF expression level (Zhou et al., 2006). In addition to repression, MeCP2 activates a number of genes by binding with the CREB (cAMP response element-binding) complex rather than the HDAC/Sin3A (histone deacetylase) repressor complex (Chahrour et al., 2008).

The role of MeCP2 and its interaction mechanisms with other molecular machineries has been studied in numerous knockout or knock-in mouse models. These animal models that lack MeCP2 globally or carry severe hypomorphic copies of MeCP2 develop characteristic Rett-like syndromes such as epilepsy, decreased motility, breathing abnormalities, and premature death (Chen et al., 2001; Guy et al., 2001) are generated to elucidate the complex etiology of RS. In accord with expectations, the restoration of MeCP2 expression in postnatal MeCP2 deficient animals using a genetic approach or an adeno-associated virus carrying the MeCP2 gene rescued the Rett-like phenotypes (Giacometti et al., 2007; Cobb et al., 2010). The findings of these rescue experiments further strengthen the relationship between MeCP2 and RS phenotypes.

Morphology and functional analysis of Rett syndrome mouse models documented a reduction in synaptic transmission, a perturbation in the excitation-inhibition balance, hyperactivation and epileptic activity, and abnormalities in dendritic spine morphology (Jian et

al., 2006). Other mechanistic studies further illustrate MeCP2 as a critical mediator of excitatory synaptic strength through the regulation of the number of glutamatergic synapses and synaptic scaling (Nelson et al., 2006; Chao et al., 2007; Blackman et al., 2012). A key player in the early developmental stage of the brain, MeCP2 is also a major contributor to the maintenance and proper functioning of mature neurons (McGraw et al., 2011). MeCP2 coding mutations by and large have a non-cell autonomous effect on neuronal properties, inducing autism-like stereotypies and Rett syndrome phenotypes.

MeCP2 duplications have been found in Rett syndrome and severe mental retardation, highlighting that any deviation in MeCP2 expression disrupts normal neurodevelopment (del Gaudio et al., 2006). This not only denotes the importance of restoring MeCP2 levels comparable to normal neurons, but also finding alternative methods in rescuing deficits downstream MeCP2 when developing therapies. A variety of therapeutic reagents such as BDNF, the BDNF activator ampakine, and IGF-1 (insulin-like growth factor 1) have been tested on RS mouse models to determine their efficacy on alleviating symptoms. Treatment of BDNF (brain-derived neurotrophic factor) to MeCP2 knockout mice rescued the stereotypical Rett phenotypes (Chang et al., 2006), while ampakine treatment rescued the pathological phenotypes in MeCP2 knock down mouse models (Ogier et al., 2007). Like BDNF, IGF-1 modulates the PI3K/Akt pathway and is considered as a promising molecular target for the treatment of RS. In a recent study, the partial rescue of survival, behavioral, and synaptic deficits after treatment of MeCP2 mutant mice with an IGF-like peptide has been documented (Tropea et al., 2009).

Despite the extensive research done on characterizing the expression, regulation, and function of MeCP2, the ultimate downstream signaling molecule that directly monitors neurotransmission remains unknown. In addition, why Rett syndrome is a progressive disorder characterized by delayed developmental regression has yet to be answered.

1.3 Linking KCC2 as a Key Player in Rett Syndrome

KCC2, a neuron specific K-Cl co-transporter, plays multiple roles in the physiology of the central nervous system. The major Cl^- transporter in neurons, KCC2 regulates intraneuronal chloride homeostasis, determines the polarity of GABAergic neurotransmission, mediates dendritic spine morphogenesis and the maintenance of glutamatergic synapses, and moderates stem cell differentiation and migration (Bortone and Polleux, 2009; Kim et al., 2012). Alterations of KCC2 function and/or expression have been documented in several neurodegenerative disorders such as Autism, Schizophrenia, and Alzheimer's disease (Arion and Lewis, 2011; Hyde et al., 2011). In light of these findings, KCC2 dysfunction may be the common denominator and the molecular underpinning for a variety of neurodevelopmental and neuropsychiatric disorders. Thus, exploring the molecular mechanisms underlying its regulation is critical in elucidating the pathology of neurological disorders.

Due to its pivotal role in brain development and function, KCC2 expression and activity are tightly regulated. For instance, transcriptional factor REST (RE1-Silencing Transcription factor) can bind to two RE-1sites in order to suppress KCC2 expression (Yeo et al., 2009), while

BDNF has been shown to activate (Uvarov et al., 2006; Boulenguez et al., 2010; Ludwig et al., 2011) and suppress KCC2 expression (Rivera et al., 2004; Coull et al., 2005). This apparent discrepancy may stem from variability across experimental methods such as the duration of BDNF application, the developmental stage of the neurons analyzed, and the source of BDNF. Neurotrophic factor IGF-1 has also been documented in regulating the activity of KCC2 through a tyrosine kinase-dependent manner (Kelsch et al., 2001).

Following a rostral to caudal order during development, KCC2 first expresses in the spinal cord and brain stem, then in the hippocampus and neocortex (Blaesse et al., 2009). During early postnatal brain development, KCC2 expression levels are generally very low and later undergo a steep increase, which strongly influences the efficacy and polarity of $GABA_A$ and glycine receptor mediated synaptic transmission (Dzhala et al., 2005; Ben-Ari et al., 2012). Specifically, the spatio-temporal regulation of KCC22 mRNA and protein expression levels catalyzes the developmental shift in synaptic GABAergic and glycinergic transmission from depolarizing to hyperpolarizing (Rivera et al., 1999; Hubner et al., 2001). This strict temporal and spatial control of KCC2 expression is critical in ensuring both proper brain development and function.

KCC2 coding mutations that disrupt either expression or function have been linked to various neurological disorders. Interestingly, animal models deficient in KCC2 developed pathological phenotypes strikingly similar to those observed in the MeCP2 knockout mouse such as respiratory dysrhythmia (Hubner et al., 2001), reduced body weight, learning and memory deficits, abnormal EEG hyperexcitability, premature lethality, and impaired motor coordination

(Tornberg et al., 2005). The brain of MeCP2 knockdown mouse models also showed deficits in BDNF, a neurotrophin that can promote the functional maturation of $GABA_A$ receptor-mediated response by upregulating KCC2 and is believed to contribute to the pathogenesis of Rett syndrome. Moreover, the recruitment of MeCP2, REST and its cofactor CoREST into a single transcriptional repressor complex that suppresses neuronal gene expression such as KCC2 at early stages of brain development has been documented (Ballas et al., 2005). These findings suggest the intriguing possibility that KCC2 may serve an important function in both the MeCP2 signaling pathway and Rett syndrome.

Before this study, whether KCC2 plays any functional role in Rett syndrome was unknown. In pursuit of an answer, we found that knocking down MeCP2 in cultured mouse cortical neurons significantly reduced KCC2 expression level as shown in Figure 1. This novel

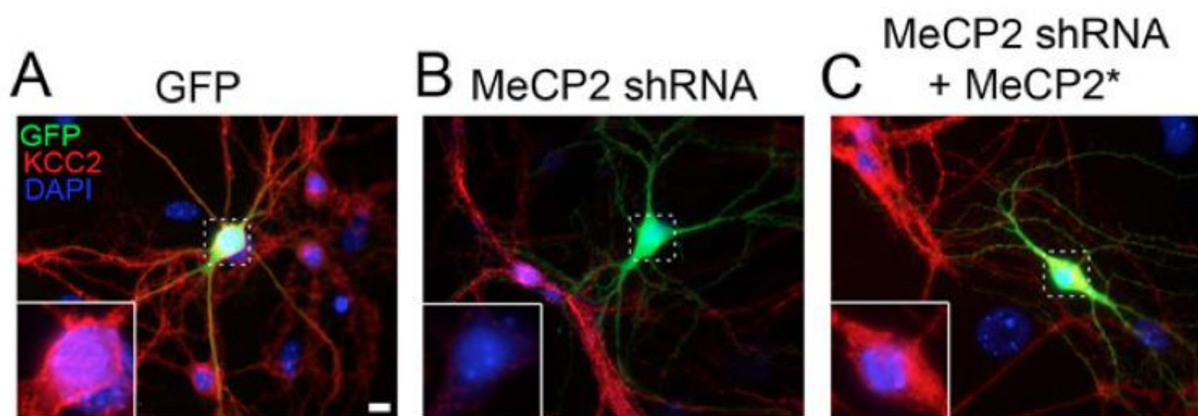

Figure 1. MeCP2 regulates KCC2 protein expression. (Xin Tang, unpublished data). A-C. Representative micrograph of mouse cortical neurons transfected with MeCP2 shRNA (C, MeCP2 KD) exhibited a reduction in KCC2 expression relative to neighboring non-transfected cells (B, GFP control). Scale bar = 10 µm.

link between KCC2 and MeCP2 was further explored in the following study to elucidate the pathogenesis of Rett syndrome and contribute to the framework for a unified theory in

understanding autism spectrum disorders. The causal relationship between MeCP2 and KCC2 was confirmed by analyzing dendritic spine morphogenesis.

1.4 Dendritic Spines in Rett Syndrome Pathology

The principal neurons in most regions of the brain are coated with small projections known as dendritic spines, the main sites of excitatory synaptic input (Gu et al., 2010; Bosch and Hayashi, 2011; Fortin et al., 2012; Fu et al., 2012; Lai et al., 2012). Alterations in dendritic spine distribution and structure are common pathological features in various neurodegenerative disorders associated with mental retardation (Penzes et al., 2011; Landi et al., 2012; Yu and Lu, 2012; Yu et al., 2012). As principal sites of synaptic input and indicators of neuronal connectivity dendritic spines have received intense study in pathology.

Since the formation and maturation of spines result from interactions between intrinsic genetic factors and external environment, the study of spine morphology and development in disorders linked to mental retardation has significant clinical relevance. Morphological studies in Rett syndrome neurons reported spine dysgenesis with a marked decrease in dendritic spine density, suggesting that impaired synaptic transmission is a likely pathogenic consequence of MeCP2 mutations in RS (Dani and Nelson, 2009). Interestingly, KCC2 also plays a role in dendritic spine morphogenesis and the maintenance of glutamatergic synapses. KCC2 deficient neurons exhibit a reduction in spine density and a prevalence of long and tortuous spines (Li et al., 2007). As mediators of spine morphogenesis KCC2 and MeCP2 may be players in the Rett

syndrome pathology that leads to spine dysgenesis. This contingency will be investigated using quantitative analyses of dendritic spine density and morphology in Rett syndrome cultured mouse neurons.

Herein this study, we report a novel link between KCC2 and MeCP2 in regulating synaptic neurotransmission. We demonstrate that by knocking down MeCP2 KCC2 expression and dendritic spine density (i.e. glutamatergic events) decreases. These functional deficits can be rescued by IGF-1 treatment or KCC2 overexpression, suggesting that MeCP2 regulates KCC2 expression. Further quantitative studies identified REST as a potential mediator that links MeCP2 to KCC2 function. Together, our results provide a mechanistic explanation to the pathogenesis of Rett syndrome and suggest that KCC2 may be a critical downstream target of MeCP2 in regulating glutamatergic function.

Chapter 2

Materials and Methods

2.1 Primary Astroglial and Neuronal Cell Culture

Astroglial cells were cultured from the cortical tissue of newborn mouse pups (postnatal day 3 to 5) similar to methods previously described (McCarthy and de Vellis, 1980; Yao et al., 2006; Deng et al., 2007; Jiang and Chen, 2009). The cortical tissue was dissected, cut into cubes with an approximate dimension of 1 mm, and incubated in 0.05% trypsin-EDTA solution (Invitrogen) for 30 min. After protein digestion, the tissue was triturated and centrifuged. The cell suspension was plated onto 25 cm^2 flasks and incubated in 5% CO_2 at 37 °C for seven days. The glial culture medium consisted of MEM, 5% FBS, 20 mM D-glucose, 2.5 mM L-glutamine, and 25 unit/ml penicillin/streptomycin. Once confluent, the astroglial cells were tripsinized and resuspended before seeded on 12 mm coverslips as a substrate for neurons to induce and promote growth.

Neuronal cells were cultured from P1 mouse cortical tissue using similar methods previously described above for glial cell culture (Yao et al., 2006; Deng et al., 2007) with the single exception that dissociated cells were plated directly on a monolayer of astrocytes on 12 mm coverslips. The neuronal seeding density was 4000 - 8000 cells/cm^2.

2.2 Plasmid constructs and transfection

The Calcium-Phosphate transfection method was performed using a protocol developed in our laboratory (Jiang and Chen, 2006). Reagents from Clonetech CalPhos Mammalian Transfection Kit were used to prepare Calcium phosphate-DNA aggregates. Neuronal cultures of mouse neurons were exposed to calcium phosphate-DNA aggregates for 30 min in a 5% CO_2, incubator set to 37 °C and were washed off using incubated transfection medium containing MEM and 30 mM Glucose, pH adjusted to 7.2 with acetic acid.

The constructs used in the following research project are: KCC2 and KCC2 shRNA constructs from Dr. Yun Wang, Fudan University, Shanghai, China, MeCP2 and MeCP2 shRNA plasmids from Dr. Michael Greenberg, Harvard University, Boston, MA (Zhou et al., 2006), REST FL and REST DN plasmids from Dr. David Anderson, California Institute of Technology, Pasadena, CA (Chen et al., 1998), and FUGW-GFP control constructs from Dr. Roger Nicoll (Shipman et al., 2011). The promoter for KCC2 plasmid is CMV with vector pIRES2-EGFP. The MeCP2 and MeCP2 shRNA constructs are LEMPRA (lentivirus-mediated protein-replacement assay). The promoters for MeCP2 and MeCP2 shRNA are pUbiquitin and pU6.

2.3 Immunostaining and imaging

After 18-20 days *in* vitro, cultured mouse neurons used for immunofluorescence staining were washed with PBS, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton in PBS for 5 min. Cells were washed three times with PBS, and then blocked with

2.5% NGS/2.5% NDS serum for 1 hr at rt to block any non-specific binding sites. The primary antibodies were diluted in the blocking solution and and incubated overnight at 4 °C. Cover slips were washed to remove any excess primary antibody and correlative fluorophore-conjugated secondary antibodies diluted in blocking solution were added to the coverslips and incubated at rt for 1 hr. After secondary antibody incubation, coverslips were rinsed 3 times with PBS for 10 min, counterstained with DAPI, and then mounted with mounting solution (50% glycerol, 50% 0.1 M NaHCO3 in water, pH 7.4). Epiluorescent images were acquired on a Nikon TE-2000-S microscope. Confocal fluorescent images were collected using an Olympus FV1000 confocal microscope with a 60x oil-immersion objective.

The antibodies used in this study were as follows: KCC2 (1:500, Millipore), MAP2 (1:500, Abcam), GFP (1:1000, Abcam), and MeCP2 (1:500, Diagennode; 1:500 Abcam).

2.4 Quantitative Spine Density and Morphology Analysis

Fluorescent images of pyramidal shaped mouse neurons were acquired with confocal microsope Olympus FV1000, using an oil immerson 60x obhective lens. Optical z-sections were acquired at 0.8 µm steps through the apical dendritic tree of the neurons. Measurements were performed on secondary and tertiary dendrites with a total length of 100 µm per neuron analyzed. Both the length and width of each dendritic spine were measured by tracing along its length and width and quantified using ImageJ software (National Institutes of Health). Dendritic spines were analyzed using the NeuroJ plugin. The image acquization and analysis were blind to the plasmids transfected. The ratio beween length and width was subsequently calculated to

generate a histogram. A range of 14-16 neurons was measured for each condition. Data were normalized per 50 μm of dendritic length.

2.5 Statistical Analysis

Data were analyzed statistically using a one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons using Prism (GraphPad; San Diego, CA). $p < 0.05$ was considered significant. Data are presented as mean ± standard error of the mean (SEM). For the histograms describing data distribution, Kolmogorov-Smirnov test was employed.

Chapter 3

Results

A disruption in the balance between glutamatergic excitation and GABAergic inhibition is often characterized in autism spectrum disorders. In this study, I focused on the interplay between KCC2 and MeCP2 in modulating gluatamatergic synapse formation in Rett syndrome mouse neurons *in vitro*. A series of quantitative analyses of dendritic spine morphology and density were conducted to explore the role of KCC2 in mediating the MeCP2 regulation of excitatory synaptic neurotransmission.

3.1 MeCP2 regulates KCC2 expression

Prior to dendritic spine analysis, we confirmed the causal relationship between MeCP2 and KCC2 in cultured mouse cortical neurons. Specifically, the molecular expression timeline of KCC2 and MeCP2 was determined by observing KCC2 expression level in response to a series of molecular manipulations (Fig. A-1). A significant reduction in KCC2 expression was observed in MeCP2 knockdown neurons. Overexpression of KCC2 and co-expression of knockdown resistant MeCP2* constructs rescued the KCC2 expression deficit induced by MeCP2 shRNA.

3.2 KCC2 is a key downstream protein that mediates MeCP2 regulation on excitatory synapse formation

In parallel with the KCC2 immunostaining analysis that established a link between MeCP2 and KCC2 expression, we investigated the role of KCC2 in mediating the MeCP2 regulation of excitatory synapse formation using quantitative analyses on dendritic spine distribution and morphology.

There has been no prior study on the role of KCC2 in mediating the MeCP2 regulation of dendritic spine morphogenesis using quantitative statistical analyses. To perform quantitative analyses of spine density and morphology, mouse neurons (18 to 20 days) transfected with a variety of MeCP2 and KCC2 molecular manipulations were imaged and compared to a GFP control. For each manipulation, cultured mouse cortical neurons comparable in shape (pyramidal-like), size, dendritic complexity, and spine density were chosen for analysis. Dendritic spines were imaged by confocal microscopy and a total length of 50 to 100 µm of secondary and tertiary apical dendrites was analyzed per neuron.

Quantitative analyses revealed significantly reduced dendritic spine density in secondary and tertiary apical dendrites of MeCP2 (20.8 ± 0.7 spines per 50 µm of apical dendrite, $n = 15$) and KCC2 (23.8 ± 3.7 spines per 50 µm of apical dendrite, $n = 15$) knockdown mouse neurons compared to the GFP control (31.8 ± 0.8 spines per 50 µm of apical dendrite, $n = 16$) as depicted in (Fig.3-1**a-c**). One-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons yielded a significant correlation (*** $p < 0.001$) between spine density in control

and knockdown neurons. This quantitative analysis demonstrates that MeCP2 and KCC2 knockdown neurons have lower spine densities than WT mouse neurons. This deficiency in spine density in MeCP2 knockdown neurons can be rescued by restoring MeCP2 expression (30.1 ± 0.8 spines per 50 μm of apical dendrite), overexpressing KCC2 (31.4 ± 0.9 spines per 50 μm of apical dendrite), or treating neurons with IGF (31.0 ± 1.1 spines per 50 μm of apical dendrite). Interestingly, when MeCP2 and KCC2 expression is both knocked down within a single neuron, IGF can no longer rescue the dendritic spine deficit (22.6 ± 0.7 spines per 50 μm of apical dendrite). A summary of the quantitative analyses of dendritic spine density under various MeCP2 or KCC2 manipulations can be found in Fig. 3-2**a**.

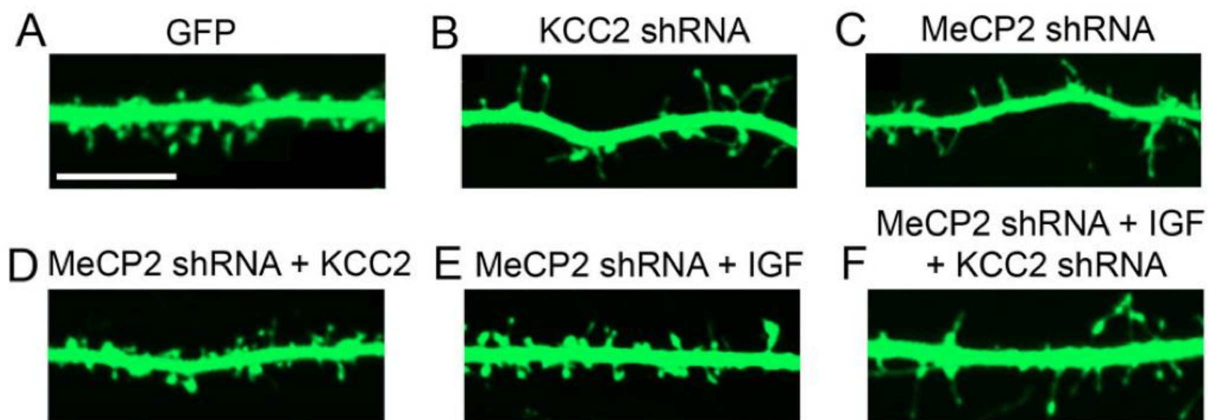

Figure 2. KCC2 and MeCP2 crosstalk in the regulation of dendritic spine development. (A-F). Representative confocal micrographs of dendritic spines under various MeCP2 or KCC2 manipulations (scale bar = 10 μm).

In addition to a reduction of spine density, MeCP2 and KCC2 knockdown neurons displayed morphological changes in dendritic spines (Fig. 3-1**b-c**). Since neuropathological studies have demonstrated a number of disease states display abnormal dendritic spine morphology, the potential role of KCC2 in the MeCP2 regulation of spine morphogenesis was

examined by quantifying the ratio of spine length to head width. The cumulative distribution of spine length/width ratio for the GFP control, KCC2 shRNA, MeCP2 shRNA, and MeCP2 shRNA + KCC2 groups is illustrated in Fig. 2-2**b**. In general, longer, filopodia-like spines can be present in higher frequency in the MeCP2 and KCC2 knockdown neurons (red and orange traces, *** p < 0.001 relative to the green GFP control curve. Kolmogorov-Smirnov test). Moreover, KCC2 overexpression in MeCP2 deficient neurons not only rescued spine density, but also dendritic spine morphology with a cumulative probability highly comparable to the GFP- control (blue trace, *** p < 0.001 relative to orange MeCP2 shRNA; p = 0.297 relative to green GFP).

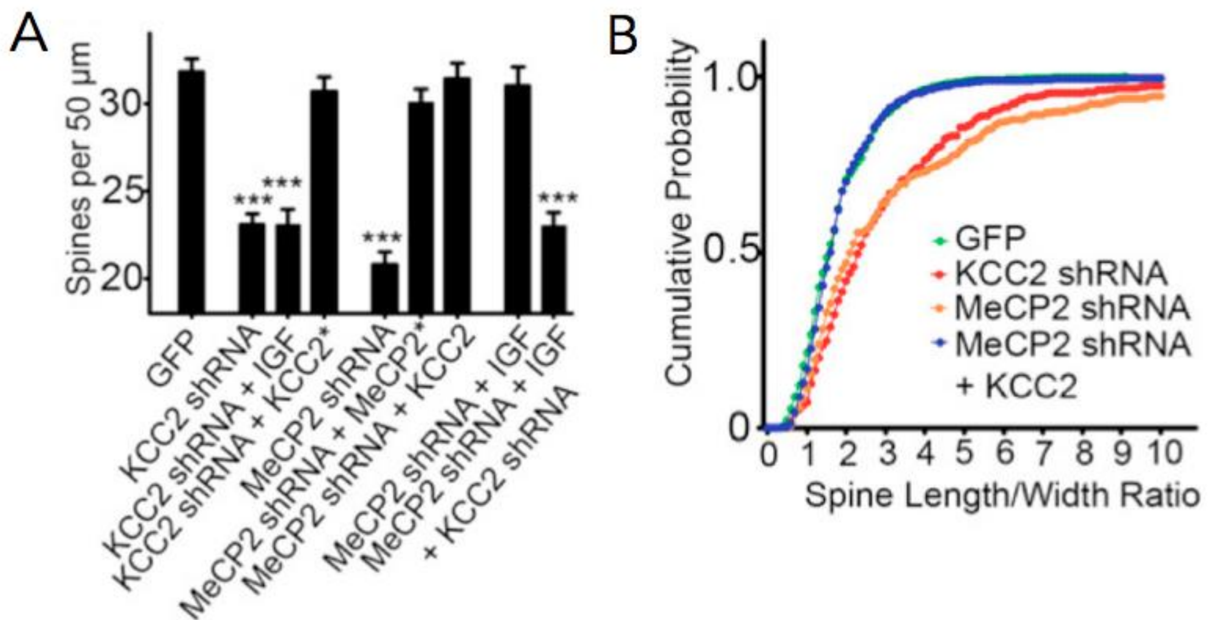

Figure 3. KCC2 acts downstream of MeCP2 to mediate dendritic spine morphogenesis. A. a bar graph that summarizes the quantitative analysis on dendritic spine density under various MeCP2 or KCC2 manipulations. **B.** a plot of the cumulative distribution of spine length/width under MeCP2 or KCC2 molecular manipulations.

The conclusions gained from the quantitative analysis on dendritic spine density and morphology was confirmed by electrophysiological recordings on mEPSCs in cultured mouse cortical neurons under the same molecular manipulations (Fig. 3-3). Complementary to the dendritic spine data, MeCP2 or KCC2 knockdown neurons exhibited a significant reduction in mEPSC frequency. This reduction could be rescued through KCC2 overexpression, providing further evidence that KCC2 is a downstream target of MeCP2's signaling cascade.

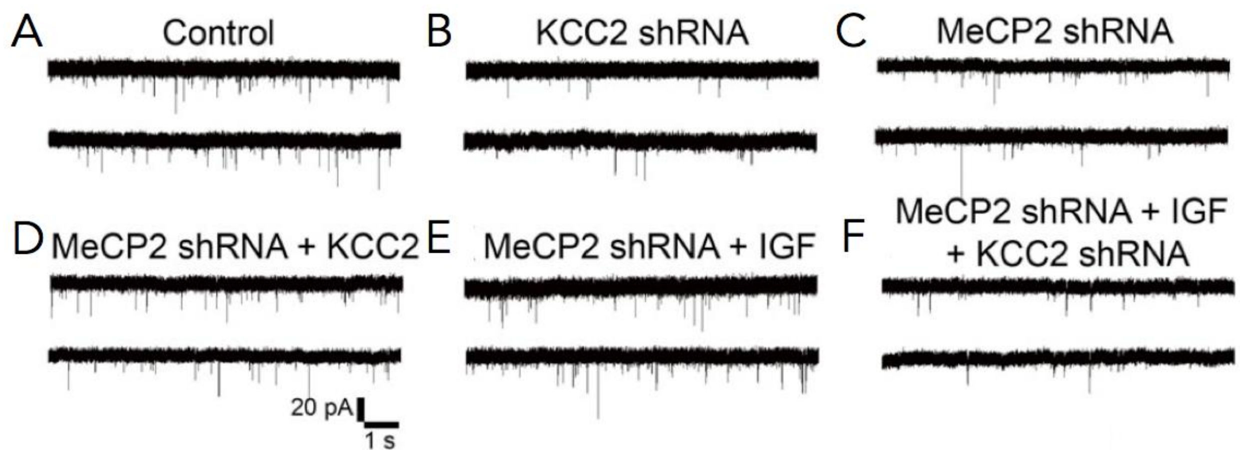

Figure 4. Representative mini EPSC recording traces of mouse neurons transfected with a variety of MeCP2 and KCC2 constructs.

3.3 MeCP2 regulates KCC2 through transcriptional repressor REST

Even though KCC2 was identified as a downstream target of MeCP2 activity, the specific molecular mechanism in which MeCP2 regulates KCC2 expression remains to be defined. As a global transcription regulator, MeCP2 affects a number of downstream signaling cascades. Thus, the hunt for a protein that directly mediates MeCP2 regulation of KCC2 began. A careful review

of previous studies identified REST as a potential mediator that links MeCP2 to KCC2 function. (Ballas et al., 2005; Abuhatzira et al., 2007). The same quantitative dendritic spine analysis approach was employed to determine the role that REST plays in MeCP2 regulating KCC2 expression.

Quantitative analyses revealed significantly reduced dendritic spine density in secondary and tertiary apical dendrites of mouse neurons transfected with REST (24.1 ± 0.6 spines per 50 μm of apical dendrite, n = 15) compared to the GFP control (35.2 ± 0.9 spines per 50 μm of apical dendrite, n = 15) as depicted in (Fig.3-4**a&b**). One-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons yielded a significant correlation between spine density in control and neurons overexpressing REST due to its ability to effectively suppress KCC2 expression. This deficiency in spine density in REST overexpressed neurons could be rescued by MeCP2 overexpression (34.8 ± 0.9 spines per 50 μm of apical dendrite). This rescue implies that MeCP2 suppresses the inhibitory effect of REST on KCC2 levels. However, IGF treatment failed to rescue the spine deficit induced by REST overexpression, which may stem from a difference their corresponding response elements in producing an effect.

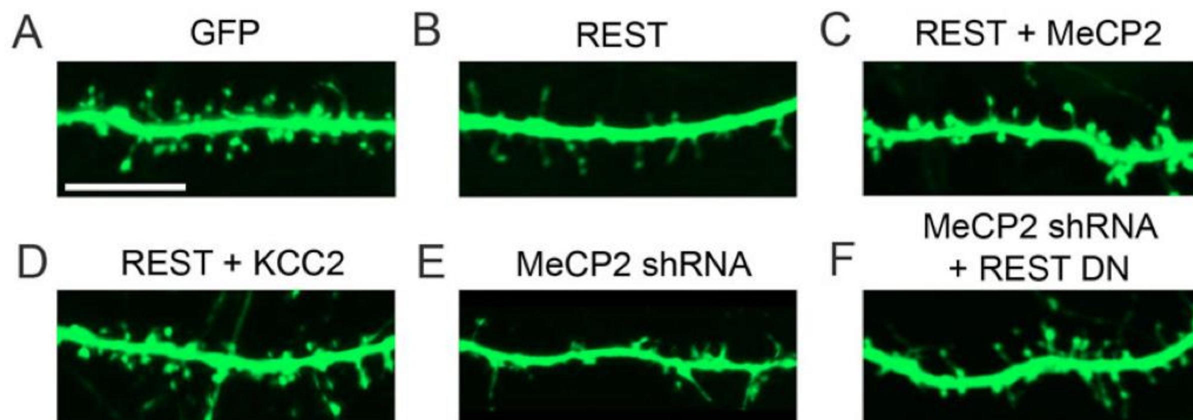

Figure 5. MeCP2 regulates KCC2 expression through REST. (A-F). Representative confocal micrographs of dendritic spines under various MeCP2 or KCC2 manipulations are depicted. (scale bar = 10 μm).

Specifically, the transcriptional repression by REST overpowers the transcriptional activation of KCC2 through IGF treatment. To further test the interplay between MeCP2 and REST and their effects on KCC2, a dominant negative mutant of REST (REST DN) was expressed in the cultured mouse neurons and found that dendritic spine density was not altered. Interestingly, coexpressing REST DN with MeCP2 shRNA significantly rescued the spine deficit induced by MeCP2 shRNA alone, lending evidence to the argument that REST is a key mediator in the MeCP2 regulation of KCC2 expression.

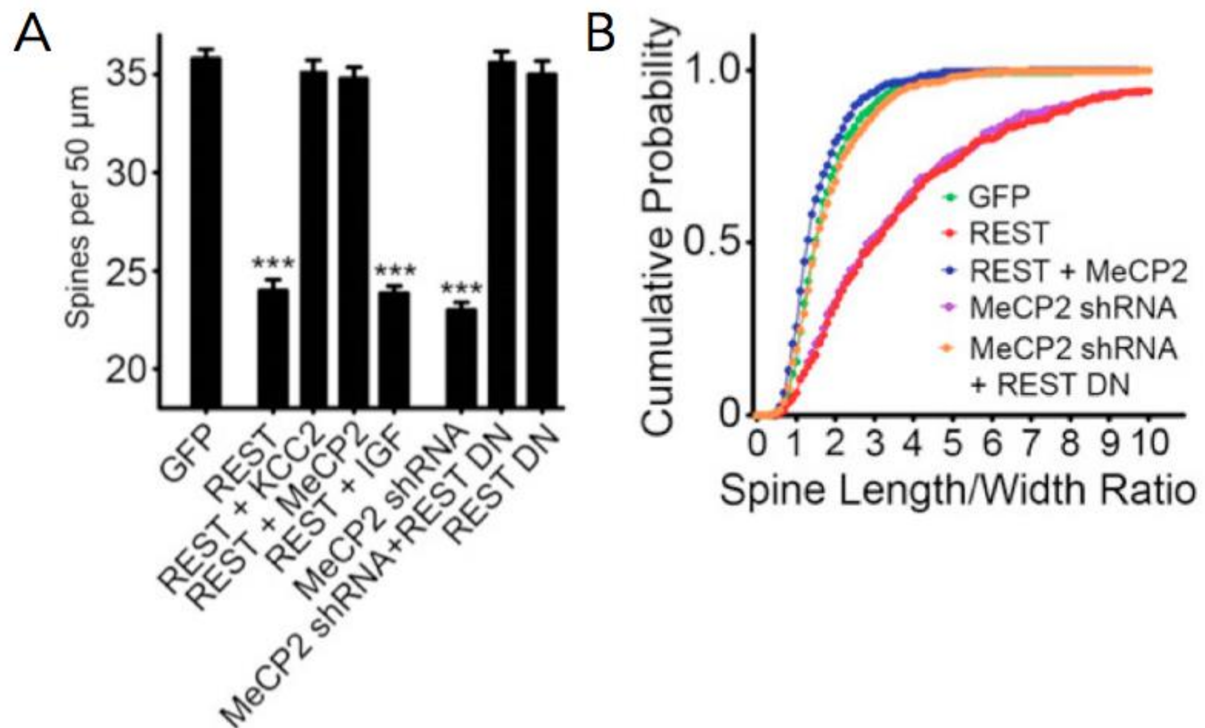

Figure 6. MeCP2 activates KCC2 through REST in dendritic spine morphogenesis. (A-B). A. a bar graph that 3-5. summarizes the quantitative analysis on dendritic spine density under various MeCP2, KCC2, and REST manipulations. B. a plot of the cumulative distribution of spine length/width under MeCP2 and REST molecular manipulations.

In addition to a reduction of spine density, KCC2 deficient neurons induced by REST overexpression displayed morphological changes in dendritic spines (Fig. 3-5**b-c**). Since a number of disease states display abnormal dendritic spine morphology, the potential role of REST in the MeCP2 regulation of spine morphogenesis through KCC2 was examined in a Rett syndrome mouse model by quantifying the ratio of spine length to head width. The cumulative distribution of spine length/width ratio for the GFP control, REST, REST + MeCP2, REST DN + MeCP2 shRNA, and MeCP2 shRNA is illustrated in Fig. 3-5**b**. In general, longer, filopodia-like spines was present in higher frequency in the REST and MeCP2 knockdown neurons (red and purple traces, *** $p < 0.001$ relative to the green GFP control curve. Kolmogorov-Smirnov test). Moreover, REST DN coexpressed in MeCP2 deficient neurons not only rescued spine density, but also dendritic spine morphology with a cumulative probability highly comparable to the GFP- control (orange trace, *** $p < 0.001$ relative to purple MeCP2 shRNA; $p = 0.325$ relative to green GFP).

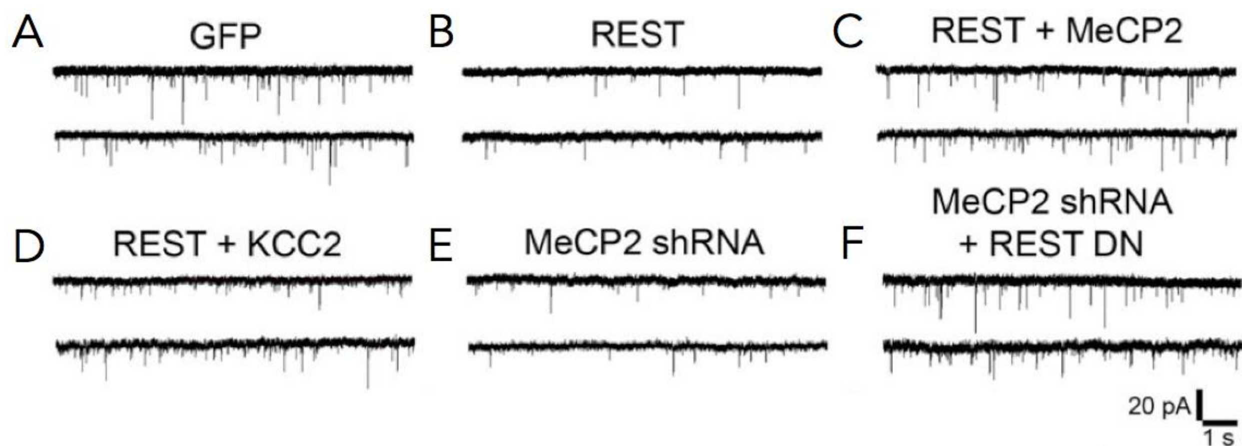

Figure 7. . Representative mini EPSC recording traces of mouse neurons transfected with a variety of MeCP2 and REST constructs (A-F). (Xin Tang, unpublished data).

The conclusions gained from the quantitative analysis on dendritic spine density and morphology was further confirmed by electrophysiological recordings on mEPSCs in cultured mouse cortical neurons under the same molecular manipulations.

Chapter 4

Discussion

In this study, we demonstrated that MeCP2 regulates KCC2 through REST in controlling dendritic spine morphogenesis and ultimately excitatory synaptic transmission. Using cultured mouse cortical neurons we discovered a novel link in MeCP2 and KCC2 expression. Through immunological assays, KCC2 expression levels were significantly reduced in MeCP2 knockdown neurons. This reduction in KCC2 expression led to spine dysgenesis with a marked decrease in dendritic spine density, which was further verified in the mEPSCs frequency of neurons under the same molecular manipulations. The MeCP2 shRNA induced impairment of dendritic spine morphogenesis can be rescued through KCC2 overexpression of IGF-1 treatment. This study suggests that KCC2 may be the ultimate downstream signal in mediating excitatory synaptic transmission after global transcriptome regulation by MeCP2 and that KCC2 may be a novel drug target for the treatment of Rett syndrome.

4.1 KCC2 is a key downstream gene that directly mediates the MeCP2 regulation on excitatory synaptic neurotransmission

A neuron specific K-Cl co-transporter, KCC2 is under the regulation of both external factors (Kelsch et al., 2001; Rivera et al., 2004; Coull et al., 2005; Boulenguez et al., 2010; Bos

et al., 2013; Yeo et al., 2013) and internal factors including REST (Uvarov et al., 2006; Yeo et al., 2009; Ludwig et al., 2011b; Rinehart et al., 2011). On the other hand, MeCP2 is highly expressed in neurons and is a global regulator of a number of neuronal genes. (Zhou et al., 2006; Skene et al., 2010b; Cohen et al., 2011). This study illustrates a novel link between MeCP2 and KCC2 in determining excitatory neurotransmission efficacy. Our data suggests that MeCP2 regulates KCC2 expression and that Rett syndrome may stem from MeCP2's inability to regulate KCC2 to mediate its control on dendritic spine morphogenesis. IGF treatment was able to rescue the spine density deficit in MeCP2 deficient neurons, but not in KCC2 shRNA expressing neurons. This finding suggests that KCC2 is a downstream effector of the IGF-1 pathway in regulating neuronal development.

Taken together, our data illustrates a potential model mechanism of action in which MeCP2 mediates KCC2 expression in controlling neuronal function and development through the transcriptional repressor REST (Fig. A-2). In normal physiological conditions, MeCP2 can bind to the RE-1 site within the KCC2 promoter region, blocking REST from binding to KCC2 and inhibiting KCC2 gene expression. In Rett syndrome neurons induced knocking down MeCP2, REST is now free to bind to the RE-1 sites located in the KCC2 promoter region and in the KCC2 intronic region as a KCC2 repressor. This suppression of KCC2 expression may result in the development of fewer and elongated dendritic spines, thus ultimately impairing glutamatergic synaptic neurotransmission. This finding suggests that MeCP2 may regulate KCC2 through REST.

However, we cannot exclude the fact that MeCP2 is a master regulator of a multitude number of genes. While MeCP2 may be mediating its control on KCC2 expression through REST, our data does not confirm this finding with definitive proof. Thus, we cannot exclude the possibility that MeCP2 affects other downstream effectors such as BDNF, which may in turn regulate KCC2 expression (Chang et al., 2006; Zhou et al., 2006).

4.2 Targeting KCC2 to treat Rett syndrome

Alterations in KCC2 expression have been linked to a number of neurological disorders including schizophrenia (Arion and Lewis, 2011; Hyde et al., 2011). This current study implicates KCC2 and its expression as a potential contributor to the pathogenesis of Rett syndrome. Together, we suggest that the MeCP2 regulation of dendritic spine morphogenesis mediated by KCC2 may be an important factor in autism spectrum disorders.

IGF-treatment of Rett syndrome neurons induced by MeCP2 shRNA rescued the spine density deficit. Moreover, treatment of MeCP2 deficient mouse models partially rescued the Rett-like phenotypes (Tropea et al., 2009). Together, our data provides a possible mechanistic explanation on how IGF treatment may rescue KCC2 expression in Rett neurons. While therapeutic strategies that manipulate the MeCP2 expression level to treat Rett syndrome yielded promising results in animal models (Garg et al., 2013; Giacometti et al., 2007), recent studies have documented the importance of maintaining MeCP2 levels within an optimum range is critical for proper neuronal development and function. Thus, therapeutic approaches that manipulate MeCP2 level may have serious adverse effects. Another potential therapeutic

approach is targeting MeCP2 downstream effectors that mediate its control on neuronal development and function. This study along with others link KCC2 expression and function to various neurological disorders, suggesting that methods aimed at restoring KCC2 expression may be a novel, valuable therapeutic approach in treating Rett syndrome.

Additional experiments could be added to strengthen our proposed model for how MeCP2 regulates KCC2 expression through REST. *In vivo* confirmation in both MeCP2 knockdown mouse models on dendritic spine morphogenesis can provide insight into the applicability of our cultured mouse neurons in the whole brain setting. Moreover, detailed experiments to investigate the molecular mechanisms on how MeCP2 interacts with REST to regulate KCC2 should be carried out such as ChIPs, gel shift assays, and RT-PCR. In addition, the mechanism of IGF rescue of KCC2 expression in MeCP2 deficient mouse neurons deserves further elucidation.

Appendix A

Supplemental Figure of MeCP2 regulation on KCC2 expression

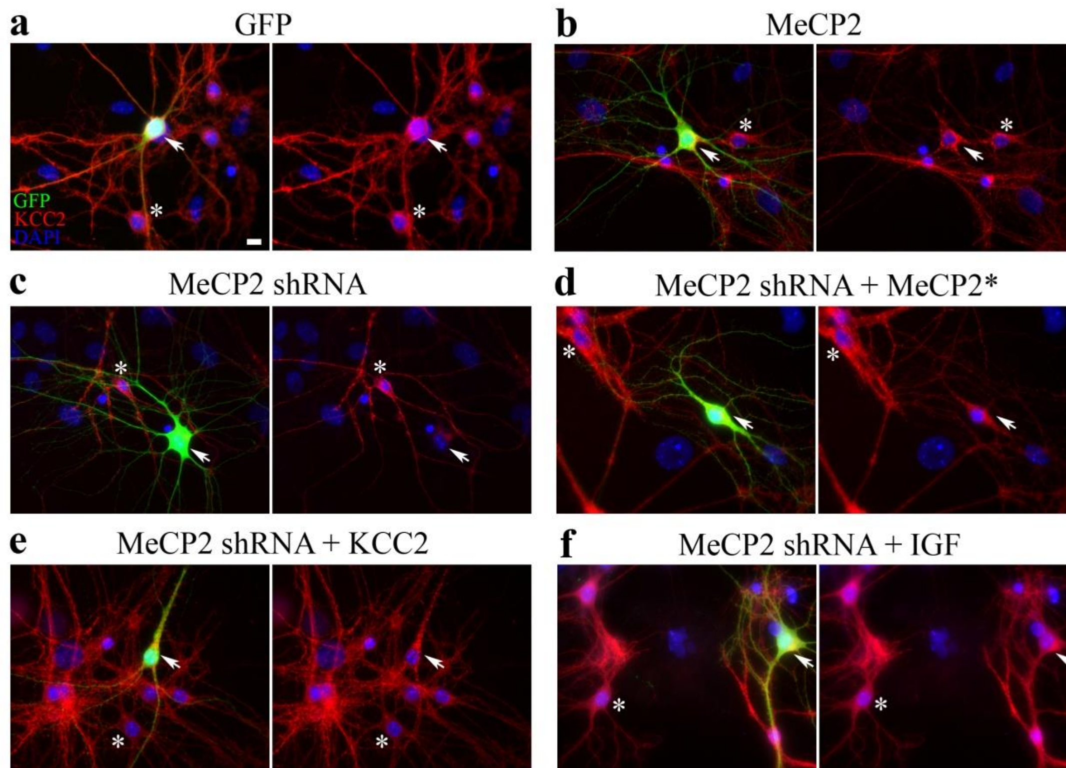

Figure 8. Representative micrographs of MeCP2 regulation on KCC2 expression. (Xin Tang, unpublished data). The cultured mouse neurons were transfected with various plasmids and probed for GFP (green), KCC2 (red), DAPI (blue). GFP expressing neurons (**a**) exhibited similar KCC2 expression relative to neighboring non-transfected neurons. MeCP2 overexpress (**b**) did not significantly alter KCC2 intensity, while MeCP2 knockdown documented a significant reduction in KCC2 expression (**c**). KCC2 deficient neurons could be rescued by restoring MeCP2 (**d**), overexpressing KCC2 (**e**), or IGF treatment (**f**).

Supplemental Figure of MeCP2 regulation on KCC2 expression through transcriptional repressor REST

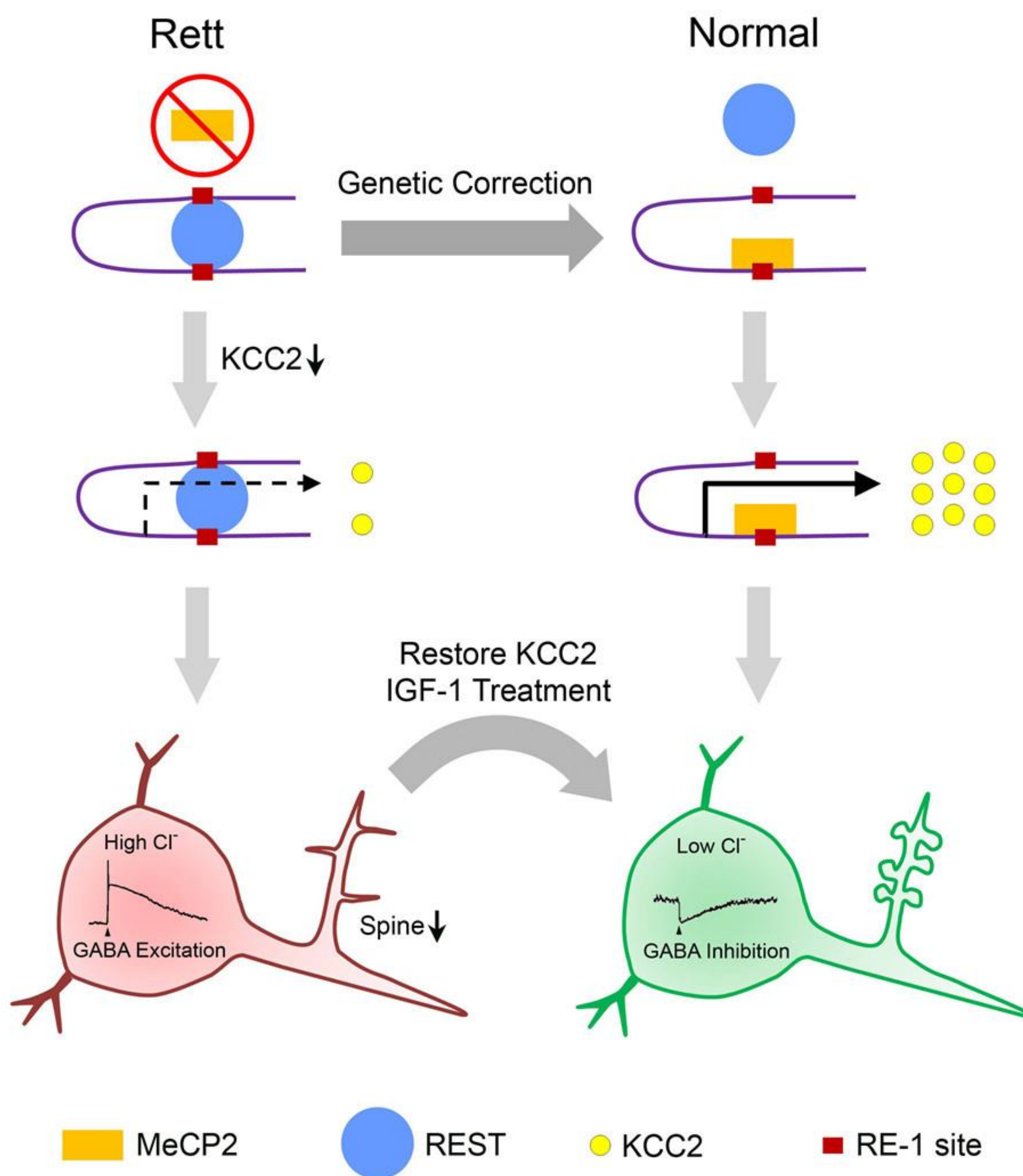

Figure 9. Graphic Abstract of the Interplay between MeCP2, REST, and KCC2.

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