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Enhanced Dense Core Granule Function and Adrenal Hypersecretion in a Mouse Model of Rett Syndrome:

Mecp2 Null Adrenal Dysfunction

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Abstract

Rett Syndrome (RTT) is a progressive developmental disorder resulting from loss of function mutations in the gene encoding MeCP2 (methyl-CpG-binding protein 2), a transcription regulatory protein. The RTT phenotype is complex and includes severe cardiorespiratory abnormalities, dysautonomia and behavioral symptoms of elevated stress. These findings have been attributed to an apparent hyperactivity of the sympathetic nervous system due to defects in brainstem development; however, the possibility that the peripheral sympathoadrenal axis itself is abnormal has not been explored. The present study demonstrates that the adrenal medulla and sympathetic ganglia of *Mecp2* null mice exhibit markedly reduced catecholamine content compared to wildtype controls. Despite this, null animals exhibit significantly higher plasma epinephrine levels, suggesting enhanced secretory granule function in adrenal chromaffin cells. Indeed, we find that *Mecp2* null chromaffin cells exhibit a cell autonomous hypersecretory phenotype characterized by significant increases in the speed and size of individual secretory granule fusion events in response to electrical stimulation. These findings appear to indicate accelerated formation and enhanced dilation of the secretory granule fusion pore, resulting in elevated catecholamine release. Our data therefore highlight abnormal catecholamine function in the sympathoadrenal axis as a potential source of autonomic dysfunction in RTT. These findings may help to explain the apparent “overactivity” of the sympathetic nervous system reported in RTT patients.

Keywords

exocytosis; chromaffin; sympathetic; neurosecretion; neuroendocrine

Introduction

Rett Syndrome (RTT) is an X-linked dominant neurodevelopmental disorder that affects approximately 1/10,000 live female births (Van den Veyver and Zoghbi, 2002; Moretti and Zoghbi, 2006). Loss-of-function mutations in the gene encoding methyl-CpG binding

protein 2 (MeCP2) account for the vast majority of RTT cases (Amir *et al.*, 1999; Moretti & Zoghbi, 2006), and mice carrying hypomorphic, truncated or null alleles at the *Mecp2* locus exhibit RTT-like symptoms (Chen *et al.*, 2001; Guy *et al.*, 2001; Shahbazian *et al.*, 2002; Pelka *et al.*, 2006; Samaco *et al.*, 2008). Classical RTT is characterized by apparently normal postnatal development until 6 to 18 months of life, followed by a marked neurological decline (Hagberg *et al.*, 1983; Shahbazian & Zoghbi, 2002; Vorsanova *et al.*, 2004; Chahrour & Zoghbi, 2007). Initial features of the disease typically include loss of acquired speech and head growth deceleration; subsequently, RTT patients develop severe cognitive and motor deficits as well as disordered and irregular breathing, cardiovascular and gastrointestinal dysautonomia, and behavioral symptoms of elevated stress (Hagberg *et al.*, 1983; Moretti & Zoghbi, 2006).

To date, studies on the pathogenesis RTT have focused primarily on identifying defects in the central neuraxis that may underlie symptoms of the disease. However, the fact that autonomic control is profoundly affected in RTT raises the possibility that the peripheral nervous system is affected as well. Although there is growing clinical evidence of an imbalance between sympathetic and parasympathetic tone in RTT patients, with an apparent “hyperactivity” of the sympathetic nervous system (Julu *et al.*, 2001; Acampa & Guideri, 2006; Acampa *et al.*, 2008), these alterations have been attributed to presumed defects in brainstem maturation (Julu *et al.*, 1997a, b).

Recent studies from our laboratory indicate that loss of MeCP2 results in an abnormal neurosecretory phenotype that affects central and peripheral neurons and adrenal chromaffin cells and is characterized by enhanced transmitter release *in vitro* (Wang *et al.*, 2006). Based on these findings, we hypothesize that symptoms of autonomic imbalance in RTT could result from elevated secretion of catecholamines from the peripheral sympathoadrenal axis *in vivo*. Therefore, the present study was designed to define the role of MeCP2 in sympathoadrenal function *in vivo* as well as mechanisms that underlie enhanced catecholamine exocytosis in *Mecp2* null cells. Our findings demonstrate that, despite reduced adrenal catecholamine content, *Mecp2* null chromaffin cells exhibit a cell autonomous, hypersecretory phenotype that is characterized by an increase in both the rate and amount of catecholamine exocytosis, and an increase in the number of secretion-ready granules competent for release upon cell stimulation. Furthermore, this hypersecretory phenotype is associated with a significant increase in the plasma level of epinephrine in isoflurane-anesthetized *Mecp2* null mice compared to wildtype controls. On the basis of these findings we propose that hypersecretion of epinephrine from the adrenal medulla could explain or contribute to clinical observations of apparent sympathetic hyperactivity in RTT patients.

Materials and Methods

Animals

Mecp2 null mice (Chen *et al.*, 2001) were bred from founder animals from the Mutant Mouse Regional Resource Center (University of California, Davis, CA). Animals were studied at post-natal day 35 (P35), an age at which they exhibit marked RTT-like symptoms (Ogier *et al.*, 2007). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Adrenal Gland Immunohistochemistry

Animals were euthanized by CO₂ asphyxiation. Adrenal glands were removed and fixed in 4% paraformaldehyde at 4°C for 2 hours. Cryoprotection was achieved by immersion in 10%, 15%, 20%, and 30% sucrose solutions over the next two days, after which the glands

were embedded in OCT and frozen at -80°C . Sections were cut with a Leica Jung Frigocut 2800N at $16\mu\text{m}$ and mounted on Fisherbrand Superfrost/Plus microscope slides. Sections were allowed to dry for one hour, and were then washed twice with PBS for 10 minutes. Slides were blocked with a fresh dilution buffer containing 0.02M NaPO_4 buffer, 50mM NaCl, 0.3% Triton X-100, and 2% BSA fraction V at room temperature for one hour. Slides were then incubated with primary antibodies against anti-MeCP2 (rabbit, Upstate #07-013) and anti-tyrosine hydroxylase (TH; chicken, Aves #TH) overnight at 4°C . Slides were then washed twice with PBS followed by a single wash with dilution buffer for 10 minutes at room temperature, then incubated with secondary antibodies conjugated to FITC and Cy3 (Goat anti-rabbit FITC (ICN/Cappel #55646), Donkey anti-chicken Cy3 (Jackson #703-166-155)). Immunofluorescence images were captured with a Q-Imaging Retiga EXi Fast1394 monochromatic CCD camera.

Adrenal Gland Morphometry

Animals were euthanized by CO_2 asphyxiation at postnatal day 35 (P35) and perfused intracardially with ice-cold PBS followed by 4% paraformaldehyde. Dissected adrenal glands were postfixed and cryoprotected as described above. Glands were serially sectioned at $30\mu\text{m}$ and directly imaged without staining. Total gland area as well as cortical and medullary area were measured from every third section of the gland. Volumes (less vascular spaces) were determined by multiplying the measured area by the thickness.

Catecholamine Measurements

HPLC with electrochemical detection was used to determine adrenal gland, superior cervical ganglion (SCG), and plasma concentrations of norepinephrine, epinephrine, and dopamine. *Mecp2* null and wildtype animals (P35) were anesthetized with isoflurane (Sigma) and euthanized by blood draw through cardiac puncture. Glutathione (GSH, 3.9mM, Sigma) and ethylene glycol tetraacetic acid (EGTA, 4.7mM, Sigma) were added to blood samples to prevent clotting and catecholamine degradation. Samples were centrifuged at $2000 \times g$ for 10 minutes at 4°C , after which the plasma supernatant was aliquoted and frozen at -80°C . Adrenal glands and SCGs were rapidly dissected and frozen at -80°C . Blood and tissue samples were analyzed by the Neurochemistry Core Lab at Vanderbilt University's Center for Molecular Neuroscience Research (Nashville, TN) as previously described (Perez & Palmiter, 2005).

Electrophysiology

Adrenal medullary chromaffin cells were isolated and cultured and perforated patch voltage clamp recordings were performed as previously described (Fulop *et al.*, 2005). Internal pipette solution contained (in mM): 135 Cs-glutamate, 10 HEPES-H, 9.5 NaCl, 0.5 TEA-Cl, and 0.53 amphotericin B, pH adjusted to 7.2, and osmolarity adjusted to 310 mOsm. During recordings, cells were constantly superfused with a Ringer's solution containing (in mM): 150 NaCl, 10 HEPES-H, 10 glucose, 2.5 CaCl_2 , 2.8 KCl, and 2 MgCl_2 , with pH adjusted to 7.2 and osmolarity adjusted to 320 mOsm. Cells were allowed to perforate until the access resistance dropped to below $30\text{M}\Omega$ before recording. All recordings were acquired with an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany) under the control of Pulse software (v8.40, HEKA Elektronik). To measure the release kinetics and fusion pore dynamics, cells were held at a -80mV command potential and stimulated with a train of simulated action potentials at 0.5 Hz or 15 Hz in conjunction with amperometric detection of released catecholamines (Chan & Smith, 2001). To assess Readily Releasable Pool (RRP) and the Reserve Pool (RP), cells were depolarized from a resting potential of -80mV to $+10\text{mV}$ with pulses of increasing duration, allowing 45 seconds in between pulses for the cell to recover (Smith *et al.*, 1998; Voets *et al.*, 1999). Evoked capacitance jumps were measured as previously described (Smith *et al.*, 1998). Briefly, cells were held in the

perforated patch configuration and clamped at a holding potential of -80 mV. Cell capacitance was measured in the frequency domain by imposing a 634 Hz sine wave of 20 mV amplitude on the holding potential. These recording conditions were previously shown to accurately measure capacitance changes due to granule fusion independent of fusion mode (Fulop & Smith, 2006).

Electrochemical Recordings

Electrochemical catecholamine detection was performed with commercially available carbon fiber electrodes (ALA Scientific Instruments, Longneck, NY) and fiber tips were always cut fresh before recordings. A $+650$ mV potential was placed on the fiber tip by a VA-10x dedicated amperometry amplifier with a $1\text{ G}\Omega$ head stage (ALA Scientific Instruments). Recordings were filtered with a 1.3 kHz Bessel filter before being sampled at 20 kHz by an ITC-1600 data acquisition system (Instrutech, Port Washington, NY). After data acquisition, amperometric data were analyzed with a macro modified from the Spike protocol (Gomez *et al.*, 2002) written in Igor Pro (WaveMetrics, Lake Oswego, OR).

Statistical Analysis

Data were analyzed by the two-tailed Student's *t*-test using the Minitab software package (version 15, Minitab, Inc., State College, PA, USA). All results are presented as the mean $\pm$ the standard error of the mean. A statistical significance criterion of $\alpha = 0.05$ was used for all tests.

Results

MeCP2 protein expression in the mouse adrenal gland

As a first step towards defining the role of MeCP2 in adrenal medullary function, we examined the distribution of MeCP2 protein in wildtype (*Mecp2*^{+/y}) adrenal glands using immunocytochemical staining. Frozen sections from postnatal day (P)35 mouse glands were double-stained for MeCP2 and the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) to identify chromaffin cells (Fig. 1A). MeCP2 immunoreactivity was strongly expressed in the nuclei of TH-positive chromaffin cells distributed throughout the adrenal medulla and was also present at lower levels in (TH-negative) cortical cells. The staining exhibited a punctuate pattern characteristic of heterochromatic foci where MeCP2 protein is concentrated (Mullaney *et al.*, 2004) whereas control sections from *Mecp2* null mice showed no specific nuclear staining (Fig. 1A).

Adrenal gland morphology in *Mecp2* null mice

To establish whether or not loss of MeCP2 alters adrenal gland structure, we performed morphometric analyses of paraformaldehyde-fixed glands from P35 wildtype and *Mecp2* null mice. These studies revealed that, in mutants, the overall size of the adrenal tends to be smaller than in wildtypes, however, this difference is not statistically significant ($t_5 = -1.60$, $P = 0.17$; Fig. 1B). In contrast, the size of the medulla alone (mutant, $0.17 \pm 0.02\text{ mm}^3$ vs. wildtype, $0.26 \pm 0.03\text{ mm}^3$; $t_5 = -2.70$, $P = 0.04$), as well as the ratio of the medulla to the whole gland (mutant, $14.1 \pm 0.5\%$ vs. wildtype, $16.7 \pm 0.8\%$; $t_5 = -2.68$, $P = 0.04$), are reduced by 35% and 15%, respectively, compared to wildtype controls (Fig. 1B). These changes could be related in part to reduction in the body weight of mutants compared to wildtypes (mutant, 12.4 ± 3.02 grams vs. wildtype, 19.6 ± 1.5 grams). The decrease in medullary tissue volume was not due to decreased vascularity, as blood vessel volume was not significantly different between mutants and wildtypes (mutant, $0.038 \pm 0.007\text{ mm}^3$ vs. wildtype, $0.067 \pm 0.014\text{ mm}^3$; $t_4 = -1.88$, $P = 0.13$).

Adrenal and sympathetic ganglion catecholamine levels in *Mecp2* null mice

We next sought to determine whether or not reduced adrenal medullary tissue volume in mutant animals was associated with parallel changes in adrenal catecholamine content. To minimize the potentially confounding effects of handling stress on adrenal catecholamine levels, animals were pre-handled for three days and then anesthetized with isoflurane during gland removal. These experiments revealed significant decreases in both whole gland catecholamine content (Fig. 2A) and in the tissue concentration of catecholamines in mutant adrenals compared to wildtype controls (% wildtype values: dopamine, 58%, $t_{36} = -4.84$, $P = 0.00001$; norepinephrine, 66%, $t_{36} = -4.80$, $P = 0.00001$; epinephrine, 76%, $t_{31} = -2.61$, $P = 0.014$), without any change in the epinephrine/norepinephrine ratio (wildtype, 1.64; mutant, 1.81; $t_{34} = 1.56$, $P = 0.13$). To determine whether or not these changes are specific to the adrenal gland, we also examined catecholamine content in the sympathetic superior cervical ganglion (SCG). As with the adrenals, dopamine and norepinephrine content in the mutant SCG were significantly decreased compared to wildtype controls ($t_{10} = -4.00$, $P = 0.003$ and $t_{11} = -2.84$, $P = 0.016$, respectively; Fig. 2B). Epinephrine levels are negligible in the SCG and were not different between wildtype and mutant animals ($t_{14} = -0.59$, $P = 0.57$; data not shown).

Plasma catecholamine levels in *Mecp2* null mice

At rest, adrenal chromaffin cells primarily secrete epinephrine, and plasma epinephrine levels primarily reflect secretion from the adrenal medulla (Malmejac, 1964). To determine whether or not circulating epinephrine levels in mutants reflected the reduced content in the adrenal medulla, we compared plasma catecholamine content in wildtype and *Mecp2* null animals. Plasma samples were obtained under isoflurane anesthesia from the same animals used for adrenal catecholamine measurements. Surprisingly, mutant animals exhibited a nearly three-fold increase in the plasma level of epinephrine ($t_{21} = 3.05$, $P = 0.006$; Fig. 2C) and a two-fold increase in the ratio of epinephrine to norepinephrine ($t_{31} = 2.65$, $P = 0.013$; Fig. 2D) compared to wildtype controls, despite the sharp reduction in adrenal catecholamine content in the same animals. In addition, plasma dopamine levels were significantly increased nearly two-fold compared to wildtypes ($t_{29} = 2.32$, $P = 0.028$; Fig. 2C).

Altered quantal secretion kinetics in isolated *Mecp2* null chromaffin cells

Plasma epinephrine levels are elevated in *Mecp2* null mice compared to wildtype controls, despite a significant reduction in epinephrine content in the adrenal gland. This finding is consistent with our previous report that *Mecp2* null chromaffin cells exhibit enhanced basal and nicotine-evoked catecholamine exocytosis *in vitro* (Wang *et al.*, 2006). To investigate the voltage-dependent processes that may contribute to enhanced secretory function in *Mecp2* null cells, quantal catecholamine release was measured by carbon fiber amperometry. Cells were held at -80 mV in perforated patch voltage clamp and stimulated with action potential equivalent waveforms as previously described (Chan & Smith, 2001). Figure 3A shows representative raw traces for each cell type stimulated at 0.5 Hz, a condition designed to mimic splanchnic input under resting sympathetic tone (Brandt *et al.*, 1976; Kidokoro & Ritchie, 1980). A section of each record is expanded in Figures 3B and 3C and illustrates that, in *Mecp2*^{-/-} cells, each stimulus is more likely to evoke a secretory event than in wildtype cells. To quantify the periodicity of the secretory behavior in mutant and wildtype cells, we compared the power spectra of amperometric recordings of each genotype. These spectra revealed that *Mecp2*^{-/-} cells exhibited a higher amplitude signal at the 0.5 Hz stimulus frequency than in wildtype cells (Fig. 3C.ii). Thus we conclude that the *Mecp2*^{-/-} chromaffin cells exhibit tighter stimulus-secretion coupling.

To further characterize the mechanisms underlying the mutant secretory phenotype, we analyzed individual amperometric spikes, representing single granule fusion events, with respect to spike amplitude, slope, half-width, and total charge (Fig. 4). Cells were again stimulated at 0.5 Hz and 15 Hz to mimic physiologic sympathetic firing patterns of basal sympathetic tone and acute sympathetic activation, respectively (Brandt *et al.*, 1976; Kidokoro & Ritchie, 1980; Fulop *et al.*, 2005). At 0.5 Hz, stimulation evokes exocytosis through a restricted secretory fusion pore in which catecholamine release is limited and quantal size is reduced compared to full collapse exocytosis (Fulop *et al.*, 2005). In contrast, at 15 Hz, the secretory fusion pore dilates rapidly and does not act to limit the rate of catecholamine exocytosis (Fulop *et al.*, 2005); therefore, the mean spike charge at 15 Hz provides a measure of maximal granule catecholamine release. Thus, comparison of amperometric spike characteristics evoked at both frequencies can provide additional information about granule fusion behavior and modulation of fusion pore dilation and thus catecholamine release (Sugita, 2008).

At 0.5 Hz, *Mecp2* null cells exhibited an increase in mean spike slope (mutant, 2.6 ± 0.6 nA/s vs. wildtype, 1.2 ± 0.2 nA/s; $t_{927} = 2.17$, $P = 0.03$), amplitude (mutant, 7.4 ± 0.8 pA vs. wildtype, 4.8 ± 0.5 pA; $t_{1177} = 2.68$, $P = 0.008$), and total charge (mutant, 0.22 ± 0.01 pC vs. wildtype, 0.149 ± 0.009 pC; $t_{1201} = 4.60$, $P = 0.000002$), with no change in spike half-width (mutant, 37.3 ± 1.5 ms vs. wildtype, 33.8 ± 1.7 ms; $t_{1203} = 1.54$, $P = 0.12$) compared to wildtype cells. Thus, during transient fusion events, the secretory granules in *Mecp2* null cells release more catecholamine (increased total charge) and release faster (increased spike slope) than in wildtype cells.

At 15 Hz, *Mecp2* null and wildtype cells exhibited nearly identical spike charge (mutant, 0.329 ± 0.007 pC vs. wildtype, 0.315 ± 0.009 pC; $t_{4439} = 1.21$, $P = 0.23$), indicating that granule catecholamine content and thus maximal quantal size is unaffected by the mutation. However, *Mecp2* null cells exhibited significant increases in spike amplitude (mutant, 12.4 ± 0.6 pA vs. wildtype, 8.9 ± 0.4 pA; $t_{5316} = 5.05$, $P = 0$) and mean rate of spike rise (mutant, 4.3 ± 0.3 nA/s vs. wildtype, 2.0 ± 0.1 nA/s; $t_{4587} = 6.77$, $P = 0$), indicating that the rate of catecholamine release per secretory event was significantly higher in mutant cells. In contrast, the mean spike half-width was decreased in *Mecp2* null cells (mutant, 29.2 ± 0.7 ms vs. wildtype, 34.8 ± 0.9 ms; $t_{3768} = -4.82$, $P = 0.0000007$), indicating that the granules were emptying faster. Both results indicate a more rapid dilation of the granule fusion pore in *Mecp2* null cells compared to wildtype. Moreover, the fact that total spike charge is the same in wildtype and *Mecp2* null cells indicates that enhanced catecholamine release at 0.5 Hz is a consequence of the increase in the speed of fusion pore opening (and, perhaps, the size of the fusion pore) rather than an increase in granule catecholamine content. We further investigated this possibility by analyzing spike foot currents. Briefly, a foot current preceding the amperometric spike is broadly interpreted as catecholamine release through the fusion pore prior to granule collapse (Chow *et al.*, 1992). We analyzed foot current duration and charge in cells stimulated at 15 Hz and found both parameters significantly decreased in *Mecp2* null mice ($t_{737} = -3.18$, $P = 0.002$ and $t_{641} = -2.55$, $P = 0.011$, respectively; Fig. 4B.v). These data indicate that the fusion pore does indeed dilate faster in null mice compared to wildtype.

Chromaffin granule trafficking to the Readily Releasable Pool (RRP)

We previously found that enhanced catecholamine exocytosis in *Mecp2* null adrenal chromaffin cells is associated with an increase in the size of the secretory granule Readily Releasable Pool (RRP) (Wang *et al.*, 2006). To test the possibility that additional mechanisms related to vesicle trafficking to the RRP also influence the *Mecp2* null secretory phenotype, we compared the influence of stimulus pulse duration on vesicle fusion and catecholamine secretion in wildtype and mutant cells. In these experiments, isolated

chromaffin cells were patch clamped to a holding potential of -80 mV and then stimulated with a series of pulse depolarizations of increasing duration while monitoring cell capacitance (an index of granule fusion) as shown in Figure 5A. At short pulse durations, capacitance changes are limited to fusion of granules in the RRP (Horrigan & Bookman, 1994). However, at long pulse durations, the RRP is consumed and further secretion occurs through additional recruitment and fusion of reserve granules (Voets *et al.*, 1999). Analysis of membrane current recordings from cells stimulated with action potential waveforms showed a typical biphasic shape corresponding to the initial influx of voltage-dependent sodium current followed by voltage-dependent calcium current (Fig. 5A.i). Five milliseconds after the onset of the stimulus the sodium current inactivated, leaving only current due to calcium influx. Change in capacitance (Fig. 5A.ii) was measured by subtracting average capacitance values before and after the pulse. Eleven pulse durations were tested in increasing order (in ms: 5.5, 16.75, 27.5, 52.25, 77, 101.75, 151.25, 228.25, 401.5, 803, 1603.25), and cells were given 45 seconds between pulses to allow full recovery of the RRP (Voets *et al.*, 1999; Chan *et al.*, 2005). Figure 5B shows the results from 12 wildtype cells and 17 mutant cells. For the first seven pulses, *Mecp2* null cells consistently showed an increased change in capacitance compared to wildtype, with the largest differences in the plateau region at pulse widths of 52.25 ms (mutant, $\Delta C_m = 36 \pm 4$ fF vs. wildtype, $\Delta C_m = 24 \pm 2$ fF; $t_{13} = 2.56$, $P = 0.024$) and 77ms (mutant, $\Delta C_m = 41 \pm 5$ fF vs. wildtype, $\Delta C_m = 25 \pm 2$ fF; $t_{14} = 3.03$, $P = 0.009$), where quantal content is limited proportionally to the number of release-ready granules (comprising the RRP). This region of the plot is shown expanded in the inset in Figure 5B. These results indicate a 45–50% increase in the RRP in *Mecp2* null cells, consistent with previous results (Wang *et al.*, 2006). However, wildtype and mutant cells showed no difference in cell capacitance in response to longer pulse durations at which recruitment of new granules from the Reserve Pool (RP) to the RRP is required to sustain continued granule fusion. These data indicate, therefore, that enhanced secretion in mutant cells is not due to increased recruitment of granules to the RRP.

Discussion

The present study reveals a complex phenotype affecting adrenal medullary function in *Mecp2* null mice. On the one hand, medullary volume and tissue concentrations of dopamine, norepinephrine and epinephrine are significantly reduced in mutants compared to wildtype controls. However, plasma epinephrine levels are significantly increased, at least under conditions of isoflurane anesthesia. These seemingly paradoxical results may be explained by our finding that isolated *Mecp2* null chromaffin cells are hypersecretory, as indicated by 1) enhanced stimulus-secretion coupling, 2) more rapid efflux of catecholamines per granule fusion event, and 3) an increase in the amount of catecholamine released per secretory event under conditions that simulate resting levels of sympathetic activity, i.e., electrical stimulation at 0.5 Hz. Thus, in addition to an increase in the size of the RRP of catecholamine granules (Wang *et al.*, 2006), our data highlight abnormal fusion pore regulation as a previously unrecognized mechanism by which loss of MeCP2 disrupts peripheral catecholamine homeostasis.

Relatively little is known about the genetic control of secretory granule kinetics. It is possible that the secretory granule phenotype of *Mecp2* null mice reflects mechanistic alterations in multiple regulatory steps linking chromaffin cell activation to elevated catecholamine release. Indeed, recent studies indicate that, in the nervous system, many hundreds of genes are dysregulated in the absence of MeCP2 (Jordan *et al.*, 2007; Chahrour *et al.*, 2008), and preliminary expression profiling studies in our laboratory indicate that the same is true in the adrenal medulla (unpublished data). One of the most striking features of granule behavior in *Mecp2* null cells is the marked increase in the rate of transmitter release,

and the more rapid opening of the fusion pore. Thus, at least one consequence of MeCP2 loss appears to be alteration of post-fusion events. The speed of pore opening is subject to regulation by diverse molecules that influence fusion of the granule and plasma membranes, including synaptobrevin 2 (Syb2; Bretou *et al.*, 2008), Munc-18 (Fisher *et al.*, 2001), Regulator of Calcineurin Activity 1 (RCAN1; Keating *et al.*, 2008), Cysteine String Protein (Csp; Graham & Burgoyne, 2000) and Calcium-dependent Activator Protein for Secretion (CAPS; Elhamdani *et al.*, 2000). It is also possible that loss of MeCP2 disrupts expression of proteins comprising the dense core matrix, thereby altering granule swelling and release kinetics (Angleon *et al.*, 1999; Amatore *et al.*, 2000). Studies in progress are attempting to define potential links between MeCP2 signaling and expression of specific molecules involved in regulation of secretory granule fusion kinetics.

We demonstrate here, for the first time, that plasma epinephrine and the epinephrine/norepinephrine ratio are increased in *Mecp2* null mice. These results are consistent with our finding that isolated chromaffin cells, devoid of any sympathetic input, are hypersecretory. This hypersecretory phenotype may also alter the sensitivity of *Mecp2* null mutants to stress, particularly to isoflurane anesthesia which has been shown to increase circulating catecholamines in human patients (Nishiyama, 2005). It is interesting in this context that mice carrying a truncation mutation in *Mecp2* exhibit elevated plasma levels of adrenal corticosteroids in response to stress (McGill *et al.*, 2006). In these animals, elevated secretion was linked to increased expression of corticotrophin releasing hormone in the hypothalamus and, thereby, increased activation of the hypothalamic-pituitary-adrenal axis. Therefore, it is possible that the elevated level of plasma epinephrine that we observe here in *Mecp2* null mutants also reflects an exaggerated response to stress resulting from enhanced secretory granule function.

Derangements of autonomic control, and cardiovascular regulation in particular, are prominent features of epinephrine excess. This has been documented particularly in patients with pheochromocytoma (Bravo, 1991; Bianchi *et al.*, 1995; Bravo & Tagle, 2003; Zapanti & Ilias, 2006) or experimental subjects infused with exogenous epinephrine (Johansson *et al.*, 1988; Hansen *et al.*, 1990). The cardiorespiratory sequelae of epinephrine excess exhibit striking overlap with several of the dysautonomic features of RTT. Most notably, common features include cardiac arrhythmias, prolonged corrected Q-T interval, and sudden death (Acampa & Guideri, 2006; Weese-Mayer *et al.*, 2006). Epinephrine excess can also be associated with paroxysmal hypertension, as in some patients with pheochromocytoma, resulting from episodic spikes in adrenal secretion (Bravo, 1991; Bianchi *et al.*, 1995; Bravo & Tagle, 2003; Zapanti & Ilias, 2006). Although resting mean arterial pressure appears to be normal in RTT patients (Julu & Witt Engerstrom, 2005), blood pressure lability over time has not been studied in these patients in detail.

In RTT patients, autonomic dysfunction has been attributed to an apparent imbalance between sympathetic and parasympathetic tone that results in a relative “hyperactivity” of the sympathetic nervous system (Julu *et al.*, 2001; Acampa & Guideri, 2006; Acampa *et al.*, 2008). This imbalance has been suggested to result from brainstem immaturity leading to decreased parasympathetic tone (Julu *et al.*, 1997b, a). Our data suggest an alternative (or complementary) hypothesis, i.e., that elevated catecholamine secretion from the adrenal glands contributes to increased peripheral sympathetic tone in RTT patients. In addition to cardiac dysautonomia, RTT patients exhibit other symptoms consistent with abnormalities in the sympathoadrenal axis, including peripheral vasoconstriction and limb hypothermia (Naidu *et al.*, 1987). Thus, our findings raise the possibility that therapeutic strategies aimed at reducing peripheral epinephrine signaling may be of value for RTT patients. However, any generalized strategy aimed at reducing catecholamine signaling could exacerbate the reduction in norepinephrine observed in the CNS of RTT patients and *Mecp2* null mice (for

review see Ogier & Katz, 2008). Thus, a more targeted approach, specific for peripheral epinephrine, may be warranted.

Although changes in brain monoamine levels or monoamine synthesizing enzymes have been documented in RTT patients (Riederer *et al.*, 1985; Zoghbi *et al.*, 1985; Brucke *et al.*, 1987; Lekman *et al.*, 1989; Zoghbi *et al.*, 1989; Saito *et al.*, 2001; see, however, Perry *et al.*, 1988; Lekman *et al.*, 1990) and *Mecp2* null mice, virtually nothing is known about peripheral catecholamine levels in RTT patients. Our data indicate that defining peripheral catecholamine homeostasis in RTT patients may be critical to understanding autonomic pathophysiology in this disease. More generally, this is to our knowledge the first genetic disease model characterized by enhanced dense core granule function and adrenal hypersecretion of catecholamines.

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Abbreviations

BSA	bovine serum albumin
HPLC	high performance liquid chromatography
MeCP2	methyl-CpG-binding protein 2
P35	post-natal day 35
PBS	phosphate-buffered saline
RP	reserve pool
RRP	readily releasable pool
RTT	Rett syndrome
SCG	superior cervical ganglion
TH	tyrosine hydroxylase

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Figure 1.

A) MeCP2 and TH immunostaining in the adrenal gland. The panel to the left shows an adrenal gland section from a wildtype mouse double-stained for MeCP2 (green) and TH (red). Magnification x10. The panel on the right shows the same adrenal section at a higher magnification (x40) near the boundary between adrenal cortex (C) and medulla (M) to better illustrate the punctate MeCP2 stain localized to the nucleus and the diffuse TH staining, which is only present in the cytoplasm of the chromaffin cells. The inset shows a corresponding exposure-matched section from a *Mecp2* null mouse, demonstrating a positive TH staining but a lack of specific MeCP2 staining. Scale bar, 250μm. B) Adrenal gland morphometric analysis showing the average volumes of the whole gland and medulla from wildtype and mutant animals. Though mutant glands tended to be smaller, the difference in whole gland volume was not statistically significant. The ratio of medulla volume to whole gland volume was calculated separately for each animal and averaged. Mutant animals showed a significant decrease in the volume of the adrenal medulla and the volume ratio. Results are mean ± SEM. * $P < 0.05$, Student's *t*-Test, $n = 4$.

Figure 2.

Tissue and plasma catecholamine levels. A) Catecholamine concentration in adrenal gland tissue from n=20 wildtype (*Mecp2*^{+/y}) and n=19 mutant (*Mecp2*^{-/y}) animals. Mutant glands had significantly reduced levels of norepinephrine (NE), epinephrine (E), and dopamine (DA) compared to wildtype controls. Note that the axis to the right with the expanded scale pertains to the DA measurements. B) Catecholamine content of superior cervical ganglion (SCG) pairs from n=9 wildtype and n=10 mutant animals. Similar to the adrenal glands, mutant SCGs had significantly reduced NE and DA compared to wildtype SCGs. C) Plasma catecholamine concentration from n=17 wildtype and n=17 mutant animals. In contrast to the tissues, mutant animals exhibited increased levels of catecholamines compared to wildtype controls, with significant increases in E and DA. D) Ratio of epinephrine concentration to norepinephrine concentration to normalize for varying levels of stress among different animals. Mutant animals had a significant increase in the E/NE ratio despite the fact that NE levels also tended to be elevated, indicating that the increase in plasma E is not due to an increase in global sympathetic discharge alone. Results are mean $\pm$ SEM.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-Test.

Figure 3.

Increased stimulus-secretion coupling in *Mecp2* null mice. A) Typical amperometric recordings from a wildtype (*Mecp2*^{+/y}) and mutant (*Mecp2*^{-/y}) adrenal chromaffin cell stimulated with a 0.5Hz train of action potential equivalents (APE). B) An expansion of the raw data (bottom traces) aligned with the stimulus template (top trace). The dotted box indicates a region with characteristic failures in the wildtype mice and tight stimulus-secretion coupling in the null mice. C,i) A further expansion of the dotted box in B provides a higher temporal resolution example of this behavior. C,ii) Spectral analysis (power spectrum) of pooled traces (n = 5 and 7 for *Mecp2*^{+/y} and *Mecp2*^{-/y}, respectively).

Figure 4.

Comparison of *Mecp2* null and wildtype spike parameters. A) Schematic of an amperometric spike illustrating the parameters used for analysis. Spike amplitude (I_{amp}) is measured as the height of the spike from baseline. Spike charge is determined by integrating the recorded current over the duration of the spike. Spike slope is measured as the slope of the line through points on the amperometric trace that are 35% and 65% of the maximum amplitude. Spike half-width ($t_{1/2}$) is the width of the spike (in ms) at the point in the amperometric trace that is at half the maximum amplitude. B) Summary of data showing that mutant cells exhibit increased spike amplitude, charge, and slope when stimulated at 0.5 Hz and increased amplitude and slope but decreased half-width when stimulated at 15 Hz. Summary data also demonstrate a smaller and shorter foot duration under 15 Hz stimulation in *Mecp2* null mice compared to wildtype. Results are the mean $\pm$ SEM from $n=10$ wildtype cells (+/y; 465 spikes at 0.5 Hz, 2068 spikes at 15 Hz) and $n=11$ mutant cells (-/y; 814 spikes at 0.5 Hz, 3538 spikes at 15 Hz). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Student's *t*-Test.

Figure 5.

Cell capacitance measurements with increasing pulse duration. A) Protocol used to measure change in cell capacitance and total calcium influx. Chromaffin cells were stimulated with a pulse depolarization, evoking the influx of both sodium and calcium currents (i). The fast sodium current (I_{Na}) decays as these channels are inactivated, leaving only the voltage-dependent calcium current (I_{Ca}). A sinusoidal waveform before and after the pulse stimulus is used to determine the cell capacitance (ii), and the change in cell capacitance (ΔC_m) is calculated to measure net exocytosis. B) ΔC_m for each pulse stimulus of increasing duration. The inset is a magnification of the area highlighted, showing the largest differences between wildtype ($Mecp2^{+/y}$) and null ($Mecp2^{-/y}$) cells in the plateau region, consistent with an increased readily releasable pool in the mutant. ΔC_m at longer pulses was not different between mutant and wildtype cells, indicating no change in vesicle trafficking from the reserve pool. Results are the mean $\pm$ SEM from $n=12$ wildtype and $n=17$ mutant cells. * $P<0.05$, ** $P<0.01$, Student's t -Test.