Intrinsic membrane properties of locus coeruleus neurons in Mecp2-null mice

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Zhang X, Cui N, Wu Z, Su J, Tadepalli JS, Sekizar S, Jiang C. Intrinsic membrane properties of locus coeruleus neurons in Mecp2-null mice. Am J Physiol Cell Physiol 298: C635–C646, 2010. First published December 30, 2009; doi:10.1152/ajpcell.00442.2009.—Rett syndrome caused by mutations in methyl-CpG-binding protein 2 (Mecp2) gene shows abnormalities in autonomic functions in which brain stem norepinephrinergic systems play an important role. Here we present systematic comparisons of intrinsic membrane properties of locus coeruleus (LC) neurons between Mecp2-/- and wild-type (WT) mice. Whole cell current clamp was performed in brain slices of 3- to 4-wk-old mice. Mecp2-/- neurons showed stronger inward rectification and had shorter time constant than WT cells. The former was likely due to overexpression of inward rectifier K+ (KIR)4.1 channel, and the latter was attributable to the smaller cell surface area. The action potential duration was prolonged in Mecp2-/- cells with an extended rise time. This was associated with a significant reduction in the voltage-activated Na+ current density. After action potentials, >60% Mecp2-/- neurons displayed fast and medium afterhyperpolarizations (FAHP and MAHP), while nearly 90% WT neurons showed only MAHP. The MAHP amplitude was smaller in Mecp2-/- neurons. The firing frequency was higher in neurons with MAHP, and the frequency variation was greater in cells with both FAHP and MAHP in Mecp2-/- mice. Small but significant differences in spike frequency adaptation and delayed excitation were found in Mecp2-/- neurons. These results indicate that there are several electrophysiological abnormalities in LC neurons of Mecp2-/- mice, which may contribute to the dysfunction of the norepinephrine system in Rett syndrome.

Rett syndrome; brain stem; norepinephrine; passive membrane properties; repetitive firing activities

RETT SYNDROME (RTT), one of the autism spectrum disorders, is caused by defects in the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2) and affects 1 in every 10,000 live births of females (4). Girls with RTT develop normally for a couple of months after birth and then start losing acquired speech and learning skills, followed by progressive development of locomotive problems, mental retardation, breathing disturbance, and autistic symptoms (14). MeCP2 deficiency underlies these neurological and neuropsychiatric disorders, which is confirmed in Mecp2-knockout mice (2, 13). MeCP2 is expressed in mature neurons. Functionally, it acts primarily as a transcriptional repressor, targeting genes essential for neuronal survival, dendritic growth, synaptogenesis, and synaptic plasticity (5, 11, 33). How MeCP2 deficiency leads to numerous brain dysfunctions in RTT remains poorly understood.

Defects in brain stem monoaminergic systems, especially the norepinephrine (NE) system, have been shown to play a role in the development of neuropsychiatric disorders in RTT. The NE system modulates a variety of systemic functions and behaviors through innervations of widespread brain regions; defects in the NE system account for a number of neurological and psychiatric diseases, and pharmacological interventions in the NE system are some of the most effective therapeutic approaches. The locus coeruleus (LC) in the anterior pons contains a cluster of NEergic neurons and provides >70% of noradrenergic innervations in the central nervous system (CNS). These NEergic neurons project to the frontal cortex, hippocampus, cerebellum, spinal cord, and other brain stem nuclei (3, 8), areas that are involved in cognition, attention, anxiety, psychosis, motor control, cardiorespiratory functions, arousal status, and stress response (10, 15, 36, 40, 41). The consistency of NEergic projections with the consequences of MeCP2 deficiency strongly suggests that defects in the NEergic neurons contribute to RTT (23). Indeed, NE metabolites and NE content are drastically reduced in cerebrospinal fluid and brain tissue of RTT patients and Mecp2-null mice (16, 48). Application of desipramine, a selective NE uptake inhibitor, extends the life span of Mecp2-null mice (28, 46). Therefore, understanding how the MeCP2 disruption affects the LC neurons can yield information of diagnosis and therapeutics for RTT.

One potential mechanism that may play a role in the NE system defects is the impairment of intrinsic properties of LC neurons. Abnormal intrinsic properties of the LC neurons may in turn lead to dysfunction of NEergic neurotransmission and neuromodulation. To test the hypothesis, systemic studies of the intrinsic properties of LC neurons were performed in brain slices of male wild-type (WT) and Mecp2-null (Mecp2-/-) mice. Our results showed that the Mecp2 disruption caused distinct defects in the intrinsic properties of the LC neurons.

MATERIALS AND METHODS

Animal Breeding and Genotyping

Female heterozygous Mecp2+/Y mice on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME) and used to generate Mecp2-/- mice. To produce Mecp2-/- mice with green fluorescent protein (GFP) expression in LC (GFP-LC) neurons, heterozygous Mecp2+/Y females were cross-bred with homozygous Prp57 males, a gift generously provided by Dr. Anthony N van den Pol (Dept. of Neurosurgery, Yale University School of Medicine, New Haven, CT). Both homozygous and heterozygous Prp57 mice express GFP specifically and stably in LC neurons. The endogenous expression of GFP does not produce any evident morphological and physiological change in these GFP-LC neurons (38). To confirm the absence of Mecp2 gene, the offspring were routinely genotyped with a PCR protocol provided by the Jackson Laboratory. Only male F1 generation mice were used in the present study. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Georgia State University Institutional Animal Care and Use Committee.

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Table 1. **RT-PCR primers**

<table>
<thead>
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<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Accession No.</th>
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<td>K_\text{v}4.1</td>
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<td>F: TGGCTGAGTATGGCTAGGAG&lt;br&gt;R: ACCAAGAAATGACGTGAG</td>
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K_\text{v}, inward rectifier K⁺; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

**Brain Slice Preparation**

Brain slices were obtained from GFP-expressing *Mecp2*-/⁻ mice and their *Mecp2*²⁻/²⁻ (wild-type) littermates. Mice at 3–4 wk of age were anesthetized by inhalation of saturated isoflurane and decapitated. The brain stem was removed rapidly and kept in ice-cold sucrose-containing artificial cerebrospinal fluid (sucrose-aCSF) oxygenated with 95% O₂-5% CO₂, containing (in mM) 200 sucrose, 3 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 d-glucose, at pH 7.40. Transverse pontine sections (300 μm) containing the LC were obtained with a vibratome (1000 Plus, Vibratome, St. Louis, MO) in sucrose-aCSF. The slices were collected in oxygenated normal aCSF with 124 mM NaCl and without 3 mM CaCl₂ at room temperature. A typical protocol started from a −80-mV holding potential with an increment of 10 mV. Series resistance was 85% compensated. Na⁺ currents were obtained by subtraction of current traces recorded with and without 3 μM tetrodotoxin (TTX). Current density was analyzed by normalizing the current amplitude to the whole cell capacitance. Steady-state activation was described as a function of normalized conductance (G/G_{max}) at different command potentials (V) with the Boltzmann equations: normalized G = G_{max}/[1 + exp [−(V - V_{1/2})/k]], where D_{max} is the maximum delay, V is the membrane voltage, V_{1/2} is the half-activation voltage, and k is the slope factor.

**Acute Dissociation of LC Neurons**

Pontine sections containing LC were obtained and maintained as described above. The sections were digested for 1 h with trypsin (0.25%, Sigma type XI) in HEPES buffer bubbled with oxygen at 35°C. The HEPES buffer contained (in mM) 140 NaCl, 2.5 KCl, 1 MgCl₂, 1 CaCl₂, 25 d-glucose, and 10 HEPES, pH 7.40. Then the sections were transferred to oxygenated HEPES buffer containing 1 mg/ml trypsin inhibitor and 1 mg/ml BSA. The LC area was micropunched and dissociated by gentle trituration with fire-polished Pasteur pipettes in HEPES buffer. Cells were plated in 35-mm petri dishes and kept at room temperature for 10 min before being observed with Hoffman modulation optics.

**Whole Cell Recording**

Whole cell current clamp and voltage clamp were performed in brain slices and acutely dissociated LC neurons, respectively. Patch pipettes were pulled with a tip resistance of 3–5 MΩ. The internal (pipette) solution for current clamp contained (in mM) 130 K-glucocinate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, and 0.4 EGTA (pH 7.3). For voltage clamp, 140 mM CsCl was substituted for K⁺ in the pipette solution. Recorded signals were amplified with an Axopatch 200B amplifier (Molecular Devices), digitized at 10 kHz, filtered at 2 kHz, and collected with the Clampex 8.2 data acquisition system (Molecular Devices).

In current clamp, only neurons with stable resting membrane potentials more negative than −40 mV and action potential (AP) amplitude >65 mV were considered to be healthy and used for further studies, because they can be stably recorded for >15 min, which is adequate for our experimental protocol (−5 min). The current-voltage (I–V) relationship was studied by injecting a series of pulse hyper- and depolarizing currents. A typical protocol started from −0.15 nA with an increment of 0.02 nA. The input resistance of individual neurons was calculated by averaging the ratio of membrane potentials at the last 100 ms of each command current. The time constant was then determined at membrane potential about −100 mV. All parameters of APs were determined after averaging at least 20 APs without any stimulus currents. AP amplitude was measured from threshold to peak. The threshold of AP was determined at the AP initiation point, and AP duration was analyzed at half of the AP amplitude (D_{1/2}). The amplitude of an afterhyperpolarization (AHP) was measured from AP threshold to the lowest hyperpolarizing potential of the AHP. The postinhibition rebound (PIR) was determined by the presence of depolarization or firing activity after a series of hyperpolarizing current pulses from −120 mV to −40 mV. Spike frequency adaptation (SFA) was examined by injecting a series of depolarizing currents. Peak firing frequency (F_p), steady-state frequency (F_s), and their ratio were then quantified in each cell. Delayed excitation (DE) was shown as the time delay between the end of hyperpolarizing command and the appearance of the first AP, and was described as a function of the conditioning hyperpolarization with Boltzmann equations with a half-maximal delay and a slope factor presented. The equation used was as follows: D = D_{max}[1 + exp [−(V - V_{1/2})/k]], where D_{max} is the maximum delay, V is the membrane voltage, V_{1/2} is the half-activation voltage, and k is the slope factor. In voltage clamp, the cells with GFP dissociated from the LC area were recorded in HEPES buffer containing 50 μM CdCl₂ at room temperature. A typical protocol started from a −80-mV holding potential with an increment of 10 mV. Series resistance was 85% compensated. Na⁺ currents were obtained by subtraction of current traces recorded with and without 3 μM tetrodotoxin (TTX). Current density was analyzed by normalizing the current amplitude to the whole cell capacitance. Steady-state activation was described as a function of normalized conductance (G/G_{max}) at different command potentials (V) with the following Boltzmann equation: normalized G = G_{max}/[1 + exp [−(V - V_{1/2})/k]], where D_{max} is the maximum delay, V is the membrane voltage, V_{1/2} is the half-activation voltage, and k is the slope factor.

**Table 2. Real-time PCR primers**

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<td>Na_1.2</td>
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<td>GAPDH</td>
<td>F: TGGGGCGGCTGATGCTGTAAC&lt;br&gt;R: GCAGGGCTGATGCTGTAAC&lt;br&gt;CAT</td>
<td>NM_008084</td>
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TASK, 2-pore domain weakly inward-rectifying K⁺; Na_1, voltage-gated Na⁺.
was expressed as a function of normalized current ($I/I_{\text{max}}$) at different conditioning potentials (150 ms) with Boltzmann equations as follows: normalized $I = I/[1 + \exp [-(V - V_\text{1/2}) / \Delta V]]$.

Electrophysiological data were analyzed with Clampfit 9.2 software (Molecular Devices). Data are presented as means ± SE. Correlation analysis was performed to assess the relationship between two parameters. The proportional difference was analyzed with the Student's $t$-test. Similar resting membrane potentials (C) and input resistance (E) were observed in both groups. The time constant ($\tau$) was reduced in $\text{Mecp}^2-\gamma$ cells ($P; *** P < 0.001$), suggesting that the surface area is smaller in these mice. Cross-sectional areas were measured in both WT (G) and $\text{Mecp}^2-\gamma$ (H) cells. Statistical analysis indicates that $\text{Mecp}^2-\gamma$ cells are significantly smaller ($P; *** P < 0.001, n = 63$).
(38), the expression of GFP was found in almost all neurons clustered in the LC area of both WT and Mecp2<sup>−/−</sup> mice, and >95% of the cells expressed tyrosine hydroxylase (Zhang X and Jiang C, unpublished observation). The GFP-LC neurons of both groups displayed similar morphology, although they appeared smaller in Mecp2<sup>−/−</sup> mice (see below).

**Passive Membrane Properties of LC Neurons in Mecp2<sup>−/−</sup> and WT Mice**

The intrinsic membrane properties of the LC neurons of Mecp2<sup>−/−</sup> and WT mice were studied in brain slices. Whole cell current clamp was performed in LC neurons identified according to brain stem location and GFP fluorescence. A total of 99 LC neurons from 10 WT and 11 Mecp2<sup>−/−</sup> mice were analyzed in the present study. Passive membrane properties of LC neurons were studied in Mecp2<sup>−/−</sup> mice and their male WT littersmates.

**Membrane potential.** Immediately after the formation of the whole cell patch configuration, LC neurons showed a brief period (1–2 min) of depolarization, and then the membrane potential was stabilized at a level for a period of up to 90 min. At steady state, the membrane potential averaged −53.8 ± 0.7 mV (n = 47) in WT neurons and −53.7 ± 0.7 mV (n = 52) in Mecp2<sup>−/−</sup> neurons. Statistically, no significant difference was found between the two groups (Fig. 1, A and C), indicating that the ionic current determining the resting membrane potential is not affected by Mecp2 knockout.

**Rectification.** The relationship of current versus membrane potential (I–V) was studied by injecting a series of hyperpolarizing current pulses through the recording pipette. Consistent with previous reports in rat LC neurons (44), a nonlinear I–V relationship was observed in most Mecp2<sup>−/−</sup> and WT neurons as shown in Fig. 1, A and B. Of 99 cells studied (47 from Mecp2<sup>−/−</sup> and 52 from WT), all but 2 cells in Mecp2<sup>−/−</sup> and 2 cells in WT showed a nonlinear I–V relationship. Inward rectification (IR) was found with hyperpolarizing currents, particularly when the membrane was more hyperpolarizing than −100 mV. The rectification ratio calculated by dividing input resistance at −70 mV by that at −100 mV was significantly greater in Mecp2<sup>−/−</sup> neurons than in WT neurons (P < 0.01, Fig. 1D). Therefore, Mecp2<sup>−/−</sup> neurons have stronger IR than WT cells.

The extra components for the IR were isolated by averaging I–V relationship records of a dozen cells as shown in Fig. 1A followed by a subtraction. The difference between Mecp2<sup>−/−</sup> and WT cells showed strong IR (Fig. 2, A–C), suggesting that Kir channels with strong IR strengths are affected with the Mecp2 deficiency. Pharmacological identification of Kir channel species was not attempted owing to the lack of specific blockers. To further understand the ionic mechanism undergoing stronger IR in Mecp2<sup>−/−</sup> neurons, we analyzed the expression of the most representative members in the Kir channel family by qPCR. In addition, we examined the expression of TASK1 and TASK3 transcripts because they are often ex-
pressed in the same brain stem neurons together with Kir channels. In agreement with previous reports (27, 37, 45), several subtypes of Kir channels and TASK channels were expressed in LC neurons. Among them, the expression of Kir4.1 and Kir5.1 was readily detected in LC with RT-PCR. The intensity of Kir4.1 was stronger in Mecp2−/− mice than in WT mice (Fig. 2D). In qPCR analysis, Kir4.1 was the most prominent Kir species in the LC area in both WT and Mecp2−/− mice. Expression of Kir3.1, Kir3.2, Kir3.3, Kir5.1, TASK1, and TASK3 was also observed, although their expression levels were 10 to 10,000 times lower than that of Kir4.1. The expression of other Kir channels was undetectable (Fig. 2E). Compared with WT, the level of Kir4.1 transcript was increased by 50% in Mecp2−/− LC (n = 22; P < 0.01). Meanwhile, significant reductions in TASK1 (P < 0.05) and TASK3 (P < 0.01) mRNA levels were observed (Fig. 2F). Since Kir4.1 is a strong inward rectifier, its overexpression thus is consistent with the stronger IR in Mecp2−/− neurons.

Input resistance. Input resistance was measured with hyperpolarizing pulses after steady membrane potential and firing activity were reached. At slightly hyperpolarizing membrane potentials (about −70 mV) produced with current injections, the input resistance of the LC neurons averaged 492 ± 26 MΩ (n = 47) in WT and 543 ± 32 MΩ (n = 52) in Mecp2−/− neurons (Fig. 1E). Although input resistance appeared greater in Mecp2−/− neurons than in WT neurons, no significant difference was found (P > 0.05). Input resistance decreased at more hyperpolarizing membrane potentials (about −80 mV to −100 mV) because of IR, while significant difference was still not found between these two groups of LC neurons (Fig. 1E).

Time constant. The time constant was measured by fitting the membrane potential trajectory during hyperpolarizing current injection with a single exponential equation. The time constant was −30% shorter in Mecp2−/− neurons (23.3 ± 1.0 ms, n = 52) than in WT neurons (33.3 ± 1.6 ms, n = 47; P < 0.001), suggesting that the surface area of the Mecp2−/− neurons is smaller (Fig. 1F). To prove this, we measured the cross-sectional area of GFP-labeled LC neurons. Our results indicated that the size of the Mecp2−/− neurons was indeed significantly smaller than that of the WT cells (Fig. 1, G–I).

Action Potential Morphology and Threshold

The majority of LC neurons in both groups (97 of 99) displayed spontaneous firing activity (Fig. 3, A and B). Several parameters of APs were measured in 89 neurons, including AP amplitude measured from threshold to peak, threshold, duration at 50% amplitude (D1/2), rise time, and decay time. Compared with WT
neurons, APs of the Mecp2−/− neurons exhibited similar amplitude (89.20 ± 1.17 mV in WT vs. 88.58 ± 1.17 mV in Mecp2−/−; $P > 0.05, n = 89$), lower threshold ($-40.29 ± 0.46$ mV vs. $-42.44 ± 0.32$ mV; $P < 0.001, n = 89$), longer $D_{1/2}$ (1.05 ± 0.02 ms vs. 1.12 ± 0.02 ms; $P < 0.05, n = 89$), and more extended rise time ($0.63 ± 0.01$ ms vs. 0.70 ± 0.02 ms; $P < 0.01, n = 89$) (Fig. 3, C–F). The decay time, however, did not show any difference between the two groups of cells (Fig. 3F). $D_{1/2}$ had strong correlation with the AP rise time (Fig. 3f). The trend was described with a linear regression with $r = 0.83$ ($P < 0.001, n = 40$) for the WT neurons and $r = 0.84$ ($P < 0.001, n = 40$) for the Mecp2−/− neurons. However, there was no significant correlation ($P > 0.05$) between $D_{1/2}$ and membrane potentials or between $D_{1/2}$ and the AP threshold (Fig. 3, G and H). Therefore, it is very likely that the longer $D_{1/2}$ of Mecp2−/− neurons is a result of the increased rise time.

The broadening of AP duration associated with an extended rise time with no significant difference in the AP decay time between WT and Mecp2−/− neurons suggests that Na+ currents are affected. Therefore, we performed whole cell voltage clamp in freshly dissociated LC neurons showing GFP fluorescence (Fig. 4, A and B). In these cells inward currents with fast onset and fast inactivation were observed (Fig. 4C). They were confirmed to be Na+ currents because they were eliminated in Na+-free extracellular solution or by 3 μM TTX. Current density was analyzed by normalizing the inward current amplitude to the whole cell capacitance. Our data indicated that the Na+ current density was significantly lower in Mecp2−/− neurons than in WT neurons ($P < 0.05, n = 15$) (Fig. 4D).

The expression level of two representative TTX-sensitive Na+ channel α-subunits that are prominently expressed in CNS and responsible for transient Na+ currents underlying AP upstroke, Na1.1 and Na1.2, was further examined. Our RT-PCR (Fig. 4E) and qPCR (Fig. 4F) analyses showed that expression of Na1.2 was significantly reduced in Mecp2−/− neurons ($P < 0.001, n = 18$), while no significant change in Na1.1 was found. In contrast, the steady-state activation and inactivation of the TTX-sensitive Na+ currents did not show evident difference between WT and Mecp2−/− neurons (Fig. 4, G and H). Together, our data suggest that the smaller Na+ currents in the Mecp2-null neurons are very likely to be due to the lower expression of Na1.2 channels rather than alternations in Na+ channel kinetics.

### Afterhyperpolarizations

Immediately after a spontaneous AP, LC neurons showed large AHPs. In more than half of the neurons (59 of 89), the AHPs had a single phase and reached maximum hyperpolarization in a few milliseconds with the decay time constant in hundred milliseconds, consistent with previously reported mAHP (34). The rest displayed two AHP components, i.e., AHPs with a fast onset (fAHP) followed by an mAHP with a brief afterdepolarization in between (Fig. 5, A–C). fAHP and mAHP have distinct kinetics and ionic bases, affecting AP morphology and repetitive firing activity of neurons (12, 19). Since the different composition of AHPs may lead to distinctive membrane properties, we tentatively divided LC neurons into two types based on the appearance of the AHPs, i.e., type A neurons with mAHP only and type B neurons with both fAHP and mAHP. Nearly 90% of WT (41/45) neurons belonged to the category of type A cells, while ~60% of Mecp2−/− cells (26/44) fell into the category of type B cells (Fig. 5D). The proportional difference of these cell types in the WT and Mecp2−/− mice was statistically significant ($P < 0.001, n = 89; \chi^2$-test).
The fAHP is known to accelerate AP repolarization and shorten AP duration (12, 19). In \textit{Mecp2}/H11002/Y mice, indeed, type B cells showed shorter AP decay time than type A cells (0.72 ± 0.02 ms vs. 0.89 ± 0.05 ms, \( P < 0.001 \), \( n = 44 \)) (Fig. 5L). Since LC neurons in both groups have similar rise times (Fig. 5K), type B cells showed a shorter \( t_{D1/2} \) (1.08 ± 0.03 ms vs. 1.18 ± 0.04 ms, \( P < 0.05 \), \( n = 44 \)) (Fig. 5J). No statistical difference in AP decay time was found in type A neurons between \textit{Mecp2}/H11002/Y and WT mice (0.89 ± 0.05 ms vs. 0.80 ± 0.02 ms, \( P > 0.05 \), \( n = 59 \)) (Fig. 5L), suggesting that the overall decay time of APs in \textit{Mecp2}/H11002/Y neurons does not seem to be affected much by the presence of fAHP.

The amplitude of mAHP was significantly smaller in \textit{Mecp2}/H11002/Y cells than in the WT neurons [24.77 ± 0.81 mV (\( n = 44 \)] vs. 27.02 ± 0.66 mV (\( n = 45 \)); \( P < 0.05 \)] (Fig. 5E), while the time constant of the mAHP did not show a significant difference (Fig. 5F). In addition, the amplitude of mAHP did not show significant correlation (\( P > 0.05 \)) with membrane potentials, AP amplitude, and the time constant of mAHP (Fig. 5, G–I).

Repetitive Firing Properties

Spontaneous firing was found in most LC cells (only 1 silent cell in WT and 2 in \textit{Mecp2}/H11002/Y). No burst firing activities were found in either group of mice. Several repetitive firing properties were studied, including firing frequency, frequency variation, SFA, delayed excitation, and postinhibition rebound.

Spontaneous firing frequency. Since most LC neurons in both groups showed spontaneous firing activity, their firing frequency was analyzed without current injection (Fig. 6A). At steady state, firing frequency of WT neurons averaged 3.6 ± 0.3 Hz (\( n = 45 \)), while it was 4.0 ± 0.4 Hz (\( n = 44 \)) in \textit{Mecp2}/H11002/Y cells. Although significant difference was not found with general grouping (Fig. 6B), type A cells in \textit{Mecp2}/H11002/Y mice had significantly higher frequency than type B cells in \textit{Mecp2}/H11002/Y mice (5.6 ± 0.7 vs. 3.2 ± 0.5 Hz, \( P < 0.01 \), \( n = 44 \)) and type A cells in WT mice (3.5 ± 0.3 Hz, \( P < 0.01 \), \( n = 59 \); Fig. 6D), respectively. Similar analysis was not attempted for type B cells in WT mice, because of the rarity of this type of cells (\( n = 4 \)).
**Firing frequency variation.** The variation in firing frequency was analyzed by dividing the standard deviation of the firing frequency by its arithmetic mean. The frequency variation of type B Mecp2−/− cells was significantly greater compared with type A cells in either WT (P < 0.05, n = 67) or Mecp2−/− (P < 0.01, n = 44; Fig. 6E) mice, indicating that the aberrant expression of fAHP might induce the instability of basal neuronal activities in Mecp2−/− cells. Similarly, with general grouping, the firing activity of Mecp2−/− neurons was slightly more variable than that of the WT neurons (Fig. 6C), which was statistically insignificant (P > 0.05, n = 89).

**Spike frequency adaptation.** SFA is a fundamental intrinsic property regulating repetitive firing dynamics in response to constant stimuli. SFA was examined in all neurons by injecting a series of depolarizing currents. The firing rate of LC neurons increased initially with depolarization, while it declined when the depolarization was maintained at a constant level (Fig. 7A). Although firing frequency increased with stronger depolarization, the SFA was retained (Fig. 7B). Compared with WT neurons, neither the Fp nor the Fs of Mecp2−/− cells showed significant difference (P > 0.05, n = 98; Fig. 7C). There was no significant difference in the ratio of Fp to Fs (or SFA ratio), either (P > 0.05, n = 98; Fig. 7D).

Since AHPs are known to regulate SFA, we further analyzed the SFA ratio in type A and type B cells. In type A neurons, the SFA ratio was significantly smaller (less adaptation) in Mecp2−/− mice than in WT mice (1.39 ± 0.04 vs. 1.51 ± 0.05; P = 0.033 in 1-tailed t-test, n = 59) (Fig. 7E) although no difference in Fs was found. In Mecp2−/− mice, the SFA ratio was also smaller in type A cells than in type B cells (1.39 ± 0.04 vs. 1.55 ± 0.09; P = 0.044 in 1-tailed t-test, n = 44) (Fig. 7F). On the other hand, type B Mecp2−/− cells displayed higher Fp than type A WT cells (35.4 ± 2.8 vs. 30.0 ± 1.0 Hz; P < 0.05, n = 67), while they showed a SFA ratio similar to WT. The alterations observed in both type A and type B cells suggest that SFA properties are affected by Mecp2 knockout. Similar data analysis was not attempted in WT neurons, because the small sample number of type B neurons does not allow quantitative analysis.

**Delayed excitation.** All LC neurons showed a delayed occurrence of AP following hyperpolarization, a property known as delayed excitation (DE). DE was analyzed with a series of conditioning hyperpolarizing pulses followed by a depolarizing current at a constant level (Fig. 8A). Step increases in hyperpolarization elongated the delay of the first spike. DE was expressed as a function of the conditioning membrane potentials (Fig. 8B), and their relationship was described with the Boltzmann equation (see MATERIALS AND METHODS) with the midpoint voltage (V1/2) and slope factor k estimated. Mecp2 knockout led to a negative shift of V1/2 by 3.4 mV (−75.9 ± 0.7 mV in WT vs. −79.3 ± 0.8 mV; P < 0.05, n = 78) and had no significant effect on k compared with WT neurons (Fig. 8, C and D). The maximal time delay averaged 393.3 ± 8.5 ms vs. 365.1 ± 13.3 ms in the WT (P = 0.038 in 1-tailed t-test, n = 78).

**Postinhibition rebound.** PIR manifests itself as depolarization or spiking following a release from hyperpolarization. PIR was investigated in all LC neurons with a series of hyperpolarizing current pulses up to −120 mV. PIR was observed in one cell only (Supplemental Fig. S1).1

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1 The online version of this article contains supplemental material.
DISCUSSION

This is the first systematic study of the intrinsic membrane properties of LC neurons in MeCP2-null mice. A number of abnormalities in passive membrane properties, APs, and repetitive firing activity have been found, providing evidence that the intrinsic neuronal activities are affected by the MeCP2 defect. The presence of many such defects in the major group of NEergic neurons in the CNS is thus consistent with the disruption of NE systems in MeCP2-null mice as well as patients with RTT.

Two passive membrane properties of LC neurons were affected by MeCP2 knockout. One is the reduction in membrane time constant, which is likely related to the reduced size of MeCP2-/- neurons, consistent with previous findings in neurons of the cortex, thalamus, basal ganglia, amygdala, and hippocampus (17), where the downregulation of brain-derived neurotrophic factor (BDNF) has been shown to play a role (5). The other abnormality is the enhanced IR in MeCP2-/- neurons, which may be a result of the alternations of Kir channels in MeCP2-/- neurons. Supporting the idea is our finding that the expression level of Kir4.1 is upregulated. The excessive expression of Kir4.1 channels has potential implications. Kir channels are known to be the major targets of neuromodulation through G protein-coupled and metabotropic receptors. Consequently, abnormalities in their surface expression may potentially affect the neuromodulation of LC neurons, consistent with the autonomic dysfunction and behavioral abnormalities of patients with RTT (23). Interestingly, TASK1 and TASK3 expressions are downregulated in the mutant mice (24), which, on one hand, may complement the overexpression of Kir4.1, thus helping maintain K+ homeostasis and membrane potentials, as the MeCP2-/- neurons indeed showed similar membrane potential to the WT. Since the TASK channels have no IR, their underexpression together with the overexpression of Kir4.1 is thus consistent with the strong IR in LC neurons.

The prolonged rise time of APs is one of the major characteristics of MeCP2-/- neurons. The waveform of APs is determined by the dynamics of Na+ and K+ channels, while the onset speed of APs is dominantly controlled by Na+ channels. The reduced availability of Na+ channels has been reported to be responsible for the slow onset of APs induced by low levels of TTX in cortical neurons (20). Similarly, reduced density of voltage-gated Na+ currents and reduced expression level of Na1.2 observed in this study seem to explain, at least in part, the elongation of AP rising time and AP duration in MeCP2-/- neurons. The duration of AP is known to account largely for
the depolarization-induced Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels. As shown in hippocampal neurons and cortical pyramidal neurons (1, 42), AP broadening induced by 4-aminopyridine (4-AP) in presynaptic neurons strengthens synaptic neurotransmitter release and postsynaptic potentials due to increase in Ca\(^{2+}\) influx. Besides regulating instantaneous communication among neurons, Ca\(^{2+}\) as a secondary messenger also mediates long-term and depolarization-dependent modulations, such as transcription and synaptic plasticity (9). Clearly, further investigations are needed to demonstrate whether the broader APs in Mecp2-null neurons lead to more Ca\(^{2+}\) influx that may have impact on neuropathology of RTT.

Another finding in our experiments is that AHPs are affected by Mecp2-null knockout. First, the fAHP seen in only a few WT neurons is present in an overwhelming proportion of Mecp2-null neurons, which we named type B cells. Compared with type A cells in Mecp2-null LC, type B cells seem to display distinctive characteristics of APs, such as the shorter half-width and decay time of AP. This effect of fAHP on AP morphology and its functional consequences has been well documented (7, 19). Additionally, type B cells display slower frequency with greater frequency variation and SFA than type A cells. Similar findings have been reported previously in neurons of the medial vestibular nucleus (VMN) (29). Neurons with a monophasic AHP (corresponding to our type A cells) have fast and regular spontaneous discharge, while those with biphasic AHP (like our type B cells) show opposite effects. Also, Gu et al. (12) demonstrated an important role of fAHP in facilitating \(F_P\) and SFA in response to depolarizing current injection in hippocampal pyramidal cells. These many distinctions between type A and type B cells in Mecp2-null mice support the heterogeneity of LC neuronal activity. Indeed, the classification of neurons according to their AHP profile has been well accepted in electrophysiological studies in several brain stem nuclei, including the VMN, nucleus gigantocellularis, and oculomotor nucleus (22, 31, 35). Although we still do not know whether type A and type B cells in Mecp2-null mice are two different populations of LC neurons, as proposed in VMN neurons and oculomotor neurons (22, 35), the classification of type A and type B cells may help to reveal the influences of MeCP2 on repetitive firing activities of LC neurons.

Why, then is such a large proportion of type B cells present in Mecp2-null mice but not in WT mice? Compared with WT cells, type A neurons showed obviously smaller mAHP amplitude in addition to significant abnormalities in time constant and AP duration. The amplitude of mAHP is a fundamental factor to modulate AP frequency and SFA, thus determining neuronal excitability. Blockage of SK channels underlying mAHP increase the firing frequency and attenuates SFA in CA1 pyramidal neurons (6, 26, 39). Consistently, the firing frequency of type A Mecp2-null neuron cells is >60% higher than its WT counterpart. In response to sustained depolarizing currents, these cells also tend to show smaller SFA than WT neurons. The increase in basal firing frequency and \(F_s\) induced by persistent depolarizing current suggests that the intrinsic excitability of type A Mecp2-null neurons is enhanced. On the
other hand, type B Mecp²⁻/⁻ cells behave more like WT type A cells with respect to membrane time constant, AP duration, firing frequency, and SFA. In this regard, the insufficiency in the mAHP might be compensated by the expression or overexpression of the fAHP in Mecp²⁻/⁻ neurons. The “compensation” hypothesis is supported by our observation that Mecp²⁻/⁻ neurons displayed repetitive firing activities similar to WT neurons with general grouping. However, such compensation is not without problems. The type B cells tend to have more frequency variations and higher F₀ than type A cells in WT neurons, indicating that the excitability of type B cells is also augmented. Given that individual LC neurons innervate specific brain regions and have heterogeneous functions (21, 32), the existence of a large number of type B cells in Mecp²⁻/⁻ LC may contribute to the high variability of Mecp²⁻/⁻ neuronal activity.

The ionic mechanisms underlying AHP abnormalities remain unclear. In VMN, several K⁺ currents underlying distinct AHP components have been identified (26, 34, 35). Whether the same currents also explain the dissimilarity between type A and B cells in LC of Mecp²⁻/⁻ mice remains to be elucidated. Previous studies as well as our present study indicate that the fAHP is rare, although mAHP is common in normal LC neurons of rodents (47). There is no evidence for the ionic basis of mAHP in WT LC neurons. In contrast, the ionic mechanisms underlying the slow component of AHP have been well studied in rat LC neurons, involving both apamin-sensitive and apamin-insensitive Ca²⁺-activated K⁺ currents (25, 30). Also, the control of neuronal excitability is not limited to AHP characteristics. For example, persistent Na⁺ currents counteracting the effect of Ca²⁺-activated K⁺ channels are also involved in controlling spontaneous firing activities (43). Therefore, further studies are needed to define the ionic mechanisms underlying the AHP abnormalities.

Small changes in DE in Mecp²⁻/⁻ neurons have been observed in our present study. In addition, several other intrinsic membrane properties have been systematically examined, including membrane potential, input resistance, AP amplitude, bursting activity, etc. Our studies suggest that there are no evident abnormalities in these membrane properties with the Mecp²⁻/⁻ knockout.

It is worth noting that the intrinsic membrane properties of LC neurons have not been systemically studied in WT mice. Since the mouse is the most common mammalian model for gene targeting, the demonstration of the intrinsic membrane properties of this important group of NEergic neurons in WT mice helps the understanding of these neurons in basic neurobiology and therapeutic designs of neuropharmacological and neuropsychiatric medicine.

In conclusion, the Mecp2 defect causes abnormalities in several intrinsic membrane properties crucial for LC neuronal firing activity, repetitive firing, and responses to presynaptic signaling. The demonstration of these abnormalities appears to constitute a significant step toward the understanding of the molecular and cellular basis of NE dysfunctions in Mecp²⁻/⁻ mice as well as patients with RTT.

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DISCLOSURES

The authors are not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

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