Disorders of the Nervous System

**Mecp2 Deletion from Cholinergic Neurons Selectively Impairs Recognition Memory and Disrupts Cholinergic Modulation of the Perirhinal Cortex**

Elizabeth C. Ballinger,1,2,3 Christian P. Schaaf,7,8,11 Akash J. Patel,7,9 Antonia de Maio,6,7,8 Huifang Tao,7,8 David A. Talmage,1,4,5 Huda Y. Zoghbi,6,7,8,10 and Lorna W. Role1,4

https://doi.org/10.1523/ENEURO.0134-19.2019

1Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, New York 11794, 2Program in Neuroscience, Stony Brook University, Stony Brook, New York 11794, 3Medical Scientist Training Program, Stony Brook University, Stony Brook, New York 11794, 4Center for Nervous System Disorders, Stony Brook University, Stony Brook, New York 11794, 5Department of Pharmacological Sciences, Stony Brook University, Stony Brook, New York 11794, 6Department of Developmental Biology, Baylor College of Medicine, Houston, Texas 77030, 7Jan and Dan Duncan Neurological Research Institute at Texas Children’s Hospital, Baylor College of Medicine, Houston, Texas 77030, 8Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, 9Department of Neurosurgery, Baylor College of Medicine, Houston, Texas 77030, 10Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030, and 11Institute of Human Genetics, Heidelberg University, 69120 Heidelberg, Germany

**Abstract**

Rett Syndrome is a neurological disorder caused by mutations in the gene encoding methyl CpG binding protein 2 (MeCP2) and characterized by severe intellectual disability. The cholinergic system is a critical modulator of cognitive ability and is affected in patients with Rett Syndrome. To better understand the importance of MeCP2 function in cholinergic neurons, we studied the effect of selective Mecp2 deletion from cholinergic neurons in mice. Mice with Mecp2 deletion from cholinergic neurons were selectively impaired in assays of recognition memory, a cognitive task largely mediated by the perirhinal cortex (PRH). Deletion of Mecp2 from cholinergic neurons resulted in profound alterations in baseline firing of L5/6 neurons and eliminated the responses of these neurons to optogenetic stimulation of cholinergic input. Both the behavioral and the electrophysiological deficits of cholinergic Mecp2 deletion were rescued by inhibiting ACh breakdown with donepezil treatment.

**Key words:** acetylcholine; Mecp2; perirhinal; recognition; Rett Syndrome

**Significance Statement**

Rett Syndrome, a developmental disorder characterized by multiple deficits including intellectual disability, is caused by mutations in the MECP2 gene. In this study, Mecp2 was selectively deleted from cholinergic neurons in mice causing a specific impairment of recognition memory that was reversed following chronic administration of the acetylcholinesterase inhibitor donepezil. As recognition memory engages the perirhinal cortex, we examined the effects of Mecp2 deletion from cholinergic neurons on the physiology of perirhinal cortical neurons and found a reduction in the variability of baseline firing and impaired responsiveness to optogenetic stimulation of cholinergic input. Our findings are consistent with a loss of encoding capacity in the perirhinal cortex and suggest a possible electrophysiological substrate for the altered profile of recognition memory performance.
Introduction

Rett Syndrome is a childhood neurologic disorder that affects 1 in 10,000 girls and is caused by mutations in a gene known as MECP2 (Rett, 1966; Hagberg et al., 1983; Lewis et al., 1992; Amir et al., 1999; Neul et al., 2010). The phenotype is complex and includes intellectual disability, breathing disturbances when awake, seizures, autonomic dysfunction, autistic features, stereotypies, locomotor defects, and gastrointestinal dysfunction. MECP2 encodes methyl CpG binding protein 2, a transcriptional regulator whose loss leads to both decreases and increases in gene expression (Chahrour et al., 2008). Discerning what cell types and Mecp2 target genes contribute to which aspects of the Rett Syndrome phenotype has posed a significant challenge. This challenge has been addressed by selectively removing Mecp2 from distinct neuronal populations, revealing which neuronal populations and brain regions contribute to the key features of the disorder. Genetically targeted approaches have previously been applied to the GABAergic, glutamatergic, Sim1-expressing, and aminergic systems; and have provided an added level of resolution in the investigation of the significance of these neurotransmitter systems to Rett Syndrome (Fyffe et al., 2008; Samaco et al., 2009; Chao et al., 2010; Meng et al., 2016).

Given that a key characteristic of individuals with Rett Syndrome is severe cognitive impairment (Rett, 1966; Hagberg et al., 1983; Neul et al., 2010) and the well-established role of the cholinergic system in cognitive functions (for review, see Ballinger et al., 2016), we have used the Mecp2 deletion approach to assess the role of Mecp2 in cholinergic neurons and the resulting phenotypes due to its loss. Prior studies in both humans and animal models have implicated alterations in acetylcholine (ACh) signaling in Rett Syndrome. Postmortem immunohistochemical studies of the brain from individuals with Rett Syndrome have shown profound cholinergic deficits: there are reduced numbers of choline acetyltransferase (ChAT)-positive cells in the basal forebrain, reduced ChAT and VChAT activity, and reduced cholinergic receptor expression (Kitt et al., 1990; Wenk and Mobley, 1996; Wenk, 1997; Wenk and Hauss-Wegrzyniak, 1999; Yasui et al., 2011). Likewise, mice lacking Mecp2 have shown reductions in both ACh and ChAT, dramatically attenuated cholinergic currents in electrophysiological experiments, and altered cholinergic receptor expression profiles (Ward et al., 2009; Ricceri et al., 2011; Oginsky et al., 2014; but see also Zhou et al., 2017). Finally, cholinergic marker reductions as evaluated by SPECT imaging in vivo have been correlated with clinical severity in patients with Rett Syndrome (Brasić et al., 2012).

To evaluate the potential contribution of the cholinergic system to phenotypes observed in Rett Syndrome in a systematic way, we have used a Cre-Lox system to selectively delete Mecp2 from cholinergic neurons only (see Fig. 2A). We evaluated the performance of these mice on a number of cognitive tasks and found specific deficits in novel object recognition—behaviors that depend on intact functioning of the perirhinal cortex (PRH; for review, see Dere et al., 2007). We then explored the electrophysiological and molecular mechanisms underlying specific cognitive deficits in novel object recognition.

Materials and Methods

Animals

For electrophysiological, behavioral, and molecular experiments transgenic male mice expressing Cre recombinase under control of the Chat promoter (Chat-Cre; stock #006410, The Jackson Laboratory; RRID:IMSR_JAX:006410) maintained on a C57 background were crossed with female mice heterozygous for a floxed Mecp2 allele (Mecp2 flox; stock #007177, The Jackson Laboratory; RRID:IMSR_JAX:007177) maintained on a 129 background. This cross generated the following four different genotypes of male offspring: mice with no transgenes, mice with the Chat-Cre transgene only, mice with the Mecp2 flox allele only, and mice with both transgenes (Chat-Mecp2<sup>-/-</sup>; see Fig. 2A). Male mice from the F1 generations of the original cross were used for experiments. Mice of all genotypes were born at the expected Mendelian ratios and were healthy appearing at birth. However, Chat-Mecp2<sup>-/-</sup> mice did exhibit a phenotype of reduced survival, with most Chat-Mecp2<sup>-/-</sup> mice dying between 16 and 36 weeks of age while all other genotypes lived for 40+ weeks. Chat-Mecp2<sup>-/-</sup> mice gained weight at rates similar to those of genetic controls and were generally healthy appearing until death, which was an acute/subacute event of unknown cause.

All mice were maintained on a 12 h light/dark cycle and allowed food and water ad libitum. Mice were either pair or group housed. No singly housed mice were used for behavioral experiments. The same cohort of mice was examined on both the partition test and the novel object recognition test. A separate cohort of mice was examined on conditioned fear testing. A subset of this second cohort also underwent Morris water maze testing. A third cohort of mice was used for electrophysiological experiments.
Context-conditioned fear and cue-conditioned fear

Fear conditioning was performed as previously described (Takeuchi et al., 2011). This test was conducted when mice were 21 weeks old. Each mouse was placed in a sound-attenuated chamber and allowed to explore freely for 2 min. An 80 dB white noise, the conditioned stimulus (CS), was presented for 30 s; this was followed by a mild (2 s, 1 mA) footshock, the unconditioned stimulus (US). Two more CS–US pairings were presented with 2 min interstimulus intervals (ISIs). Context testing was conducted 1 d after conditioning in the same chamber. Cued testing with altered context was conducted on the same day, following the context testing, using a triangular box made of white opaque Plexiglas and vanilla scent presented behind the separation to change olfactory stimulus. Data acquisition, control of stimuli (i.e., tones and shocks), and data analysis were performed automatically using the Actimetrics FreezeFrame3 System (Coulbourn Instruments; RRID:SCR_014429). For context-conditioned fear, the percentage of time spent freezing in the conditioned context on testing day was calculated and compared between groups. For cue-conditioned fear, on testing day the cue-specific time spent freezing was calculated as follows: cue-specific freezing = (% time freezing during cue) – (% time freezing before cue). Cue-specific freezing was then compared between groups.

Morris water maze

Morris water maze was performed as previously described (Takeuchi et al., 2011). This test was conducted when mice were 17 weeks old. A circular pool (120 cm in diameter) was filled with water (21 ± 1°C), in which non-toxic white tempera paint was mixed to make the surface opaque. For the invisible platform test, a white-colored platform (5 cm in diameter) was filled with water (21°C), and placed on a surgical stereotax (Kopf Instruments) with a heated stage. An incision in the scalp was made, and a small hole was drilled in the skull above the left nucleus basalis magnocellularis [NBM; coordinates from bregma: anteroposterior (AP), −0.7 mm; mediolateral (ML), 1.7 mm; z-axis, −4.0 mm]. A total of 0.5 μl of either AAV9-Ef1a-DIO-ChETA-eYFP or AAV9-CAG-DIO-oChIEF-tdTomato was injected using a microsyringe (Hamilton). Mice were used for electrophysiological recording 3 weeks after infection.

Viral injection

Before the electrophysiological recording, a subset of mice underwent viral injection to facilitate optogenetic stimulation of cholinergic neurons. To target cholinergic neurons, we used a Cre-dependent virus, and the experiments were limited to Chat-Cre and Chat-Mecp2−/− mice. Mice for optogenetic experiments were anesthetized with isoflurane at 11 weeks of age and mounted on a stereotaxic frame (Kopf Instruments) with a heated stage. An incision in the scalp was made, and a small hole was drilled in the skull above the left nucleus basalis magnocellularis [NBM; coordinates from bregma: anteroposterior (AP), −0.7 mm; mediolateral (ML), 1.7 mm; z-axis, −4.0 mm]. A total of 0.5 μl of either AAV9-Ef1a-DIO-ChETA-eYFP or AAV9-CAG-DIO-oChIEF-tdTomato was injected using a microsyringe (Hamilton). Mice were used for electrophysiological recording 3 weeks after infection.

Electrophysiological recording

For electrophysiological experiments, mice that were at least 13 weeks of age were anesthetized with isoflurane and placed on a surgical stereotax (Kopf Instruments) with a heated stage. A craniotomy over the left perihinal cortex was performed and a tungsten electrode of either 1 or 5 MΩ (A-M Systems) was positioned into the posterior
PRH (coordinates from bregma: AP, −3.25 mm; z-axis, −3.35 to −3.85 mm; ML from temporal ridge, −200 to +500 μm). Extracellular recordings were preamplified by the head stage of A-M Systems amplifier. For optogenetic experiments, mice (i.e., Chat Cre mice with or without Mecp2 flox) received an additional craniotomy over the left NBM through which a 1 MΩ parylene-C-insulated tungsten electrode (A-M Systems) and a 200 μm optical fiber (Thorlabs) coupled to a 473 nm laser (Shanghai Dream Lasers Technology) were positioned in the NBM.

Signals were acquired at a sampling rate of 40 kHz and bandpass filtered at 100–1000 Hz by the amplifier (A-M Systems) before being passed through a Humbug Noise Eliminator (A-M Systems) and then displayed on a Tektronix TDS 2014B oscilloscope and fed to a Cambridge Electronic Design 1401 data board for visualization and collection using Spike 2 software (Cambridge Electronic Design). Laser stimuli used for optical stimulation of cholinergic neurons consisted of 20 laser pulses of 1 ms duration delivered at a frequency of 10 Hz.

Relocalization of recording site

At the end of each recording session, an electrolytic lesion was created by passing 100 μA of current for 45 s through the recording electrode to facilitate relocalization of the recording site. The mouse was then perfused transcardially and brain slices were obtained as discussed below.

For a subset of mice, slices containing the perirhinal cortex were stained using NeuroTrace (Thermo Fisher Scientific) blue fluorescent Nissl stain. The perirhinal cortex was defined histologically as per Beaudin et al. (2013). In short: the medial border was defined by the external capsule, the dorsal border was distinguished by the loss of the prominent layer IV seen in the dorsally adjacent temporal association cortex, the ventral border was distinguished by loss of the prominent layer II seen in the ventrally adjacent entorhinal cortex.

For all mice, slices containing the PRH were imaged on a stereoscope (Zeiss). For mice used for optogenetic experiments, slices containing the NBM were imaged to confirm viral expression.

Sample preparation for light and confocal microscopy

Mice were anesthetized with a 9:1 mixture of ketamine and xylazine and transcardially perfused with 1× PBS followed by 4% PFA. Brains were removed and postfixed overnight in 4% PFA before being sucrose equilibrated and frozen in OCT (optimal cutting temperature) compound. Brains were then cryosectioned (Leica Biosystems) at 50 μm thickness.

Immunohistochemistry

For Nissl staining brain slices were blocked and permeabilized for 30 min in 1× PBS with 5% donkey serum and 0.1% Triton X-100, and then incubated for 90 min in a 1:200 dilution (in blocking/permeabilization solution) of the NeuroTrace blue fluorescent Nissl stain (Thermo Fisher Scientific) followed by three 5 min washes in 1× PBS. Slices were mounted with DAPI Fluoromount-G (Southern Biotech). All steps were performed at room temperature.

For MeCP2 and Chat staining, brain slices were blocked and permeabilized for 1 h at room temperature in 1× PBS with 5% donkey serum and 0.5% Triton X-100 and then incubated for 48 h on a shaker at 4°C in 1:200 rabbit anti-MeCP2 (catalog #3456, Cell Signaling Technology; RRID:AB_2143849) or 1:200 goat anti-ChAT (EMD Millipore; RRID:AB_2079751) diluted in blocking solution. Slices were then washed three times for 10 min each in 1× PBS and then incubated overnight in 1:500 Invitrogen goat anti-rabbit (Thermo Fisher Scientific) or 1:1000 Invitrogen donkey anti-goat (Thermo Fisher Scientific). After three 10 min washes in 1× PBS, slices were mounted with Vectashield (Vector Laboratories; RRID:AB_2336788) with DAPI and imaged on a confocal microscope.

Electrophysiological data analysis

Extracellular recordings were sorted off-line using the Offline Sorter (Plexon). Features of the waveforms were extracted, and individual units were demarcated by manually identifying clusters of waveforms in a two-dimensional feature space of spike properties (Gray et al., 1995). The quality of each sort was rated according to the isolation distance between clusters within the recording. Only recordings of high sort quality, with <5% overlap with other clusters, were used for further analysis. Units with firing rates <0.05 Hz were excluded from further analysis.

The variability of the baseline firing rate was quantified by calculating the Fano factor (FF) of the firing rate computed for each 10 s bin during the 300 s preceding optical stimulation.

Responses to optogenetic stimulation were evaluated using a permutation test of the F statistic with 10,000 permutations comparing interspike intervals occurring within the 140 s immediately before and after stimulation. To identify delayed responders, the same analysis was performed comparing the 140 s immediately before stimulation and a sliding 140 s window following stimulation. Each slide step was 70 s. A response was detected if the resultant p value from these permutation tests was <0.05. A unit was considered to exhibit an “early laser” response if a response was detected in the first 140 s window following stimulation. A unit was considered to exhibit a “delayed” response if a response was detected at any later window within the first 560 s following stimulation. If no responses were detected within the first 560 s following stimulation, the unit was considered to have no response.

Donepezil pump implantation

A subset of Chat-Mecp2+/y mice generated using the same breeding strategy as described above underwent subcutaneous implantation of an osmotic minipump (model 2006, Alzet). Mice were anesthetized with isoflurane, and an incision was created over the left shoulder or caudal skull. Hemostats were lubricated with saline and then passed through the incision and used to open the subcutaneous space by separating the skin from the subcutaneous fascia. The pump was then implanted in this
space, and the mouse was allowed to recover for 2 weeks. Pumps delivered either sterile saline or donepezil HCl (Biotang) in saline at a dose of 0.3 mg/kg/d.

**Statistics**

Performance on the partition test and Morris water maze was compared using a repeated-measures ANOVA design with genotype and testing session included as factors. Post hoc pairwise comparisons were performed using the Tukey’s test. Performance on conditioned fear tests, baseline firing rate, firing rate variability, and performance on the test day of Novel Object Recognition for non-drug-treated mice were compared using the Kruskal–Wallis test. Performance on conditioned fear and poststimulation with familiar on test day were compared using the Wilcoxon rank sum test. The results of analysis were summarized in Table 1.

**Results**

**Cognitive phenotyping of Chat-Mecp2+/y mice**

As cholinergic signaling plays a vital role in mediating cognition (Ballinger et al., 2016) and intellectual disability is a central phenotype of MECP2 disorders, we first asked whether selective MeCP2 deletion from cholinergic neurons altered performance in several learning-related cognitive tasks. We used a Cre-lox system to selectively delete MeCP2 from cholinergic neurons (Figs. 1, 2A). This system has previously been shown to effectively reduce MeCP2 expression, as measured by immunofluorescence (Ito-Ishida et al., 2015; Zhang et al., 2016).

**Chat-Mecp2+/y** mice performed at control levels on the Morris water maze, an assay of spatial memory (**Fig. 2B**; n = 12 for WT, Chat Cre, and Mecp2 flox mice; n = 11 for Chat-Mecp2+/y mice; repeated-measures ANOVA: interaction effect for genotype × testing day: Wilks’ lambda = 0.827, $F_{(9,100)} = 0.902, p = 0.527$). Similarly, Chat-

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**Table 1: Statistical methods by figure**

| Set of data | Type of analysis | Results of analysis |
|-------------|------------------|---------------------|
| **Figure 2** |                  |                     |
| B: time to reach platform in Morris water maze: group × testing session interaction | Two-way repeated-measures ANOVA $F_{(9,100)} = 0.902$ | $p = 0.527$ |
| C: comparison by genotype of the percentage of time spent freezing to context | Kruskal–Wallis test $H_{(3)} = 4.64$ | $p = 0.20$ |
| D: comparison by genotype of the percentage of time spent freezing to cue | Kruskal–Wallis test $H_{(3)} = 6.46$ | $p = 0.09$ |
| E: comparison by genotype of time with novel/time with familiar on test day | Kruskal–Wallis test $H_{(3)} = 22.97$ | $p < 0.0005$ |
| F: time spent at partition: group × testing session interaction | Two-way repeated-measures ANOVA $F_{(6,118)} = 4.908$ | $p < 0.0005$ |
| **Figure 3** |                  |                     |
| C: comparison by genotype of Fano factor of firing rate | Kruskal–Wallis test $H_{(3)} = 8.92$ | $p = 0.03$ |
| D: comparison by genotype of firing rate | Kruskal–Wallis test $H_{(3)} = 6.62$ | $p = 0.085$ |
| **Figure 5** |                  |                     |
| C: within-unit comparison of variance of ISI prestimulation and poststimulation | Permutation test of $F$ statistic |                     |
| D: comparison by genotype of response rates | $\chi^2$ test of homogeneity CR(2) = 6.02 | $p = 0.049$ |
| **Figure 6** |                  |                     |
| A: comparison by treatment type of time with novel/time with familiar on test day | Wilcoxon rank sum test Rank sum = 120 | $p = 0.03$ |
| B: comparison by treatment type of firing rate variability | Wilcoxon rank sum test Rank sum = 293 | $p = 0.087$ |
| D: within unit comparison of variance of ISI prestimulation and poststimulation | Permutation test of $F$ statistic |                     |
| E: comparison by treatment type of response rates | $\chi^2$ test of homogeneity CR(2) = 4.15 | $p = 0.126$ |
Figure 2. continued

mice were also impaired on the partition test (repeated-measures ANOVA: interaction effect for genotype \times behavior session; Wilks’ lambda = 0.64; F\textsubscript{110} = 4.908; p < 0.0005). Pairwise comparisons revealed that Chat-Mecp2\textsuperscript{+/y} were significantly different from both no transgene mice (p < 0.0005) and Chat Cre mice (p = 0.001), although the difference between Chat-Mecp2\textsuperscript{+/y} and Mecp2 flox mice did not reach significance (p = 0.079). On the partition test, Chat-Mecp2\textsuperscript{+/y} mice were impaired in their ability to recognize a familiar mouse and spent longer interacting with the familiar mouse on re-presentation (mean, 178.05 s; SD, 63.02) than any of the genetic controls (no transgene: mean, 86.58 s; SD, 47.92; Chat Cre: mean, 82.38 s; SD, 51.89; Mecp2 flox: mean, 111.31 s; SD, 43.97). Error bars represent the SEM, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0005, ns = non significant.

Mecp2\textsuperscript{+/y} mice performed at control levels on the context-conditioned fear assay (Fig. 2C; n = 18 for WT and Chat Cre mice; n = 17 for Mecp2 flox and Chat-Mecp2\textsuperscript{+/y} mice; Kruskal–Wallis test, H\textsubscript{03} = 4.64, p = 0.20) and cue-conditioned fear assay (Fig. 2D; n = 18 for WT and Chat Cre mice; n = 17 for Mecp2 flox and Chat-Mecp2\textsuperscript{+/y} mice; Kruskal–Wallis test, H\textsubscript{03} = 6.46, p = 0.09).

Chat-Mecp2\textsuperscript{+/y} mice were impaired in recognition memory, as measured by the novel object recognition task (Fig. 2E; n = 13 for no transgene, Chat Cre, and Mecp2 flox mice; n = 12 for Chat-Mecp2\textsuperscript{+/y} mice; Kruskal–Wallis test, H\textsubscript{03} = 22.97, p < 0.0005). Post hoc comparisons revealed that Chat-Mecp2\textsuperscript{+/y} mice showed significantly reduced preference for the novel object compared with all three genetic controls (no transgene, p = 0.0001; Chat Cre, p = 0.0004; Mecp2 flox; p = 0.0079; no transgene novel/familiar object ratio: mean, 5.6; SD, 4.30; Chat Cre novel/familiar object ratio: mean, 4.63; SD, 2.68; Mecp2 flox novel/familiar object ratio: mean, 4.18; SD, 3.62). No other significant differences were found. Chat-Mecp2\textsuperscript{+/y} mice also performed abnormally in the partition test assay of social interaction and memory (Fig. 2F; n = 16/group; repeated-measures ANOVA: interaction effect for genotype \times behavior session; Wilks’ lambda = 0.64, F\textsubscript{110} = 4.908, p < 0.0005). Although Chat-Mecp2\textsuperscript{+/y} mice originally interacted with the familiar mouse and the novel mouse at control levels, when the familiar mouse was reintroduced at the end of the trial, Chat-Mecp2\textsuperscript{+/y} mice spent more time interacting with the familiar mice (mean, 178.05 s; SD, 63.02) than the control mice did (no transgene: mean, 86.58 s; SD, 47.92; Chat Cre: mean, 82.38 s; SD, 51.89; Mecp2 flox: mean, 111.31 s; SD, 43.97). These data are consistent with the idea that selective deletion of Mecp2 from cholinergic neurons alters the ability of the animals to distinguish between novel and familiar stimuli but did not affect social interactions per se. Testing on a comprehensive behavioral battery revealed no other behavioral deficits (Table 2).

Electrophysiological recording of the PRH

Recognition memory is thought to engage circuits including the PRH, which receives cholinergic projections from neurons in the basal forebrain. Given that Chat-Mecp2\textsuperscript{+/y} mice are selectively impaired in assays of recognition mem-
ory, we next asked whether there were any overt changes in electrophysiological profile of PRH neurons.

In vivo extracellular recordings were collected from L5/6 of the posterior portion of the PRH (Fig. 3A–C). Although there was no significant difference in baseline firing rate (Fig. 3D; no transgene: $n = 24$ units from 6 mice; Chat Cre: $n = 22$ units from 11 mice; $\text{Mecp2 flox}: n = 6$ units from 3 mice; Chat-Mecp2$^{+/y}$: $n = 20$ units from 8 mice; Kruskal–Wallis test, $H_{(3)} = 6.62, p = 0.085$), PRH units from control mice (WT, Chat Cre, and Mecp2 flox mice) had highly variable firing patterns. In contrast, PRH units from Chat-Mecp2$^{+/y}$ mice exhibited very regular and rhythmic firing (Fig. 3C). Comparison of the variability of the firing rate as measured by the Fano factor revealed a significant difference between groups (Kruskal–Wallis test, $H_{(3)} = 8.92, p = 0.03$). PRH units from Chat-Mecp2$^{+/y}$ mice had the lowest firing rate variability (Fig. 3E; no transgene, mean FF = $0.612 \pm 1.250$; Chat Cre, mean FF = $0.474 \pm 0.650$; Mecp2 flox, mean FF = $0.447 \pm 0.365$; Chat-Mecp2$^{+/y}$, mean FF = $0.248 \pm 0.431$).

### Optogenetic stimulation of cholinergic input to the PRH

The above results indicate that Mecp2 deletion from cholinergic neurons has an important functional effect on PRH firing at baseline. We next asked whether Mecp2 deletion from cholinergic neurons affected the response of the PRH to the stimulation of endogenous acetylcholine release. The PRH receives the majority of its cholinergic innervation from neurons in the NBM (Woolf, 1991; Kondo and Zaborszky, 2016). To acutely stimulate acetylcholine release in the PRH, we infected Chat Cre and Chat-Mecp2$^{+/y}$ mice with a Cre-dependent AAV (adeno-associated virus) expressing the channelrhodopsin variants oChIEF or ChETA fused to tdTomato (Fig. 4A,B). Cholinergic neurons in the NBM of both Chat Cre and Chat-Mecp2$^{+/y}$ mice expressed functional oChIEF, as indicated by fluorescent imaging of tdTomato (Fig. 4C) and by optically evoked action potentials (Fig. 4D).

We next recorded PRH units before, during, and after laser activation of oChIEF expressed in NBM cholinergic neurons (20X 1 ms pulses at 10 Hz). In control animals, the stimulation of NBM cholinergic neurons changed the variance of the interspike intervals (Fig. 5A) as measured by the $F$ statistic (variance$_{pre}$/variance$_{post}$). PRH units in Chat-Mecp2$^{+/y}$ did not respond to optical stimulation of cholinergic input; there was no change in ISI variance (Fig. 5B) before versus after optical stimulation of cholinergic input.

Figure 5C shows a heat map of response for each PRH unit as a function of time before and after optical stimulation of cholinergic input for control mice (Fig. 5C, left) and Chat-Mecp2$^{+/y}$ mice (Fig. 5C, right). While 22.7% of control PRH units (5 of 22) exhibited a change in firing rate variability in the first 140 s time period following laser stimulation, there were no immediate laser stimulation-associated responses detected in Chat-Mecp2$^{+/y}$ PRH units. The proportion of units exhibiting delayed responses to laser stimulation was similar between control and Chat-Mecp2$^{+/y}$ mice (Chat Cre mice: 36.4%, 8 of 22; Chat-Mecp2$^{+/y}$: 35%, 7 of 20). In contrast, the proportion of units with no detectable response to optical stimulation was higher in Chat-Mecp2$^{+/y}$ mice (65%, 13 of 20) than in controls (40.9%, 9 of 22). These distributions of responses were statistically significantly different (Fig. 6D; $\chi^2$ test for homogeneity, CR(2) = 6.02, $p = 0.049$), which is consistent with a general loss of ACh modulation of PRH activity in the Chat-Mecp2$^{+/y}$ mice.

### Effect of donepezil on Chat-Mecp2$^{+/y}$ phenotype

Mecp2 deletion from cholinergic neurons impaired recognition memory performance and altered PRH cell firing at baseline and after the stimulation of cholinergic projection neurons. If these deficits resulted from impaired cholinergic signaling per se, then we predicted that these phenotypes would be reversed by pharmacological inhibition of acetylcholine degradation by acetylcholinesterase (AChE).

Chronic administration of the AChE inhibitor donepezil (0.3 mg/ml/d, delivered via a subcutaneous minipump) rescued the performance of Chat-Mecp2$^{+/y}$ mice in the novel object recognition task (Fig. 6A). Chat-Mecp2$^{+/y}$ mice treated with donepezil (Chat-Mecp2$^{+/y}$ + Dpz) spent significantly more time exploring a novel object than a familiar object (Fig. 6A; Chat-Mecp2$^{+/y}$ + saline mice, $n = 8$; Chat-Mecp2$^{+/y}$ + Dpz mice, $n = 10$). In fact, their behavior was qualitatively similar to that of control mice, which is consistent with the idea that donepezil blockade of AChE activity was sufficient to rescue the ability of the mice to distinguish between novel and familiar stimuli.

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**Table 2: Chat-Mecp2$^{+/y}$ Mice have reduced survival but are not impaired on other behavioral phenotypes**

| Test                        | Phenotype tested | Phenotype present in Chat-Mecp2$^{+/y}$ mice |
|-----------------------------|------------------|---------------------------------------------|
| General health exam         | Weight           | NS from no transgene; Mecp2 flox            |
| General health exam         | Stereotypes      | No                                          |
| Elevated plus maze          | Anxiety-like behavior | No                                        |
| Light/dark box              | Anxiety-like behavior | No                                        |
| Open field arena            | Anxiety-like behavior | No                                        |
| Open field arena            | Hypo/hyperactivity | No                                          |
| Rotarod                     | Motor learning    | NS from no transgene; Mecp2 flox            |
| Rotarod                     | Motor coordination | No                                          |
| Grip strength meter         | Motor strength    | No                                          |
| Prepulse inhibition         | Sensory gating    | No                                          |
| Passive avoidance           | Contextual learning | No                                        |
| Aging of animal             | Reduced survival  | Yes                                          |

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References

Kondo, and Zaborszky, 2016

Woolf, 1991

Chat-Mecp2$^{+/y}$ did not respond to optical stimulation of cholinergic input; there was no change in ISI variance (Fig. 5B) before versus after optical stimulation of cholinergic input.
We next tested whether the electrophysiological effects of cholinergic MeCP2 deletion could similarly be rescued by donepezil treatment. Figure 6B (top) shows a representative extracellular record obtained from a PRH L5/6 neuron in an anesthetized Chat-Mecp2^−/y mouse treated with donepezil. Units recorded in Chat-Mecp2^−/y + Dpz mice had highly variable firing patterns reminiscent of the electrophysiological profiles of control mice (Fig. 5B, bottom).

We also tested the effect of donepezil treatment on the PRH response to the stimulation of cholinergic input. Figure 6C shows a representative PRH unit from a Chat-Mecp2^−/y + Dpz mouse in which the cholinergic input to the PRH has been optically stimulated. The box plot of ISIs (Fig. 6C, left) obtained before and after stimulation shows a change in the variance of ISI. Figure 6D shows a heat map of responses of Chat-Mecp2^−/y + Dpz PRH units as a function of time since laser stimulation. The early laser response rate of Chat-Mecp2^−/y + Dpz PRH units is partially rescued when compared with untreated Chat-Mecp2^−/y, increasing from a value of 0 to 16.7% (2 of 12 PRH units). The proportion of units with delayed responses was slightly higher than in control or Chat-Mecp2^−/y mice (Chat-Mecp2^−/y: 41.7%, 5 of 12 PRH units).

Figure 3. Neuronal firing in the PRH is highly variable, and this variability is lost in Chat-Mecp2^−/y mice. A, In vivo recordings were collected from layers 5 and 6 of the PRH. B, Sample Nissl staining and electrolytic lesion marking recording sites in the PRH (white dotted line). C, Representative recordings show the highly variable baseline firing in controls that is lost in Chat-Mecp2^−/y mice. D, There was no difference between genotypes in baseline firing rates in the PRH (Kruskal–Wallis test: H(c3) = 6.62; p = 0.085). E, Variability of firing rate as measured by the Fano factor was significantly different between groups (Kruskal–Wallis test: H(c3) = 8.92; p = 0.03). Chat-Mecp2^−/y mice had a lower firing rate variability than all three controls (no transgene: mean FF = 0.612 ± 1.250; Chat Cre: mean FF = 0.474 ± 0.650; MeCP2 flox: mean FF = 0.447 ± 0.365; Chat-Mecp2^−/y: mean FF = 0.248 ± 0.431). No transgene: n = 24 units from 6 mice; Chat Cre: n = 22 units from 11 mice; MeCP2 flox: n = 6 units from 3 mice; Chat-Mecp2^−/y: n = 20 units from 8 mice. *p ≤ 0.05, ns = non significant.
where we found subtle alterations in baseline firing of L5/6 neurons in mice with selective knockout of MeCP2 from cholinergic neurons (Dere et al., 2007). Selective deletion of MeCP2 from cholinergic neurons also ablated responses to the stimulation of cholinergic input to PRH. Finally, we demonstrated that both the behavioral and the electrophysiological profiles of cholinergic MeCP2 deletion were rescued by inhibiting ACh breakdown with donepezil.

Chat-Mecp2−/− mice were selectively impaired in recognition memory tasks—both in tests of novel versus familiar object and in novel versus familiar conspecific recognition. This is consistent with the results of the study by Zhang et al. (2016), who showed that Chat-Mecp2−/− mice were impaired on the recognition of a familiar conspecific, although they did not assay novel object recognition. The selective nature of the observed cognitive deficit is an intriguing result as cholinergic signaling is known to be vital for performance on both spatial and emotional memory tasks, such as the Morris water maze and the cue-conditioned and/or context-conditioned fear assays (McNamara and Skelton, 1993; Gould, 2003; Jiang et al., 2016). The fact that Chat-Mecp2−/− mice are not impaired on these tasks implies one of two possibilities. First, not all cholinergic neurons are functionally dependent on MeCP2 expression. Cholinergic neurons that project to brain areas involved in recognition memory may be functionally dependent on MeCP2 expression and are therefore functionally impaired by its deletion, while cholinergic neurons that project to areas involved in emotional and spatial memory are not. Although originally conceptualized as homogeneous, cholinergic basal forebrain neurons are actually quite diverse in terms of receptor and neurotransmitter expression and exhibit intricate topographical and functional organization that has only recently begun to be appreciated (Allen et al., 2006; Chandler and Waterhouse, 2012; Chandler et al., 2013; Saunders et al., 2015; Zaborszky et al., 2015a,b; Kondo and Zaborszky, 2016). The second possibility is that the loss of MeCP2 partially impairs cholinergic neurons and that different degrees of impairments might produce different phenotypes. Partial impairment impacts novel object recognition, but it might take more severe impairment to impact other learning phenotypes.

The fact that the deletion of MeCP2 from cholinergic neurons alters PRH firing both at baseline and after the stimulation of cholinergic input suggests that disruption of cholinergic signaling has several distinct effects on excitability over differing time scales. This is not surprising as ACh is thought to exert its effects via both tonic and transient signaling mechanisms (for review, see Ballinger et al., 2016). The degree to which these different modes of signaling contribute to different or overlapping cognitive functions is not well understood. Our observation that deleting MeCP2 in cholinergic neurons affects both the baseline rate and pattern of firing of L5/6 PRH neurons as well as the response to stimulation of cholinergic input supports the idea that cholinergic transmission via both of these signaling mechanisms may be involved in the synaptic regulation of PRH neuron excitability.

**Figure 4.** Strategy for optogenetic stimulation of cholinergic neurons. **A**, Schematic of the experimental paradigm. **B**, A viral vector encoding an optically activated excitatory ion channel is injected into the NBM. The viral vector is of a flip excision switch design such that it will be expressed only in the presence of Cre recombinase. **C**, Representative images of virally labeled cholinergic neurons (white arrowheads) from a control mouse (blue, top) and a Chat-Mecp2−/− mouse (red, bottom). **D**, Representative optically evoked action potentials in the NBM of a control mouse (top) and a Chat-Mecp2−/− mouse (bottom). The timing of laser pulses delivered into the NBM is indicated by light blue hash marks.
At baseline, the effect of impairing cholinergic signaling via Mecp2 deletion is to reduce the variability of firing. This may represent a loss of dynamic range over which individual neurons can encode. The function of ongoing, tonic ACh release in the PRH may therefore be to increase this dynamic range. In addition, the effect of Chat-Mecp2$^{-/-}$ on the response of PRH neurons to optogenetic stimulation of cholinergic input is to ablate any changes in firing of the target PRH neurons. This may represent reduced functional connectivity of the NBM–PRH circuit. This loss of functional connectivity and dynamic range may impair novel object recognition encoding and therefore underlie the behavioral impairment. As our recordings have all been collected from anesthetized mice, a critical next step is to record from awake animals during behavior performance to clarify the relationship between these electrophysiological phenotypes and behavioral impairment. Furthermore, the current experiments have used cholinergic cell body stimulation to investigate the integrity of the NBM–PRH circuit. However, cholinergic NBM neurons are known to project broadly to a variety of brain areas (see Wu et al., 2014), and it is therefore impossible to determine whether the effects shown here are specific to the PRH or are downstream effects from other cholinergically innervated areas. In fact, when Mecp2 is re-expressed in cholinergic neurons in Mecp2 knock-out

**Figure 5.** PRH response to the stimulation of endogenous cholinergic signaling is impaired in Chat-Mecp2$^{-/-}$ mice. A, B, Representative data from a PRH unit in a control mouse exhibiting a response to stimulation of cholinergic input (A) and a PRH unit from an Chat-Mecp2$^{-/-}$ mouse (B). Top, Representative raster plot of spikes. Vertical light blue bar indicates timing of optical stimulation. Bottom, Box plot of interspike intervals. C, Heat map of $p$ values as a function of time since optical stimulation for PRH units from control mice (left) and from Chat-Mecp2$^{-/-}$ mice (right). Responses either occurred in the first time period following laser stimulation or were delayed. Each row represents a separate unit. The results are summarized in pie charts at bottom. D, Summary of differing response rates between control and Chat-Mecp2$^{-/-}$ units ($\chi^2$ test for homogeneity: CR(2) = 6.02; $p = 0.049$). $\ast p \leq 0.05$. 
mice, the deficit in recognition of a familiar conspecific persists, suggesting that, although the present results implicate cholinergic MeCP2 expression as necessary for intact recognition memory, it is not sufficient (Zhou et al., 2017). Thus, there are likely other relevant brain areas through which the cholinergic basal forebrain exerts its effect on recognition memory performance. Future analysis of the functional connectivity of other brain areas in the context of cholinergic MeCP2 deletion may clarify their contribution to the behavioral phenotype documented here. Further work investigating cholinergic input to the PRH in Chat-Mecp2<sup>-/-</sup> mice using terminal field optogenetic stimulation and neurotransmitter release measurements is also needed to clarify the role of cholinergic signaling in this brain area and the effects of MeCP2 disruption on it.

It is possible that the electrophysiological and behavioral deficits discussed above are a consequence of a
“sick neuron” syndrome induced by the catastrophic effects of MeCP2 deletion in a subpopulation of neurons or that these effects are mediated by a neurotransmitter other than ACh, as cholinergic neurons are known to synthesize many different neurotransmitters (Tkatch et al., 1998; Allen et al., 2006; Saunders et al., 2015). If these deficits are truly due to a cholinergic signaling impairment, we would expect that they might be rescued by the inhibition of ACh breakdown and boosting of the cholinergic signal. This was indeed the case: the fact that the inhibition of ACh breakdown with donepezil rescued these impairments demonstrates an essential role of ACh per se in mediating the phenotype. The donepezil rescue of behavior was seemingly more robust than the donepezil inhibition of ACh breakdown with donepezil rescued these deficits are truly due to a cholinergic signaling impairment, Cholinergic signaling is therefore an important mediator of cognitive deficits in mice lacking MeCP2, and MeCP2 expression is vital for cholinergic mediation of recognition memory.

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