Acta Neuropathol (2008) 116:425–437
DOI 10.1007/s00401-008-0407-x

ORIGINAL PAPER

Neurotrophic effects of Cerebrolysin in the Mecp2$^{308/Y}$ transgenic model of Rett syndrome

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Received: 8 April 2008 / Revised: 13 June 2008 / Accepted: 14 June 2008 / Published online: 4 July 2008

Abstract Rett syndrome is a childhood neurodevelopmental disorder caused by mutations in the gene encoding for methyl-CpG-binding protein (MeCP2). Neuropathological studies in patients with Rett syndrome and in MeCP2 mutant models have shown reduced dendritic arborization and abnormal neuronal packing. We have previously shown that Cerebrolysin (CBL), a neurotrophic peptide mixture, ameliorates the synaptic and dendritic pathology in models of aging and neurodegeneration. This study aimed to determine whether CBL was capable of reducing behavioral and neuronal alterations in Mecp2$^{308/Y}$ mutant mice. Two sets of experiments were performed, the first with 4-month-old male Mecp2$^{308/Y}$ mutant mice treated with CBL or vehicle for 3 months (Group A) and the second with 1-month-old mice treated for 6 months (Group B). Behavioral analysis showed improved motor performance with CBL in Group A and a trend toward improvement in Group B. Consistent with behavioral findings, neuropathological analysis of the basal ganglia showed amelioration of dendritic simplification in CBL-treated Mecp2$^{308/Y}$ mutant mice. CBL treatment also ameliorated dendritic pathology and neuronal loss in the hippocampus and neocortex in Mecp2$^{308/Y}$ mutant mice. In conclusion, this study demonstrates that CBL promotes recovery of dendritic and neuronal damage and behavioral improvements in young adult Mecp2$^{308/Y}$ mutant mice and suggests that CBL may have neurotrophic effects in this model. These findings support the possibility that CBL may have beneficial effects in the management of Rett syndrome.

Keywords Neurotrophic peptides · Neurodevelopmental disorders · Dendrites

This work was partially funded by a grant from EBEWE Pharmaceuticals and by NIH grants NS057096, AG18440 and AG10435.

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Introduction

Rett syndrome is a childhood neurodevelopmental disorder characterized by normal early development followed by gait abnormalities, seizures and mental retardation. It is an X-linked disorder affecting 1 in 15,000 female children and is caused by sporadic loss-of-function mutations of the gene encoding the transcriptional repressor methyl-CpG-binding protein (*MECP2*) [2, 3, 62]. Since this gene is located on the X-chromosome, Rett syndrome in its classic form only appears in girls, in males symptoms are more severe and usually lethal [8, 61]. A large number of mutations in the *Mecp2* gene and X-chromosome inactivation patterns lead to a broad phenotypic spectrum [20, 47].

Classic Rett syndrome first appears after 6–18 months of apparently normal development when girls begin to regress intellectually in a predictable pattern [28]. Neuropathological studies have shown reduced brain size with decreased dendritic arborization and synaptic formation in the frontal,

motor and temporal cortices [4, 6]. There is also increased neuronal packing density of small neurons and reduced expression of the dendritic protein-MAP2 [29]. The synapto-dendritic arbor of pyramidal neurons in the cortex appears to be selectively damaged, while calbindin-expressing interneurons are preserved. Alterations in cholinergic, dopaminergic and serotonin systems have also been reported [44].

Supporting a role for Mecp2 in the pathogenesis of Rett syndrome, previous studies have shown that male transgenic mice expressing a targeted truncated *Mecp2* (premature stop codon after codon 308) develop progressive neurological alterations beginning at 6 weeks that include tremor, motor impairment as assessed by pole test, hypoactivity and increased anxiety by 8 months mutant mice appear disheveled and are prone to seizures [38, 56]. The brains of the *Mecp2*$^{308/y}$ mutant mice show increased levels of acetylated histone 3 and expression of the c-terminus truncated Mecp2; however, no conclusive neuropathological features have been described in this model. The Mecp2$^{308/Y}$ model might mimic the behavioral symptoms of patients with Rett syndrome closer than mouse models carrying null mutations [9, 19]. Though is important to remember that no animal model of a human disorder can completely mimic the condition seen in humans and that the neuropathology seen in models may be more subtle than that found in humans; together these Rett mice models can, in addition to mimicking behavioral characteristics of Rett such as deficits in locomotion [56], provide important information regarding the neuropathology of Rett syndrome including altered hippocampal and amygdala volumes [58] and altered post-synaptic density length in the hippocampus [39].

Although considerable progress has been made at understanding the genetics and molecular mechanisms of Rett syndrome and the effects of mutations in Mecp2, no therapies are currently available.

Cerebrolysin (CBL) is a brain-derived peptide preparation produced by a standardized enzymatic breakdown of purified brain proteins and consists of low-molecular weight neuropeptides and free amino acids. We have previously shown that CBL ameliorates the synaptic and dendritic pathology in models of aging and neurodegeneration [23, 24, 35, 49–52, 64]. CBL has been shown to display nerve growth factor (NGF)-like effects on cholinergic neurons [55] and to protect synapses and dendrites by blocking glycogen synthase kinase3β (GSK3β) and cyclin-dependent kinase 5 (CDK5) hyperactivation [54]. Recent studies have shown that CBL reduces the memory deficits in patients with dementia [1, 40, 63] and an open-pilot trial [16] in nine female Rett syndrome patients (2–7 years) showed that CBL normalized the electroencephalograph parameters, decreased seizures and improved behavioral and motor function.

Taken together these studies suggest that CBL may have beneficial effects in Rett syndrome by ameliorating the synaptic and dendritic pathology. In this context, and to investigate the potential influence of CBL on the pathogenesis of Rett-like phenotype, young and mature male Mecp2$^{308/Y}$ mice were treated with CBL and behavior and neuropathological studies were performed (Fig. 1). The findings suggest that CBL may help to ameliorate the pathological progression of Rett syndrome and therefore may have beneficial effects not only in neurodegenerative disorders such as Alzheimer's disease but also in neurodevelopmental disorders such as Rett syndrome.

Materials and methods

Breeding and maintenance of Mecp2$^{308/Y}$ mouse model

Breeding pairs of the Mecp2$^{308/Y}$ mouse model were obtained from The Jackson Laboratory, Bar Harbor/ME. This model was selected because it has previously been shown to reproduce some aspects of the behavioral alterations in Rett syndrome [56, 57]. Female mutant homozygous mice die at a very young age and heterozygous females display an unbalanced pattern of X-chromosome inactivation which favors the expression of the wild-type allele and results in a high degree of phenotypic variability [66], in an effort to avoid these complications male mutant mice were used for these experiments. Young mutant female heterozygous mice and wild-type male littermates were bred over several generations to obtain a total of 48 male Mecp2$^{308/Y}$ mutant mice. A subset ($n = 8$) of wild-type male mice were used as controls. Mutant offspring were identified by PCR analysis of tail DNA. Briefly, genomic DNA was extracted as previously described [37] and amplified

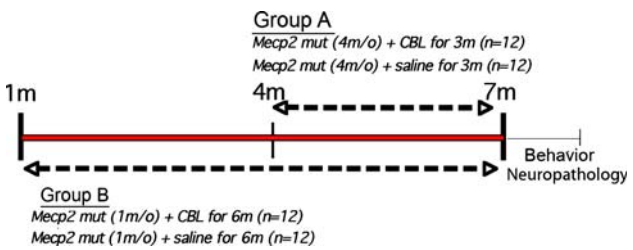

Fig. 1 Experimental design. Mecp2$^{308/Y}$ (Premature stop after codon 308) mutant mice, previously shown to display some behavioral characteristics associated with Rett syndrome [40] were obtained from the Jackson Laboratory and were divided into two experimental groups, differing in their length of treatment with Cerebrolysin. Group A mice were treated from 4 till 7 months of age with either Cerebrolysin (5 ml/kg, $n = 12$) or vehicle (saline, $n = 12$). Group B mice were treated from 1 month of age till 7 months with either Cerebrolysin (5 ml/kg, $n = 12$) or vehicle (saline, $n = 12$). At 7 months mice were analyzed behaviorally and then for neuropathology

in 30 cycles (93°C × 30 s, 49°C × 30 s, 72°C × 1 min) with a final extension at 72°C × 5 min.

Cerebrolysin treatment

In order to assess possible beneficial effects of CBL on behavioral and neuronal alterations in young and mature Mecp2$^{308/Y}$ mutant mice, two sets of experiments were performed. For the first, a total of 24 (4-month-old) male Mecp2$^{308/Y}$ mutant mice were treated for 3 months with CBL (n = 12; 5 ml/kg) or vehicle (n = 12) (Group A) (Fig. 1). For the second set, a total of 24 (1-month-old) male Mecp2$^{308/Y}$ mutant mice were treated for 6 months with CBL (n = 12; 5 ml/kg) or vehicle (n = 12) (Group B) (Fig. 1). CBL (and vehicle) were administered via i.p injection.

The two groups were designed to enable a comparison of the effects of Cerebrolysin when given as a therapeutic measure, once the disease has become established (Group A, treatment from 4 till 7 months) versus its effects when administered as an early intervention prior to the frank onset of the disease (Group B, treatment from 1 to 7 months).

At the end of the treatment period, mice were tested behaviorally in the open field and pole test. These tests were designed to assess the motor function of the mice. The open-field test is the most standardized measure of general motor function [11], while the pole test is a well-documented test used to assess basal ganglia-related movement in mice [12, 13, 27, 36, 45, 46].

In order to assess the baseline levels of neuropathology, groups (n = 3) of untreated Mecp2$^{308/Y}$ mutant mice were killed at 1, 4 and 7 months of age. These animals were compared to age-matched wild-type controls.

Behavioral analysis

For the open field, spontaneous activity was measured in Plexiglas cages (42 × 22 × 20 cm) placed into frames (25.5 × 47 cm) mounted with two levels of photocell beams at 2 and 7 cm above the bottom of the cage (San Diego Instruments,

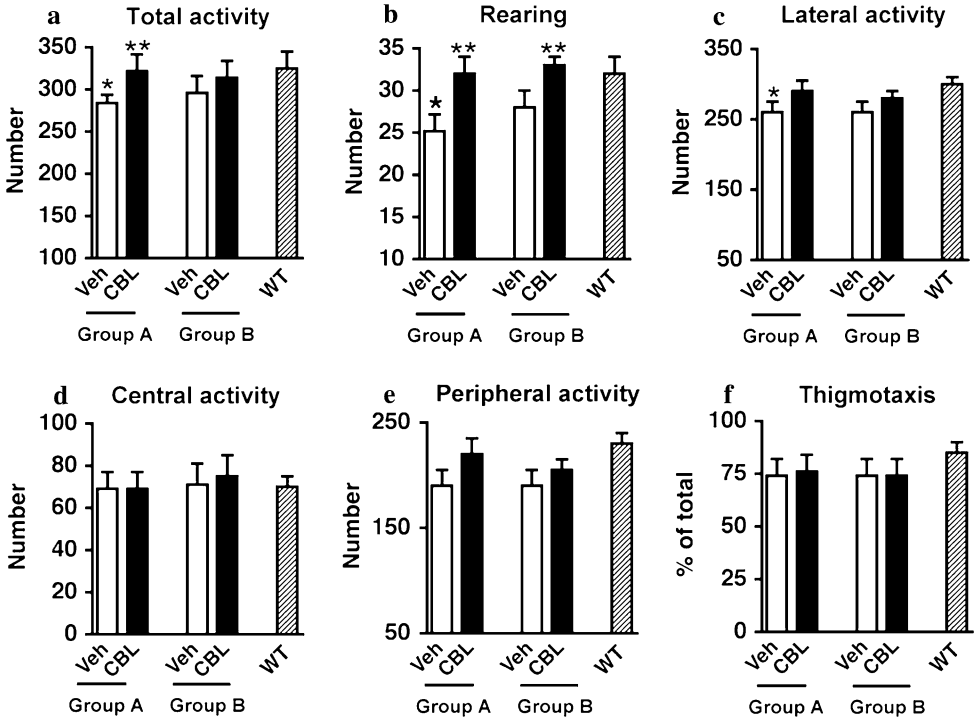

Fig. 2 Effects of Cerebrolysin on open-field activity in Mecp2$^{308/Y}$ mutant mice. Spontaneous activity of the mice was analyzed via the open-field test. Mice were placed in a Plexiglass cage and two photocell beams were used to measure vertical and horizontal activity. Mice were tested for 15 min. Activity was analyzed as total activity (**a**), rearing (**b**), lateral activity (**c**), time spent in the centre of the open field (**d**), time spent at the periphery (**e**), and thigmotaxis (**f**). Compared to wild-type controls, total (**a**), rearing (**b**) and lateral activity (**c**) were all significantly decreased in vehicle-treated Group A Mecp2$^{308/Y}$ mutant mice. Cerebrolysin treatment in Group A increased total, rearing and lateral activity to levels comparable with wild-type controls ($P < 0.05$). Rearing activity was also reduced in Group B vehicle-treated Mecp2$^{308/Y}$ mutant mice in comparison to wild-type controls; Cerebrolysin treatment increased rearing activity in these mice to a level comparable to wild-type control mice. Analysis of the time spent in the centre of the cage versus time spent at the perimeter (**d**–**f**) showed no differences between wild-type controls and vehicle- or Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice. *indicates a significant difference between vehicle-treated Mecp2$^{308/Y}$ mutant mice (n = 12) and wild-type controls (n = 8) ($P < 0.05$, by one-way ANOVA and post hoc Fisher) **indicates a significant difference between Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice (n = 12) and wild-type controls (n = 8) ($P < 0.05$, by one-way ANOVA and post hoc Fisher)

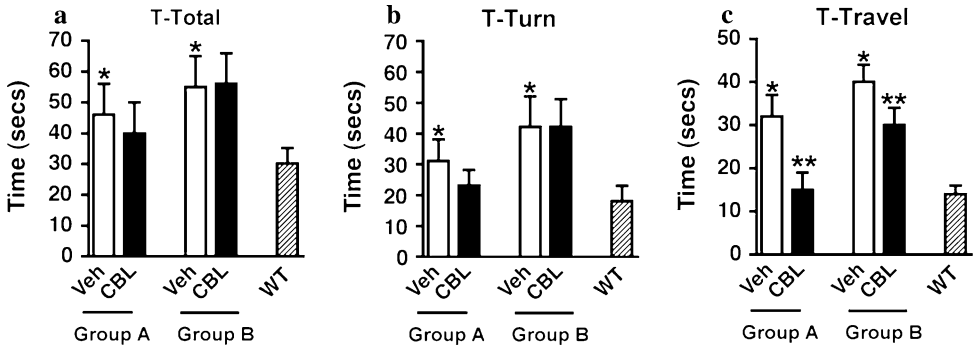

Fig. 3 Effects of Cerebrolysin on Pole Test performance in Mecp2$^{308/Y}$ mutant mice. Motor behavior was assessed using the pole test. Mice were placed at the top of a vertical pole and total time to descend (T-total, **a**), time taken orient themselves (T-turn, **b**) and time to travel down the pole (T-travel, **c**) were measured. Mice received 2 days of training, five trails each day and were analyzed on the third day. Group A and B vehicle-treated Mecp2$^{308/Y}$ mutant mice displayed significantly longer T-Total times in comparison to age-matched wild-type controls (**a**). CBL treatment did not result in any significant change in T-Total, though a trend toward decreased T-Total was observed in Group A Mecp2$^{308/Y}$ mutant mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mutant mice (**a**). T-Turn was significantly increased in both Groups A and B vehicle-treated Mecp2$^{308/Y}$ mutant mice in comparison to wild-type controls. CBL treatment did not result in any significant change in T-Turn, though a trend toward decreased T-Turn was observed in Group A Mecp2$^{308/Y}$ mutant mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mutant mice (**b**). Vehicle-treated Mecp2$^{308/Y}$ mutant mice from both Groups A and B displayed significantly increased T-travel times in comparison to wild-type controls, CBL treatment significantly lowered T-travel time in both Groups A and B in comparison to vehicle-treated Mecp2$^{308/Y}$ mutant mice (**c**). *indicates a significant difference between vehicle-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher), **indicates a significant difference between Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher)

San Diego, CA). These two sets of beams allow for the recording of both horizontal (locomotion) and vertical (rearing) behavior. Specific variables derived included horizontal activity, vertical activity, total distance traversed, total number of movements and time spent in the center versus time spent in the perimeter of the cage (thigmotaxis). Mice were tested for 5 min as this is usually sufficient to evaluate gross abnormalities in locomotion [11].

For the pole test, animals were placed head upward on top of a vertical wooden pole 50 cm long (1 cm in diameter). The base of the pole was placed in the home cage. When placed on the pole, animals orient themselves downward and descend the length of the pole back into their home cage. Groups of mice received 2 days of training that consisted of five trials for each session. On the test day, animals received five trials, time to orient downward (T-turn), time to travel down the pole (T-travel) and total time to descend (T-total) were measured.

Tissue processing and analysis of neurodegeneration

Following NIH guidelines for the humane treatment of animals, under anesthesia mice were killed and brains removed. The right hemibrain was immersion-fixed in 4% paraformaldehyde in pH 7.4 PBS and serially sectioned at 40 μm with the Vibratome (Leica, Deerfield, IL) for subsequent analysis of neurodegeneration. The left hemibrain was kept at −80°C. To investigate the effects of CBL in Mecp2$^{308/Y}$ mutant mice, vibratome sections were immunolabeled overnight with antibodies against the neuronal marker NeuN (1:1,000, Chemicon) and the dentritic marker MAP2 (1:250, Chemicon), followed by incubation with species-appropriate secondary antibodies (1:200, Vector Laboratories). Sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, CA) and mounted under glass coverslips with anti-fading media (Vector Laboratories). The immunolabeled blind-coded sections were analyzed with the laser scanning confocal microscope (LSCM) (MRC1024, BioRad) to evaluate the area of the neuropil covered by MAP2 immunoreactive dendrites in the basal ganglia, frontal cortex, dentate gyrus, CA3 and CA1 regions of the hippocampus. Stereological analysis was also conducted using the Stereo Investigator software package (MBF Biosciences), as previously described [32] to examine the neuronal density in the basal ganglia, frontal cortex (layers II/III, 3 sections, 4 fields per section) and dentate gyrus, CA3 and CA1 regions of the hippocampus, neurons were determined by NeuN immunoreactivity, this measure is an estimate of total cell number.

The frontal cortex and hippocampus were investigated on the basis of previous studies reporting dendritic abnormalities in the neocortex [29, 30] and impaired learning, memory and post-synaptic density morphology [39], the basal ganglia were chosen as motor activity linked to basal ganglia function was being assessed.

Western blot analysis

Protein levels of acetylated histone H3 and total H3 histone in Mecp2$^{308/Y}$ mutant mice were analyzed by immunoblot.

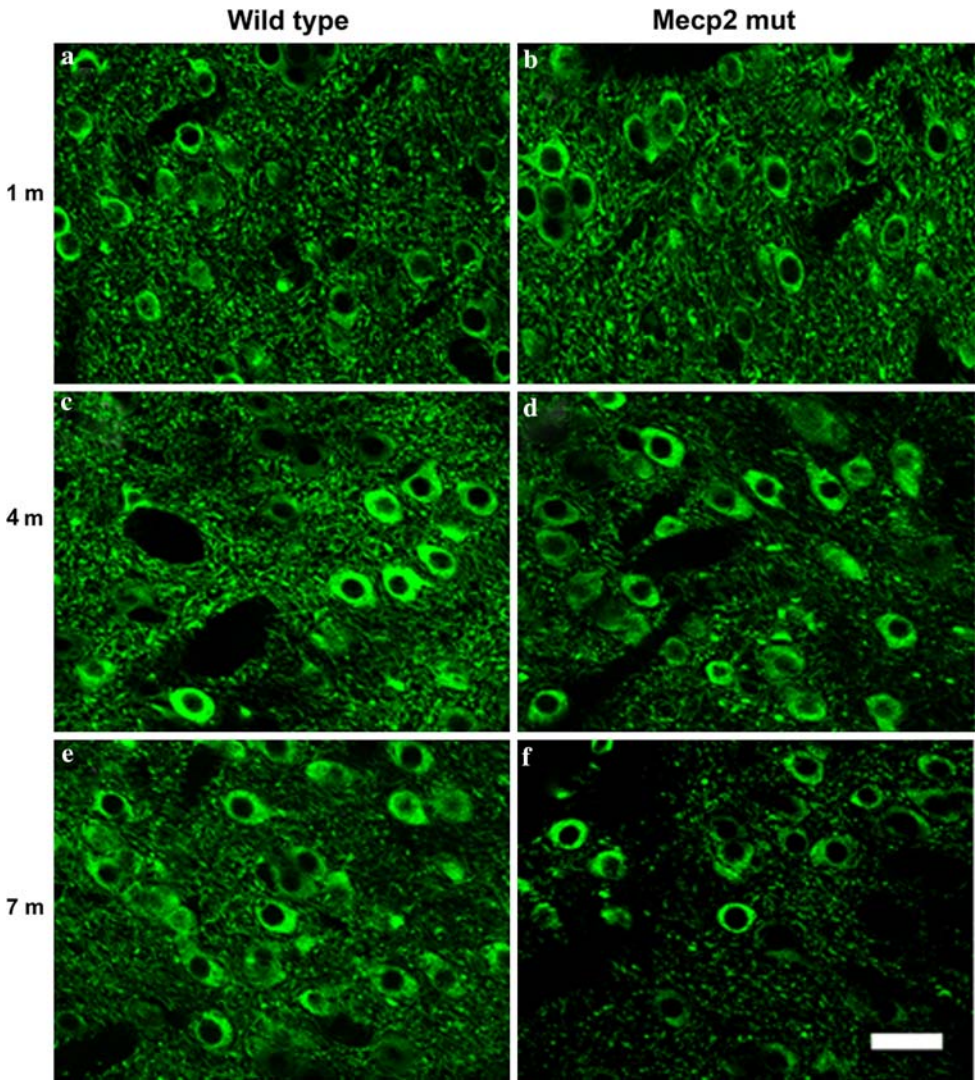

Fig. 4 Baseline neuropathology in Mecp2$^{308/Y}$ mutant mice. Laser scanning confocal microscopic images of the basal ganglia of wild-type mice at 1-month (**a**), 4-months (**c**) and 7-months (**e**) of age and of untreated Mecp2$^{308/Y}$ mice at 1 month (**b**), 4 months (**d**) and 7 months (**f**) of age immunostained with an antibody against the dendritic marker MAP2. *Scale bars* represent 20 μM. Dendritic complexity between Mecp2$^{308/Y}$ mutant mice and age-matched wild-type controls is similar at 1 month of age (**a**, **b**). Loss of dendritic complexity in the Mecp2$^{308/Y}$ mutant mice in comparison to age-matched wild-type controls becomes evident at 4 months of age (**c, d**) and is markedly increased at 7 months (**e**, **f**)

Brain homogenates were obtained as previously described [53]. Twenty micrograms of total protein per mouse were loaded onto 10% Bis–Tris (Invitrogen) SDS-PAGE gels, transferred onto Immobilon membranes, incubated with antibodies against anti-acetyl-histone H3 rabbit polyclonal (1:1,000, Upstate Biotechnology) and anti-histone H3 mouse monoclonal antibody (1:1,000, Upstate Biotechnology). After overnight incubation with primary antibodies, membranes were incubated in appropriate secondary antibodies, reacted with ECL and developed on a VersaDoc gel-imaging machine (Bio-Rad, Hercules, CA). Anti-beta-actin (1:1,000, Sigma) was used to confirm equal loading.

Statistical methods

Differences between groups were tested using one and two factor ANOVA with Fisher PLSD posthoc tests. Additional preliminary analysis between control and treated groups was by unpaired, two-tailed, Student's *t* test. All the results are expressed as mean ± SEM.

Results

Cerebrolysin effects on behavioral performance in mature and young Mecp2 mutant mice

Previous studies have shown that *Mecp2* mutant mice older than 6 weeks display alterations in the open field and pole test [19, 56]. Compared to age-matched wild-type controls, vehicle-treated *Mecp2*$^{308/y}$ mutant mice in Group A displayed decreased total, rearing and lateral activity (Fig. 2a–c). CBL treatment of Group A Mecp2$^{308/y}$ mutant mice increased total, rearing and lateral activity in comparison to vehicle-treated Group A *Mecp2*$^{308/y}$ mutant mice and to a level comparable to wild-type controls (Fig. 2a–c). In Group B, where treatment was started at a younger age, there was a trend toward decreased total ($P = 0.3$), rearing ($P = 0.09$) and lateral ($P = 0.08$) activity in vehicle-treated *Mecp2*$^{308/y}$ mutant mice in comparison to wild-type controls and an amelioration of this phenotype with CBL treatment (Fig. 2a–c). No significant differences in thigmotaxis

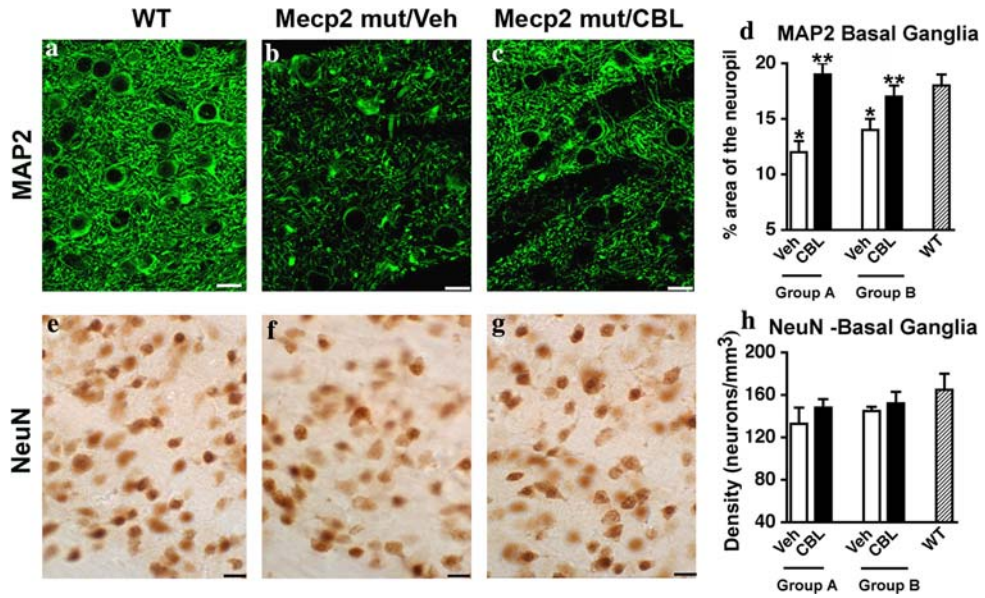

Fig. 5 Cerebrolysin ameliorates dendritic damage in the Basal Ganglia of Mecp2$^{308/Y}$ mutant mice. Laser scanning confocal microscopic images of the basal ganglia of wild-type mice (**a**) vehicle-treated Mecp2$^{308/Y}$ mice (**b**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**c**) immunostained with an antibody against the dendritic marker MAP2. Area of the neuropil covered by MAP2 immunoreactivity was analyzed (**d**). The lower panel depicts bright light microscopic images of the basal ganglia of wild-type mice (**e**), vehicle-treated Mecp2$^{308/Y}$ mice (**f**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**g**) immunostained with an antibody against the neuronal marker, NeuN. Stereological analysis of neuronal density was also performed (**h**). Images (**b**), (**c**), (**f**) and (**g**) are from Group A Mecp2$^{308/Y}$ mice (treated from 4 till 7 months of age with either Cerebrolysin or saline). *Scale bars* represent 10 μM, a decrease in MAP2 immunoreactivity was evident in vehicle-treated Mecp2$^{308/Y}$ mice in comparison to wild-type controls (**b**) compared to (**a**) and (**d**). A significant increase in MAP2 immunoreactivity was observed following Cerebrolysin treatment in Mecp2$^{308/Y}$ mice in comparison to vehicle-treated mice (**c**) (*inc stats p number, etc.*) making MAP2 levels in these mice more analogous to those seen in wild-type mice. No differences were noted in basal ganglia NeuN levels between the different groups. *indicates a significant difference between vehicle-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher), **indicates a significant difference between Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher)

where observed between wild type and vehicle- or CBL-treated Mecp2$^{308/Y}$ mutant mice (Fig. 2d–f).

In the pole test, vehicle-treated Mecp2$^{308/Y}$ mutant mice from both Groups A and B displayed significantly longer T-total times in comparison to age-matched wild-type controls ($P < 0.05$). There were no significant differences in T-Total upon CBL treatment, though a trend toward decreased T-Total ($P = 0.08$) was observed in Group A Mecp2$^{308/Y}$ mutant mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mutant mice (Fig. 3a). T-Turn was significantly increased in both Groups A and B vehicle-treated Mecp2$^{308/Y}$ mutant mice in comparison to wild-type controls. There were no significant differences in T-Turn upon CBL treatment, though a trend toward decreased T-Turn ($P = 0.08$) was observed in Group A Mecp2$^{308/Y}$ mutant mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mutant mice (Fig. 3b). Vehicle-treated Mecp2$^{308/Y}$ mutant mice from both Groups A and B displayed significantly increased T-travel times in comparison to wild-type controls, CBL treatment significantly lowered T-travel time in both Groups A and B in comparison to vehicle-treated Mecp2$^{308/Y}$ mutant mice (Fig. 3c).

Cerebrolysin effects on patterns of neurodegeneration in mature and young Mecp2$^{308/y}$ mutant mice

In order to establish a baseline for the neuropathology observed in the Mecp2$^{308/y}$ mutant mice, untreated Mecp2$^{308/y}$ mutant mice were killed at 1, 4 and 7 months and compared to age-matched wild-type mice. No real difference in dendritic complexity in the basal ganglia, as evidenced by MAP2 immunoreactivity, was evident between wild-type mice and untreated Mecp2$^{308/y}$ mutant mice at 1 month of age (Fig. 4a, b). By 4 months of age the untreated Mecp2$^{308/y}$ mutant mice begin to show a reduction in dendritic complexity in comparison to age-matched wild-type mice (Fig. 4c, d), this becomes increasing marked at 7 months of age (Fig. 4e, f). Similar results were observed in the neocortex and hippocampus (data not shown).

Consistent with behavioral data, neuropathological analysis of the basal ganglia at 7 months showed a decrease in dendritic complexity as evidenced by MAP2 immunoreactivity in vehicle-treated Mecp2$^{308/Y}$ mice in comparison to age-matched wild-type controls in both Groups A and

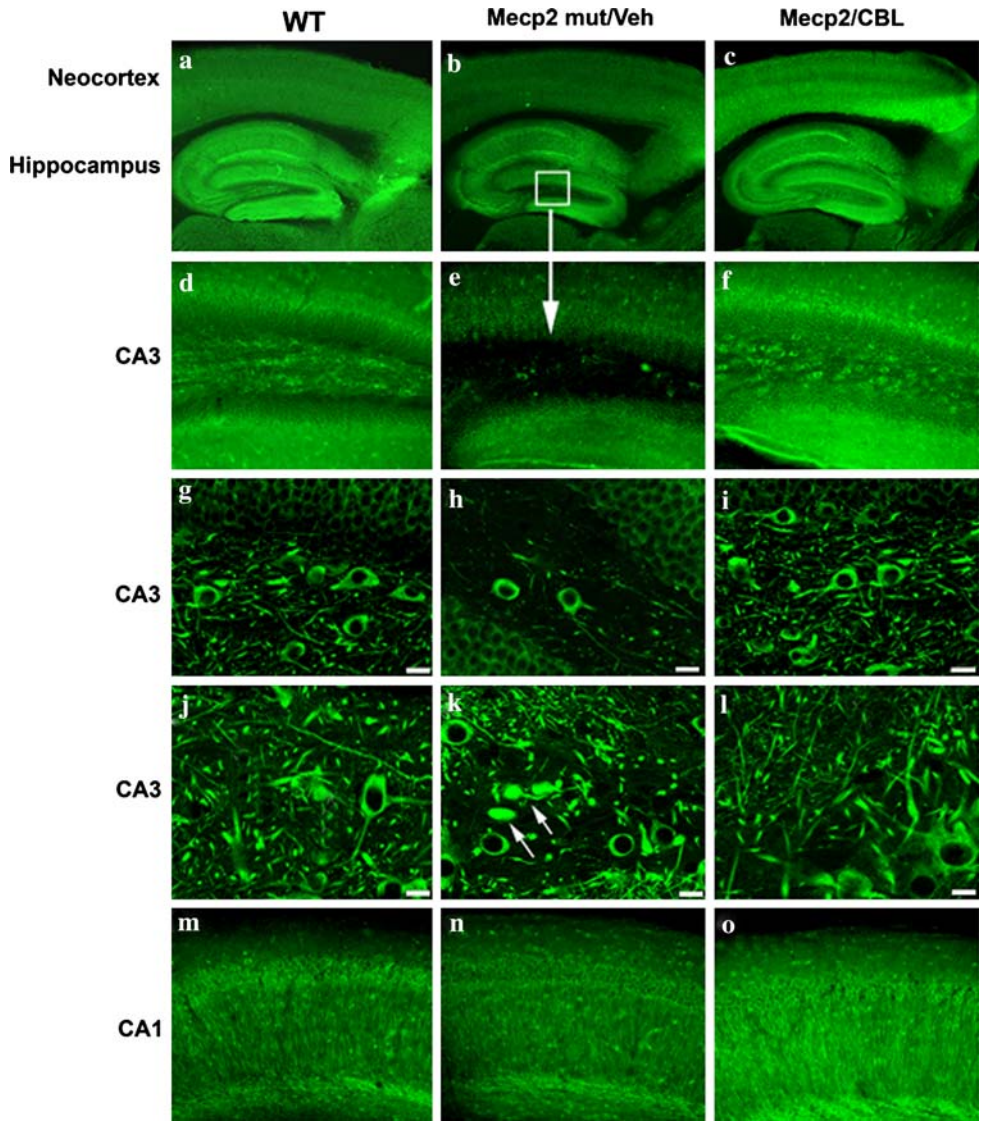

Fig. 6 Cerebrolysin ameliorates dendritic damage in the hippocampus of Mecp2$^{308/Y}$ mutant mice. Low-power laser scanning confocal microscopic images of MAP2 immunoreactivity in the hippocampus of wild-type mice (**a**) vehicle-treated Mecp2$^{308/Y}$ mice (**b**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**c**). High-power confocal microscopic images of the CA3 in wild-type mice (**d**, **g**, **j**) vehicle-treated Mecp2$^{308/Y}$ mice (**e**, **h**, **k**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**f**, **i**, **l**). Dendrite morphology was analyzed in wild-type mice (**j**) vehicle-treated Mecp2$^{308/Y}$ mice (**k**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**l**). In vehicle-treated Mecp2$^{308/Y}$ mice dendrites appeared tortuous and irregular (*arrows* in **k**). MAP2 immunoreactivity was also analyzed in the CA1 of wild-type mice (**m**) vehicle-treated Mecp2$^{308/Y}$ mice (**n**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**o**). Images (**b**, **c**), (**e**, **f**), (**h**, **i**), (**k**, **l**) and (**n**, **o**) are from Group A Mecp2$^{308/Y}$ mice (treated from 4 till 7 months of age with either Cerebrolysin or saline). *Scale bars* represent 10 μM. A decrease in MAP2 immunoreactivity in the dentate gyrus and CA3 region was evident in vehicle-treated Mecp2$^{308/Y}$ mice in comparison to wild-type controls (**e**) compared to (**d**) and (**h**) compared to (**g**). An increase in MAP2 immunoreactivity was observed in Cerebrolysin-treated Mecp2$^{308/Y}$ mice making MAP2 levels in these mice more analogous to those seen in wild-type mice (**e**, **f**). No overall differences were noted in MAP2 levels in the CA1 (*arrows* in **m**–**o**)

Group B (Fig. 5a, b). CBL treatment significantly ameliorated this dendritic pathology in Mecp2$^{308/Y}$ mutant mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mice (Fig. 5c, d). Levels of MAP2 immunoreactivity in CBL-treated Mecp2$^{308/Y}$ mutant mice were comparable to those seen in wild-type mice (Fig. 5d).

Stereological analysis of neuronal density in the basal ganglia, as evidenced by NeuN immunoreactivity, revealed no significant differences in NeuN immunoreactivity between wild type and vehicle- or CBL-treated Mecp2$^{308/Y}$ mutant mice (Fig. 5e–h).

In order to further characterize the patterns of neurodegeneration and response to CBL, dendritic pathology and neuronal number were analyzed in the hippocampus and neocortex. Compared to age-matched wild-type controls, vehicle-treated Mecp2$^{308/y}$ mutant mice displayed a significant decrease in the levels of MAP2 immunoreactivity in pyramidal neurons in the CA3 region of the hippocampus

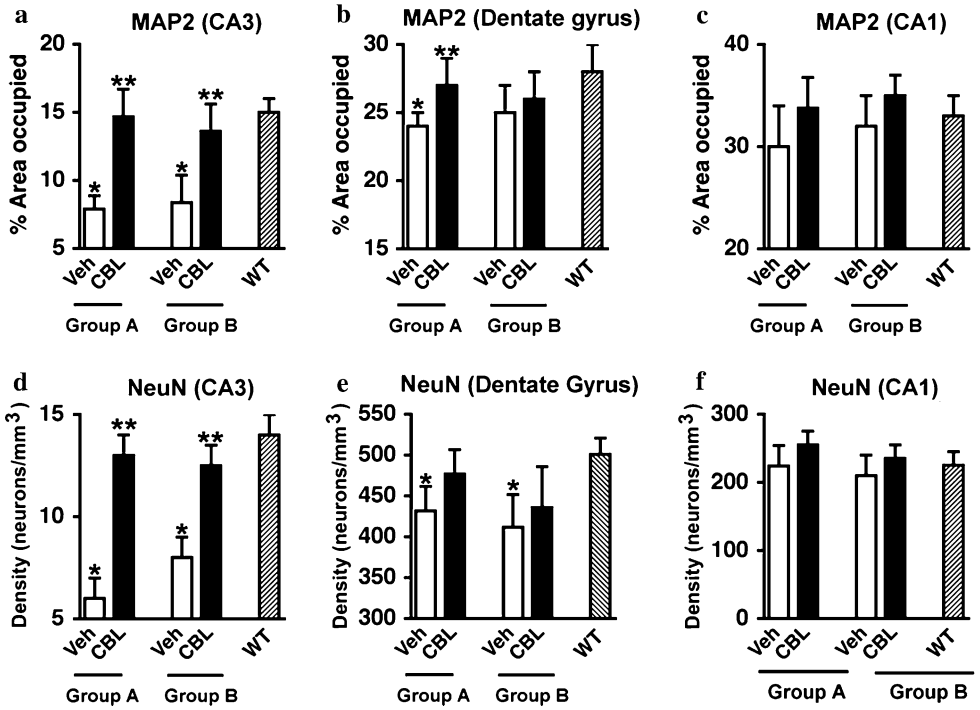

Fig. 7 Analysis of Cerebrolysin effects in the hippocampus of Mecp2$^{308/Y}$ mutant mice. Analysis of hippocampal MAP2 immunoreactivity in the CA3 region (**a**), dentate gyrus (**b**) and CA1 (**c**) of wild type and vehicle- or Cerebrolysin-treated Mecp2$^{308/Y}$ mice. Stereological analysis of neuronal number as evidence by NeuN immunoreactivity in hippocampal CA3 region (**d**), dentate gyrus (**e**) and CA1 (**f**) of wild type and vehicle- or Cerebrolysin-treated Mecp2$^{308/Y}$ mice. Analysis of hippocampal MAP2 immunoreactivity showed a significant increase in MAP2 immunoreactivity in the CA3 region of Cerebrolysin-treated Group A and Group B Mecp2$^{308/Y}$ mice (**a**). A significant increase in MAP2 immunoreactivity was also evident in the dentate gyrus of Group A Cerebrolysin-treated mice (**b**). No significant difference was noted in CA1 region between wild-type controls and vehicle- or Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice (**c**). Neuronal density as evidenced by NeuN immunoreactivity showed a significantly higher proportion of NeuN-immunoreactive neurons in the CA3 region of Cerebrolysin-treated Group A and Group B Mecp2$^{308/Y}$ mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mice (**d**). A higher proportion of NeuN-immunoreactive neurons were also noted in the dentate gyrus of Group A Cerebrolysin-treated mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mice (**e**). No significant differences in NeuN immunoreactivity were noted in CA1 region between wild-type controls and vehicle- or Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice (**f**). *indicates a significant difference between vehicle-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher), **indicates a significant difference between Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher)

(Figs. 6d–h, 7a). The dendritic arbor of these neurons was considerably simplified and in many instances was tortuous and irregular (arrows in Fig. 6k). CBL treatment ameliorated the dendritic pathology in the hippocampus and increased the levels of MAP2 immunoreactivity in the CA3 region in both Groups A and B, (Figs. 6e, f, h, 7a). In comparison to age-matched wild-type controls, the dentate gyrus of Group A vehicle-treated Mecp2$^{308/y}$ mutant mice also showed a significant loss of MAP2 immunoreactivity (Fig. 7b). CBL administration ameliorated this loss, with levels of MAP2 immunoreactivity in the dentate gyrus of Group A CBL-treated Mecp2$^{308/y}$ mutant mice being comparable to those observed in wild-type controls (Fig. 7b). No significant differences in MAP2 levels were observed in the CA1 between wild type and vehicle- or CBL-treated Mecp2$^{308/Y}$ mutant mice (Figs. 6m–o, 7c).

Stereological analysis of pyramidal neurons with the antibody against NeuN, showed a reduction in NeuN-immunoreactive cells in hippocampal region CA3 of Group A and Group B vehicle-treated Mecp2$^{308/y}$ mutant mice in comparison to wild-type controls (Fig. 7d). CBL treatment significantly ameliorated this loss in both Groups A and Group B mice with a larger proportion of NeuN-immunoreactive neurons in CBL-treated Mecp2$^{308/y}$ mutant mice, with the number of NeuN-immunoreactive cells in these mice being comparable to that in wild-type controls (Fig. 7d). Compared to age-matched wild-type controls, Group A and Group B vehicle-treated Mecp2$^{308/y}$ mutant mice also displayed a reduced number of NeuN-immunoreactive cells in the dentate gyrus. CBL treatment ameliorated this loss in Group A Mecp2$^{308/y}$ mutant mice (Fig. 7e), with a similar trend observed in Group B CBL-treated Mecp2$^{308/y}$ mutant mice ($P = 0.0548$). No significant difference in the number of NeuN-immunoreactive cells was evident between wild type and vehicle- or CBL-treated Mecp2$^{308/Y}$ mutant mice in the CA1 (Fig. 7f).

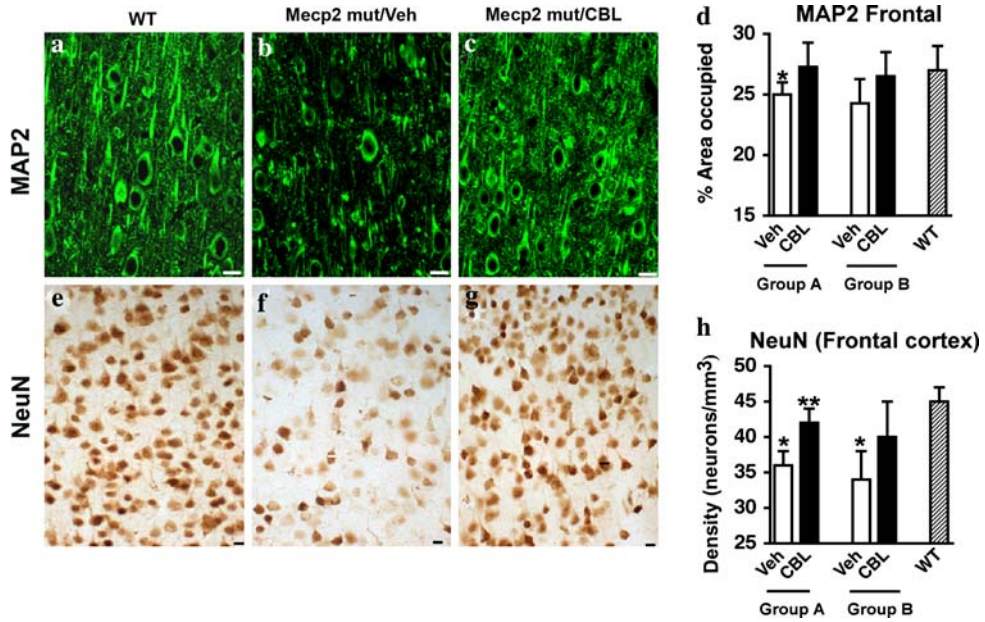

Fig. 8 Cerebrolysin ameliorates dendritic damage in neocortex of Mecp2$^{308/Y}$ mutant mice. Laser scanning confocal microscopic images of the neocortex of wild-type mice (**a**) vehicle-treated Mecp2$^{308/Y}$ mice (**b**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**c**) immunostained with an antibody against the dendritic marker MAP2. Area of the neuropil covered by MAP2 immunoreactivity was analyzed (**d**). The lower panel depicts bright light microscopic images of the neocortex of wild-type mice (**e**), vehicle-treated Mecp2$^{308/Y}$ mice (**f**) and Cerebrolysin-treated Mecp2$^{308/Y}$ mice (**g**) immunostained with an antibody against the neuronal marker, NeuN. Stereological analysis of neuronal density was also performed (**h**). Images (**b**), (**c**), (**f**) and (**g**) are from Group A Mecp2$^{308/Y}$ mice (treated from 4 till 7 months of age with either Cerebrolysin or saline), *scale bars* represent 10 μM. Decreased MAP2 immunoreactivity was observed in vehicle-treated Mecp2$^{308/Y}$ in comparison to wild-type mice (**a**, **b**). Cerebrolysin treatment resulted in a significant increase of neocortical MAP2 immunoreactivity in Group A mice (**c**, **d**). A reduction of NeuN immunoreactivity was observed in vehicle-treated Mecp2$^{308/Y}$ in comparison to wild-type mice (**e**, **f**). Cerebrolysin treatment resulted in a significantly higher proportion of NeuN-immunoreactive neurons in the neocortex of Group A Mecp2$^{308/Y}$ mice in comparison to vehicle-treated Mecp2$^{308/Y}$ mice (**g**, **h**). *indicates a significant difference between vehicle-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher), **indicates a significant difference between Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice ($n = 12$) and wild-type controls ($n = 8$) ($P < 0.05$, by one-way ANOVA and post hoc Fisher)

The neocortex of Group A and Group B vehicle-treated Mecp2$^{308/y}$ mutant mice showed a moderate loss of MAP2 immunoreactivity in comparison to wild-type controls (Fig. 8a, b). CBL treatment significantly ameliorated this loss of MAP2 immunoreactivity in Group A Mecp2$^{308/y}$ mutant mice in comparison to vehicle-treated Mecp2$^{308/y}$ mutant mice (Fig. 8c, d) and to a lesser extent in CBL-treated Group B Mecp2$^{308/y}$ mutant mice (Fig. 8d).

In comparison to wild-type control mice, NeuN immunoreactivity was significantly reduced in the neocortex of Group A and Group B vehicle-treated Mecp2$^{308/y}$ mutant mice (Fig. 8e, f). CBL treatment significantly increased the proportion of NeuN-immunoreactive neurons in Group A Mecp2$^{308/y}$ mutant mice (Fig. 8g, h) and to a lesser extent in Group B mice in comparison to vehicle-treated Mecp2$^{308/y}$ mutant mice.

Cerebrolysin effects on H3 histone acetylation

Mecp2 is proposed to function as a global transcriptional repressor by binding methylated DNA and interacting with histone de-acetylase-containing complexes that remodel the chromatin structure to repress gene transcription [15, 33, 41–43]. Previous studies with Mecp2$^{308/Y}$ mutant mice have shown that these mice display a hyperacetylation of histone H3 [56]. In an effort to pinpoint the mechanism by which CBL exerts its effect we sought to determine if CBL had any possible effect on Mecp2-regulated H3 histone acetylation. Consistent with the previous studies on these mice [42], compared to wild-type controls, levels of acetylated H3 were higher in Mecp2$^{308/Y}$ mutant mice (Fig. 9a). Interestingly, no effects of CBL administration on levels of H3 acetylation were observed (Fig. 9a). Western blot analysis of total H3 histone levels (Fig. 9b) revealed no differences between wild type and vehicle- or CBL-treated Mecp2$^{308/Y}$ mutant mice. These results indicate that the beneficial effects of CBL seen in this study are independent of the known transcription-regulating effects of *Mecp2* and are suggest that they likely to be related to the previously reported neurotrophic action of CBL [14, 21, 23, 26, 55].

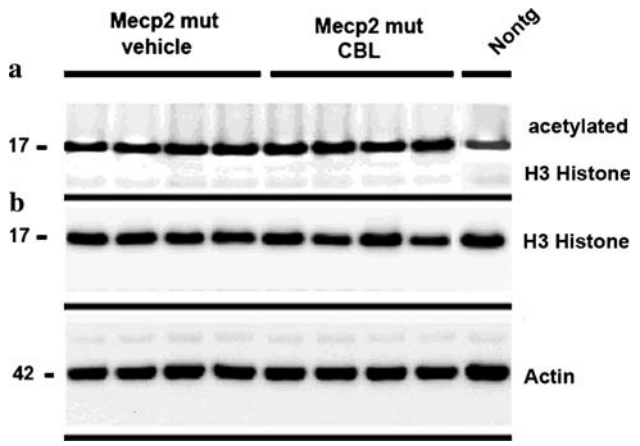

Fig. 9 Cerebrolysin does not effect H3 histone acetylation in Mecp2$^{308/Y}$ mutant mice. Western blot analysis of acetylated H3 histone (**a**) and total H3 histone (**b**) levels in vehicle- and Cerebrolysin-treated Mecp2$^{308/Y}$ mutant mice and non-transgenic mice. Actin was used as a loading control. Levels of acetylated H3 histone were higher in Mecp2$^{308/Y}$ mutant mice in comparison to non-transgenic controls. Cerebrolysin treatment had no effect on levels of H3 histone acetylation. No differences were observed in total levels of histone H3

Discussion

The present study demonstrates that CBL ameliorates the behavioral alterations in the open field and pole test of Mecp2$^{308/Y}$ mutant mice. The performance alterations in the open field and pole test are consistent with the previous studies in this model [56] and are reminiscent of the behavioral disturbances in patients with Rett syndrome [31]. Consistent with these improvements, neuropathological examination of the basal ganglia in these mice found that CBL administration ameliorated the dendritic simplification observed in the Mecp2$^{308/Y}$ mice. Moreover, we found that CBL treatment ameliorated the extensive dendritic damage and neuronal loss observed in pyramidal neurons in the CA3 region of the hippocampus and in the neocortex of vehicle-treated Mecp2$^{308/Y}$ mutant mice, such that levels of MAP2 and NeuN immunoreactivity in CBL-treated Mecp2$^{308/Y}$ mutant mice were comparable to those seen in wild-type mice. These results are consistent with the previous studies that have reported impaired learning and memory and neuropathological alterations in the hippocampus of Mecp2$^{308/Y}$ mutant mice [39] and dendritic abnormalities in the neocortex of Rett patients [29, 30] and suggest that CBL may have a beneficial effect in these regions. It is unclear why there are such differences between the different hippocampal subfields; we would speculate that it may be related to the fact that different subfields arise during different periods of development and express different macromolecules [17, 60]; any insult or gene effect occurring during neurodevelopment may only affect a particular subfield and its connectivity. In the adult, the subfields receive afferent and efferent connections from different regions; these connections may also be differentially susceptible in Rett syndrome and in turn to treatment with CBL.

The effects of CBL in ameliorating the behavioral alterations in the Mecp2$^{308/Y}$ mutant mice may be related to its ability to promote dendritic regeneration. In Rett syndrome, a decreased number of dendritic spines are observed in neocortical areas [7] and neurons in the frontal, motor and temporal cortex show shortened basal and apical dendrites [4, 6]. These brain areas are responsible for some of the significant motor and behavioural symptoms observed in Rett syndrome [5]. A temporal correlation exists between the time point of normal maturation of layers III and V, when projection and association circuits of these layers are organized [48], and the observation of clinical deficits in Rett syndrome. These manifest as motor delays and emotional instability [5] and occur at the age of 1–3 years [31]. This observation suggests a possible relationship between the failure of the dendritic arborisation and the apparent failure of cortical processing leading to functional deficits [5]. In addition to the morphological abnormalities noted in the Rett dendrites, biochemical changes such as a reduction in the expression of the dendritic marker MAP2 have also been reported in Rett patients [29]. Dendritic changes are also a consistent feature in diseases with mental retardation, such as Down syndrome [59] or fragile X syndrome [10].

The effects of CBL on dendritic arborisation have been investigated in a chronic low serum cell stress model [23]. After 4 days in cell culture, quantification of spontaneous outgrowth of embryonic chicken telencephalon neurons demonstrated an outgrowth promoting effect of CBL. In contrast to the observed neuronal degeneration after eight days in cells treated with a synthetic amino acid solution, CBL-treated cells presented a well-differentiated neuronal network that was more pronounced than those seen in control cultures treated with brain-derived neurotrophic factor (BDNF) or serum supplementation. These results suggest a neurotrophic role of CBL, which may in turn be due to the stabilizing effect of CBL on the microtubule-associated protein MAP2.

Consistent with its role in promoting dendritic network formation, CBL has been shown to increase, in a dose-dependent manner, levels of MAP2 expression in models of excitotoxicity [25, 26]. The exact mechanism by which CBL exerts its effect on MAP2 remains unclear though a recent study found that CBL protects against loss of MAP2 in translation-inhibited cell cultures treated with cycloheximide independently from de novo synthesis [65].

It is worth noting that the beneficial effects of CBL in the Mecp2$^{308/Y}$ mutant model were consistently more prominent in Group A (mature mice, 4-month-old, treated for 3 months) in comparison to mice in Group B (young

mice, 1-month-old, treated for 6 months). It is interesting to note that at 1 month of age (when treatment began for Group B mice) there did not appear to be a difference in dendritic complexity between the Mecp2$^{308/Y}$ mutant mice and age-matched wild-type control. Loss of dendritic complexity became more evident as the Mecp2$^{308/Y}$ mutant mice aged, being noticeable at 4 months and more so at 7 months, the age bracket at which CBL seem to have exerted a greater effect. This feature of the results may suggest a possible therapeutic window for the effects of CBL and could indicate that it has a trophic rather than preventative mode of action. Consistent with this hypothesis, CBL has been reported to have neurotrophic effects in vitro on cultured neurons from chicken embryos [40] and synaptotrophic effects on neuronal cells [34]. Consistent with in vitro data, in vivo demonstration of the neurotrophic effects of CBL were observed in studies examining cell death in rat spinal motoneurons following avulsion of the ventral spinal roots [21, 22]. Though the molecular mediators of the neurotrophic effect of CBL remain to be definitively determined, CBL had been reported to reduce levels of CDK5 and GSK3beta [53], and this may be related to the beneficial effects of CBL on neuronal survival and its anti-apoptotic effects [24]. This is consistent with the findings that CBL has no effect on histone acetylation, as had this been the case it would have affected both groups equally.

The neuropeptide mixture Cerebrolysin is a well-established compound used in the management of cognitive alterations in patients with dementia [1, 40, 63] and both in vitro and in vivo studies have shown that it has neurotrophic effects and promotes synaptic and neuronal plasticity [18, 24, 49–51] and cytoskeletal stability [65]. The results from this study suggest that in addition to its documented beneficial effects in neurodegenerative conditions such as Alzheimer's disease it may also have potential as a treatment of neurodevelopmental disorders such as Rett syndrome.

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