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Toxicity of overexpressed MeCP2 is independent of HDAC3 activity

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Duplication of the X-linked MECP2 gene causes a severe neurological syndrome whose molecular basis is poorly understood. To determine the contribution of known functional domains to overexpression toxicity, we engineered a mouse model that expresses wild-type or mutated MeCP2 from the Mapt (Tau) locus in addition to the endogenous protein. Animals that expressed approximately four times the wild-type level of MeCP2 failed to survive to weaning. Strikingly, a single amino acid substitution that prevents MeCP2 from binding to the TBL1X(R1) subunit of nuclear receptor corepressor 1/2 (NCOR1/2) complexes, when expressed at equivalent high levels, was phenotypically indistinguishable from wild type, suggesting that excessive corepressor recruitment underlies toxicity. In contrast, mutations affecting the DNA-binding domain were toxic when overexpressed. As the NCOR1/2 corepressors are thought to act through histone deacetylation by histone deacetylase 3 (HDAC3), we asked whether mutations in NCOR1 and NCOR2 that drastically reduced their ability to activate this enzyme would relieve the MeCP2 overexpression phenotype. Surprisingly, severity was unaffected, indicating that the catalytic activity of HDAC3 is not the mediator of toxicity. Our findings shed light on the molecular mechanisms underlying MECP2 duplication syndrome and call for a re-evaluation of the precise biological role played by corepressor recruitment.

[Keywords: histone deacetylase 3; MeCP2; NCOR]

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that interfere with either NID or MBD function failed to register deleterious phenotypes, suggesting that these two domains must be intact for overexpression to be detrimental [Heckman et al. 2014]. To test this with increased stringency, we used overexpression of the coding region of MeCP2 from the autosomal Mapt [Tau] locus, which achieves higher MeCP2 abundance and leads to a severe phenotype. Luikenhuis et al. (2004) used this method previously to show that mice lacking the endogenous Mecp2 gene could be rescued by the Tau-Mecp2 fusion protein expressed heterozygously but that high expression in homozygous animals was severely detrimental. Extending this approach, we found that ∼3.8-fold overexpression of MeCP2 is lethal in mice, but a missense mutation in the NID exogenously expressed at a similarly high level is benign. Our results emphasize that excess NID is an essential mediator of overexpression toxicity. In contrast, less extreme overexpression of hypomorphic MBD mutants that cause Rett syndrome was deleterious, arguing that the enhanced expression of these hypomorphic forms of MeCP2 in the presence of the wild-type protein may not offer therapeutic benefit [Lamonica et al. 2017]. As the NID interacts with a shared subunit of the NCoR1/2 corepressors, it seemed plausible that overrecruitment might cause excessive repression mediated by a catalytically active component of these complexes: histone deacetylase 3 (HDAC3) [Guenther et al. 2001]. To test this hypothesis genetically, we asked whether toxicity associated with overexpression of MeCP2 in mice could be ameliorated by mutation of the deacetylase activation domains (DADs) of both NCoR1 and NCoR2 [You et al. 2013]. Our results do not support the hypothesis, as the phenotypes of mice overexpressing MeCP2 were no less severe when the double-DAD mutation was introduced into these animals. Our results suggest that other aspects of corepressor function, distinct from HDAC3 activity, are deleterious when excessive. Thus, while underlining the importance of the NID as the primary underlying cause of MECP2 duplication syndrome, the findings raise questions about downstream mechanisms that mediate this effect.

**Results**

**MeCP2 is expressed from the Tau locus at wild-type levels in neurons and glia**

In order to create a model of MECP2 duplication syndrome, we chose to replace the endogenous Tau-coding region with an Mecp2 cDNA using constructs similar to those described previously [Fig. 1A; Luikenhuis et al. 2004]. Specifically, we inserted a cDNA encoding the entire e2 isoform of MeCP2 in-frame downstream from the N-terminal 31 codons of the Tau-coding region [Supplemental Fig. S1A–D]. Western blot assays of MeCP2 in Tau-Mecp2 heterozygote brains confirmed similar expression levels of Tau-Mecp2 and the slightly shorter wild-type MeCP2 [Fig. 1B,C]. Although Tau was initially thought to be a neuron-specific protein, it is also expressed at lower levels in glia [LoPresti et al. 1995]. Accordingly, MeCP2 expression derived from the Tau-Mecp2 recom-bined locus was detected by flow cytometry of both neuronal [NeuN-high] and glial [NeuN-low] nuclei in mice lacking an endogenous Mecp2 gene [Fig. 1D]. The wild-type pattern of high MeCP2 levels in neurons and lower levels in nonneuronal cells was recapitulated in mouse brains heterozygous for Tau-Mecp2 on an Mecp2+/y mouse background, although we noted slightly elevated levels compared with wild-type in both cell types. Immunostaining with an anti-MeCP2 antibody confirmed that MeCP2 signal was abundant in Tau-Mecp2 neurons [Fig. 1E]. We conclude that the expression profile of Mecp2 from a heterologous autosomal promoter and locus is remarkably similar to that seen from the endogenous locus in wild-type mice. Importantly, we confirmed a previous report [Luikenhuis et al. 2004] that the Rett-like phenotypes observed in Mecp2-null mice can be rescued by exogenous expression of MeCP2 from the Tau locus [Fig. 1F–H].

**A 2.4-fold overexpression of MeCP2 is well tolerated, but 3.8-fold overexpression is lethal**

Having established that Tau-Mecp2 is expressed at appropriate levels and can phenotypically rescue Mecp2-null mice, we asked whether overexpression gave a phenotype that mimics MECP2 duplication syndrome. Mice wild type for the endogenous Mecp2 gene, but additionally expressing one Tau-Mecp2 allele, expressed, in total, ∼2.4 times wild-type levels of the protein [Fig. 1C]. Remarkably, despite significant overexpression, these mice were viable and fertile and survived for >1 yr [Fig. 2A–C]. However, they exhibited reduced body weight, and we additionally noted a very late-onset (∼40 wk) breathing phenotype manifest as “panting.” Further analysis using the elevated plus maze, a test for anxiety, showed no difference in time spent in open versus closed arms, although mobility was somewhat reduced in this test [Supplemental Fig. S2A,B]. A 3-d learning paradigm on the accelerating rotarod revealed no significant difference compared with wild-type animals [Supplemental Fig. S2C].

In contrast, mice homozygous for the Tau-Mecp2 allele in the presence of the endogenous Mecp2 locus, which were anticipated to express 3.8-fold more MeCP2 protein than wild-type [1 + 1.4 + 1.4] were not recovered [Fig. 2D]. We expected ∼40 animals with this genotype among 159 progeny, but only two animals reached weaning, one of which had to be culled due to a severely runted phenotype and hydrocephalus. However, analysis of litters at 18.5 d post-coitum [dpc] revealed the expected Mendelian proportions of this genotype, indicating that death occurred at or soon after birth [Fig. 2D]. As this genotype results in a Tau-null mouse, we wished to confirm that the phenotype was not due to lack of Tau. In line with previous reports [Harada et al. 1994; Dawson et al. 2001], we found that homozygous Tau-null mice were phenotypically normal, as weight, survival, and phenotypic score [an aggregate score analyzing general appearance, activity, gait, tremor, breathing, and hindlimb clamping] were indistinguishable from wild-type over 1 yr [Supplemental Fig. S2D–G].
Molecular analysis confirmed increased expression of Tau-MeCP2 in the single surviving animal, as expected (Supplemental Fig. S2H). In line with the reduced weight observed in Tau-Mecp2 heterozygous animals, the Tau-Mecp2 homozygous animal had even further decreased weight (Supplemental Fig. S2I). It is notable that Tau-Mecp2 homozygous mice lacking endogenous MeCP2 expression were recovered at normal Mendelian frequencies. Thus, the combination of Tau deletion and 2.8-fold MeCP2 overexpression does not lead to synthetic lethality, ruling out an interaction between these mutations (Supplemental Fig. S2J). The results indicate that expression of MeCP2 in mice at \( \sim 2.4 \)–\( 2.8 \) times the wild-type level is unexpectedly benign, but \( \sim 3.8 \) times the wild-type level is unexpectedly lethal.
type level is severely toxic. The observed homozygous lethality in our model represented a more severe phenotype than reported previously [Luikenhuis et al. 2004], which may be due to differences in genetic background in this mouse strain or husbandry differences.

Mutating the NID rescues the lethality caused by MeCP2 overexpression

We next asked whether mutations that cause Rett syndrome alter toxicity when overexpressed in this system. Initially, the relatively frequent mutation R306C, which disrupts the interaction between MeCP2 and the NCoR1/2 corepressor complexes, was introduced at the Tau locus [Supplemental Fig. S3A,B]. Heterozygous expression of this allele in mice deficient for endogenous MeCP2 gave rise to typical features of Rett syndrome similar to those observed when the endogenous locus is mutated [Supplemental Fig. S3C–F; Lyst et al. 2013; Brown et al. 2016]. We noted that a double dose of the Tau-Mecp2[R306C] allele in homozygotes on an Mecp2-null background improved survival compared with homozygotes [Supplemental Fig. S3C]. This argues that this mutation retains weak MeCP2 function, in agreement with its somewhat less severe phenotype in mice and humans [Cuddapah et al. 2014; Brown et al. 2016]. It is noticeable that an increased dose of this hypomorphic allele improved survival but not phenotypic scoring. Expression of the Tau-Mecp2[R306C] allele in a wild-type background raised total MeCP2 to the same total abundance as seen with wild-type Tau-Mecp2, reaching ∼3.6-fold in Tau-Mecp2[R306C] homozygotes (Fig. 3A,B). Remarkably, in contrast to the neonatal lethal phenotype seen with overexpressed wild-type MeCP2, mice expressing transgenic R306C at the same high level were indistinguishable from wild-type littermates [Fig. 3C–F]. In the elevated plus maze, homozygous Tau-MeCP2[R306C] mice showed normal avoidance of the open arms but modestly reduced distance traveled during the test [Supplemental Fig. S3G,H]. The accelerating rotarod learning paradigm also revealed no significant differences compared with wild-type littermates [Supplemental Fig. S3I]. We conclude that this single amino acid substitution effectively abolishes the toxicity caused by MeCP2 overexpression.

Overexpressing MBD mutants in the presence of wild-type MeCP2 causes neurological defects

We next performed the equivalent experiment with mutations that affect DNA binding via the MBD. We first introduced the most common missense Rett syndrome mutation, T158M (which compromises DNA binding but also reduces MeCP2 stability), into the Tau locus [Supplemental Fig. S4A,B; Brown et al. 2016]. As anticipated, mice heterozygous for the Tau-Mecp2[T158M] allele that also lacked endogenous MeCP2 exhibited Rett syndrome-like phenotypes similar in severity to equivalent mutations at the endogenous MeCP2 locus. Although mice homozygous for the Tau-Mecp2[T158M] allele in an Mecp2-null background showed improved survival compared with Mecp2-null mice, phenotypic scoring was not improved [Supplemental Fig. S4C–F; Brown...
et al. 2016). Due to instability of the mutant protein, we found that overexpression from the Tau-Mecp2[T158M] locus led to only a modest increase in MeCP2 expression, reaching ~1.8 times wild type in Tau-Mecp2[T158M] homozygotes in the presence of endogenous MeCP2 (Fig. 4A,B). This low level of additional mutant protein did not severely affect survival or weight of mice, but hindlimb clasp- ing was strikingly elevated from ~10 wk of age, indicative of a significant brain pathology. Other phenotypic aspects detected by weekly scoring were not significantly altered [Fig. 4C–F, Supplemental Fig. S4G].

To determine whether MBD mutants consistently caused overexpression phenotypes, we introduced the R133C mutation into the Tau locus. R133C is another common Rett syndrome mutation that reduces HDAC3 activity and is benign even at high levels, relatively modest overexpression of wild-type protein in Tau-Mecp2 heterozygotes, which have no obvious phenotype, we found that excess R133C protein led to pronounced hindlimb clasp- ing beyond ~6 wk of age. These animals also displayed sub- tly reduced body weight, although no significant effect on survival was detected [Fig. 4J–L, Supplemental Fig. S5G]. Thus, when expressed in addition to wild-type MeCP2, the toxicity observed previously for T158M commenced earlier with the R133C mutation. We conclude that, in contrast to overexpression of the R306C mutant protein, which is benign even at high levels, relatively modest overexpression of MeCP2 with missense MBD mutations in a brain that additionally has normal levels of wild-type MeCP2 is markedly deleterious.

The toxicity of MeCP2 overexpression is independent of HDAC3 activity

Our finding that inactivation of the NID via an R306C mutation abolishes the toxicity caused by MeCP2 overexpression focuses attention on the function of this domain. Previous evidence supports the hypothesis that its primary role is to bind the TBL1X(R1) subunit and thereby recruit NCoR corepressors [Lyst et al. 2013; Kruusvee et al. 2017]. A key shared subunit of NCoR1/2 complexes is HDAC3 (You et al. 2013). HDAC3 is considered to be the component of NCoR1/2 complexes that is responsible for inhibiting transcription via deacetylation of
Figure 4. Overexpression of MBD mutants in addition to wild-type MeCP2 is deleterious. [A] Western blots of three brains of animals heterozygous and homozygous for Tau-Mecp2[T158M] and wild-type littermates (males, 7 wk) probed for MeCP2 and γ-tubulin. [B] Quantification of blots shown in A (mean and standard deviation). Genotypes are +/-[black], +/-Tau-Mecp2[T158M][light green], and Tau-Mecp2[T158M]/Tau-Mecp2[T158M][dark green]. A two-tailed unpaired t-test was used for statistical analysis. P = 0.0748 for wild type versus heterozygous; P = 0.0053 for wild type versus homozygous. [C] Genotypes derived from Tau-Mecp2[T158M] heterozygous intercrossings observed at weaning. A χ² test was used to test for normal Mendelian ratios of genotypes. P = 0.1554. [D] Survival of a cohort comprising +/-[black; n = 9], +/-Tau-Mecp2[T158M][bright green; n = 10], and Tau-Mecp2[T158M]/Tau-Mecp2[T158M][dark green; n = 10]. A Mantel-Cox test was used to analyze survival. P = 0.5767 for wild type versus heterozygous; P = 0.2918 for wild type versus homozygous. [E] Body weights of the mice shown in D (mean and standard deviation). Two-way repeated measures of ANOVA were performed (weeks 3 and 40 were excluded, as not all animals were analyzed; one wild-type and two heterozygous animals that died were also excluded). n = 8 wild type; n = 8 heterozygous; n = 10 homozygous. Genotype effect: F[2,23] = 0.3047. P = 0.7403. [F] Clasping score of the mice shown in D. Two-way repeated measures of ANOVA were performed (weeks 3 and 40 were excluded, as not all animals were analyzed; one wild-type and two heterozygous animals that died were also excluded). n = 8 wild type; n = 8 heterozygous; n = 10 homozygous. Genotype effect: F[2,23] = 4.964. P = 0.0161. Overall scores are in Supplemental Figure S4G. [G] Western blots of three brains of mice heterozygous and homozygous for Tau-Mecp2[R133C] and wild-type littermates (males, 7 wk) probed for MeCP2 and γ-tubulin. [H] Quantification of blots shown in G (mean and standard deviation). The genotypes used were +/-[black], +/-Tau-Mecp2[R133C][cyan], and Tau-Mecp2[R133C]/Tau-Mecp2[R133C][dark blue]. A two-tailed unpaired t-test was used for statistical analysis. P = 0.0028 for wild type versus heterozygous; P = 0.0002 for wild type versus homozygous. [I] Clasping score of the mice shown in G. Two-way repeated measures of ANOVA were performed (week 3 was excluded as not all animals were analyzed; one wild-type and three heterozygous animals that died were also excluded). n = 11 wild type; n = 9 heterozygous; n = 9 homozygous. Genotype effect: F[2,20] = 5.937. P = 0.0075. [J] Body weights of the mice shown in I (mean and standard deviation). Two-way repeated measures of ANOVA were performed (week 3 was excluded as not all animals were analyzed; one heterozygous and three homozygous animals that died were also excluded). n = 11 wild type; n = 9 heterozygous; n = 9 homozygous. Genotype effect: F[2,20] = 16.6. P < 0.0001.
histones. To determine genetically whether the activity of HDAC3 mediates the toxic effects of MeCP2 overexpression, we used mutants in NCoR1 and NCoR2 proteins that reduce HDAC3 catalytic activity (Fig. 5A; You et al. 2013). HDAC3 is catalytically inactive in isolation but becomes active upon interaction with the DADs of NCoR1 and NCoR2 proteins (Guenther et al. 2001; You et al. 2013). The DAD mutations in NCoR1/2 greatly reduce HDAC3 activation, but the reported phenotype of homozygous double mutants (NS-DAD mutants) is mild without overt neurological abnormalities (You et al. 2013). If HDAC3 activity within the NCoR1/2 complexes is the primary downstream effector of MeCP2, these mice should at a minimum exhibit Rett syndrome-like features but in fact are viable with normal survival. As HDAC3-null mutations are embryonically lethal, we considered the possibility that DAD mutations retain some HDAC3 activity. We recovered NCoR1/2 complexes from brain extracts using affinity for a NID peptide and found that HDAC3 was retrieved equally from wild-type and NS-DAD mutant extracts, indicating no detectable effect of the mutations on the binding of HDAC3 to NCoR1 or NCoR2 complexes (Fig. 5B). However, the amount of recovered HDAC activity was drastically reduced in NS-DAD mutant brains (Fig. 5C). We conclude that the DAD mutations significantly reduce HDAC activity associated with NCoR complexes by >70%.

The finding that the DAD mutations show severely reduced HDAC activity made it possible to test whether the toxicity caused by MeCP2 overexpression is due to excessive HDAC3 activity. According to this hypothesis, DAD mutations would be expected to significantly reduce the severity of this phenotype. The experiment requires MeCP2 overexpression in mice that are homozygous for knock-in DAD mutations in both NCoR1 and NCoR2. Despite the reduced ability of the DAD mutant corepressors to activate HDAC3, we observed no discernible improvement in the early lethal phenotype resulting from MeCP2 overexpression (Fig. 5D; Supplemental Fig. S6A). Therefore, in contrast to overexpression of the R306C mutant, which resulted in effectively wild-type mice, the inability of NCoR1/2 to fully activate HDAC3 did not ameliorate the overexpression phenotype. Our results suggest that while toxicity very likely requires excessive TBL1XR1 and hence NCoR complexes by >70%

Discussion

A goal of the present work was to determine which domains of the MeCP2 protein underlie the severe phenotype caused by its overexpression. To achieve this, we drove transcription of an MeCP2 cDNA from the endogenous Tau locus in mice that also expressed the X-linked wild-type gene. Levels of Tau-MeCP2 closely mimicked those of endogenous MeCP2 in both neurons and glia. In agreement with a previous report (Luikenhuis et al. 2004), mice with only Tau-MeCP2 as a source of MeCP2 protein were viable, fertile, and overtly normal in

Figure 5. Reducing HDAC3 activity does not rescue lethality of Tau-Mecp2 overexpression. [A] Schematic overview of the experiment. In wild-type brains, MeCP2 [red cylinder] recruits the core NCoR1/2 complexes (green circles) by binding to TBL1X [R1] [bright-green circle]. If the NID of MeCP2 is mutated (R306C), MeCP2 cannot bind and recruit the NCoR complexes. In NS-DAD mutants, the mutated DADs of both NCoR1 and NCoR2 fail to fully activate HDAC3, allowing removal of histone acetyl groups. [B] An MeCP2 peptide containing either the wild-type or a mutated [R306C] NID was used to pull down TBL1XR1 and HDAC3 from brain nuclear extracts from wild-type or NS-DAD animals. Wild-type MeCP2 pulls down NCoR1/2 components from both wild-type and NS-DAD brain extracts with similar efficiency, whereas the R306C peptide is unable to pull down the corepressor subunits from either extract. [C] Proteins isolated in B were assayed for HDAC activity. Shown are mean and standard deviation of three biological replicates as well as background luminescence readings. HDAC activity of proteins pulled down using an MeCP2 wild-type peptide from NS-DAD brains is reduced to ~27% of wild-type brains. The R306C peptide recovered ~5% HDAC activity irrespective of brain genotype. [D] The number of animals surviving to weaning when wild-type, heterozygous or homozygous for Tau-Mecp2, and either heterozygous or homozygous for N-DAD and S-DAD mutations. The failure of animals homozygous for Tau-Mecp2 to survive was not rescued by reducing HDAC3 activity via DAD mutations either singly or on both NCoR1 and NCoR2. For survival of additional genotypes, see Supplemental Figure S6A. Note that as Ncor and Tau genes are located on the same chromosome (42 Mb apart), +/Tau-Mecp2 is observed at a reduced frequency if N-DAD is homozygous [43 cM]. A χ² test was used to test for normal Mendelian ratio of genotypes. P < 0.0001 for homozygous–heterozygous, P = 0.0001 for heterozygous–homozygous, P = 0.0025 for homozygous–homozygous, indicating a non-Mendelian distribution.
phenotype despite lacking the native promoter and the unusually long (~8.5-kb) 3′ untranslated region that is an evolutionarily conserved feature of the endogenous transcript (Singh et al. 2008). When this autosomal transgene was expressed heterozygously in mice with an endogenous MeCP2 gene, animals were also phenotypically relatively normal despite a 2.4-fold increase in brain MeCP2 [Supplemental Fig. S6B]. The results raise the possibility that mice are more tolerant than humans to MeCP2 overexpression. It was reported previously that expression of the human MECP2 locus in mice with normal levels of the endogenous protein also leads to a relatively mild phenotype, although survival and motor function are compromised to a somewhat greater extent than seen here (Collins et al. 2004). The difference may be due to the altered genetic background or brain region-specific expression in those experiments.

A recent study provided evidence that twofold overexpression of MeCP2 carrying either the R306C mutation, which abolishes NID function, or the R111G mutation, which abolishes binding to methylated DNA, is phenotypically neutral (Heckman et al. 2014). The severe phenotype caused by higher expression of Tau-MeCP2 provides a more stringent test of the toxicity of mutant MeCP2 overexpression. Using this approach, we establish here that ~3.6-fold overexpression of MeCP2[R306C] leads to a wild-type phenotype, in stark contrast to the lethal phenotype caused by equivalent levels of wild-type MeCP2 (Supplemental Fig. S6B). It follows that toxicity is intimately dependent on excess of this domain, whose only well-characterized function is to bind the WD40 domain of TBL1X and its close relative, TBL1XR1. Indeed, a recent crystal structure of the NID bound to the TBL1XR1 subunit, which is shared by NCoR1/2 complexes, showed that four adjacent amino acids that are individually mutated in Rett syndrome [one of which is R306] all make intimate molecular contacts with the WD40 domain of TBL1X and its close relative, TBL1XR1. Indeed, a recent crystal structure of the NID bound to the TBL1XR1 subunit, which is shared by NCoR1/2 complexes, showed that four adjacent amino acids that are individually mutated in Rett syndrome [one of which is R306] all make intimate molecular contacts with the WD40 domain of TBL1X and its close relative, TBL1XR1. These data suggest that the MeCP2–TBL1X[R1] interaction, which is critical for avoidance of Rett syndrome, is also of pivotal importance as a cause of MECP2 duplication syndrome. It seems that Rett syndrome is caused by too little recruitment of TBL1X[R1] to DNA, whereas in MECP2 duplication syndrome, TBL1X[R1] recruitment is excessive.

TBL1X[R1] proteins are best known as components of the NCoR1/2 corepressor complexes, where they form a tetramer that associates with the NCoR scaffolds (Oberoi et al. 2011). As MeCP2 pulls down major subunits of NCoR1/2 complexes by immunoprecipitation [Lyst et al. 2013], we infer that the primary consequence of interaction with TBL1X[R1] is recruitment of these corepressors. While it is conceivable that TBL1X[R1] is involved in additional functional complexes that are relevant to Rett syndrome, none has so far been characterized. We therefore sought to establish what features of NCoR1/2 might account for toxicity. The primary mediator of transcriptional inhibition by NCoR corepressors is thought to be histone deacetylation via HDAC3, which is inactive unless intimately associated with these complexes [Guenther et al. 2001; You et al. 2013]. In view of evidence that occupancy of gene bodies by MeCP2 in vivo correlates positively with relative down-regulation of gene expression (Kinde et al. 2016; Lagger et al. 2017), an attractive possibility was that this effect is mediated through histone deacetylation by HDAC3. Based on our evidence that hyperrecruitment of TBL1X[R1] underlies the MeCP2 overexpression phenotype, it follows that reducing HDAC3 activity associated with NCoR1/2 complexes might ameliorate this phenotype. Strikingly, though, we saw no reduction in the severe consequences of Tau-MeCP2 overexpression in NS-DAD mice. Although absence of a genetic rescue must be interpreted with caution, the fact that DAD mutations in both NCoR1 and NCoR2 (You et al. 2013) reduced MeCP2-associated HDAC activity by ~72% [Fig. 5C] strongly suggests that the MeCP2 overexpression phenotype is independent of HDAC3 activity. It is likely that even a small reduction in severity would have been detected in this experiment, as MeCP2-overexpressing mice are born but fail to survive until weaning. For example, our Tau-MeCP2 homozygous mice on a wild-type background [total of 3.8-fold overexpression] fail to survive until weaning, whereas Tau-Mecp2 homozygous mice on a Mecp2-null background [2.8-fold overexpression] were recovered at Mendelian ratios [Fig. 2D; Supplemental Fig. S2J]. The finding that this ~26% reduction in MeCP2 dosage is sufficient for a striking improvement in phenotypic outcome suggests that the phenotype is sensitive enough to detect subtle decreases in the level of MeCP2 functionality. Taken together, the results lead us to conclude that HDAC3 activity does not play a major part in MeCP2 toxicity. It follows that inhibitors of HDAC3 are unlikely to be therapeutically useful for treatment of MECP2 duplication syndrome. An alternative strategy might be to inhibit the interaction of MeCP2 with NCoR1/2 complexes themselves using small molecules.

Assuming that the interaction between MeCP2 and TBL1X[R1] predominately recruits NCoR1/2, we may ask what other features of these corepressors might be responsible for the adverse phenotype. Interestingly, the HDAC3 protein itself has been reported to affect transcription in a deacetylation-independent manner [Sun et al. 2013], which probably would not be affected by the DAD mutations. Also, it is possible that other enzymes, notably class IIA HDACs, associate with NCoR either directly or indirectly and cause deacetylation [Fischle et al. 2001; Fischle et al. 2002; Hudson et al. 2015]. A further possibility is that the large NCoR1/2 complexes have other functions that mediate the deleterious consequences of overrecruitment. These could include recruiting activities such as the ubiquitin protease USP44 (Lan et al. 2016) or the ubiquitin-conjugating enzyme UbcH5 (Perissi et al. 2004). Distinguishing these possibilities is a priority for future work.

Unlike MeCP2[R306C] overexpression, which was phenotypically neutral, mild overexpression of Rett syndrome-causing MBD mutants on top of wild-type MeCP2 was detrimental. Both T158M and R133C mutations are defective in DNA binding but also somewhat unstable. To our knowledge, all MBD mutants of MeCP2 reported
so far are unstable to varying degrees. Because of this, overexpression did not yield the high levels seen with wild-type or R306C mutant proteins. Despite very mild overexpression, we observed a strong hindlimb-clasping phenotype in each case, indicative of pathologies affecting the brain and/or spinal cord [Lalonde and Strazielle 2011]. The toxic effect was stronger in the case of R133C, which retains more DNA-binding affinity, is more stable than T158M, and gave ~2.6 times wild-type levels of total MeCP2 in brain. In contrast, animals expressing equivalent levels of wild-type protein (2.4 times wild type) were phenotypically normal apart from reduced weight. These findings raise the possibility that overexpression of a protein that cannot effectively bind DNA causes dominant-negative effects. In this connection, it may be relevant that both of these proteins contain an intact NID, which may still be available to interact with NCoR1/2.

A previous study using a different MBΔ mutation, R111G, expressed on top of wild-type MeCP2 led to a different conclusion, as no adverse phenotype was observed [Heckman et al. 2014]. The disparity is unexplained but may be due to differences in genetic background, the alternative mutants that were analyzed, or the nonoverlapping assay paradigms used in the two cases. Whether the toxicity of MBΔ mutated MeCP2 requires HDAC3 activity has yet to be formally tested, although we consider this unlikely given that wild-type MeCP2 appears not to function via this pathway.

A separate study suggested that overexpression of T158M in an MeCP2-null background ameliorated phenotypic severity and may therefore offer a potential therapeutic strategy for Rett syndrome [Lamonica et al. 2017]. Due to the instability of the mutant protein, the risk of toxicity caused by MeCP2 overexpression would arguably be reduced. Our results cast doubt on the validity of this approach, as mice expressing a combination of wild-type and mutant protein were adversely affected compared with mice expressing the same level of only wild-type MeCP2. As the Rett syndrome brain is mosaic for functionally wild-type and mutant cells, there is a risk that delivery of excess T158M or R133C mutant proteins would cause toxic side effects. Another concern raised by these results is that future gene therapy in which wild-type MeCP2 would be introduced into neurons expressing an MBΔ mutant form (e.g., T158M) would mimic the protein combination that gives a hindlimb-clasping phenotype in our study. Further work with model systems is needed to determine whether gene therapy for individuals with MBΔ mutations might carry a risk and whether this would be outweighed by clinical benefit.

Materials and methods

Generation of knockout embryonic stem cells and mice

The targeting vector containing a homology region around Tau exon 2 and an in-frame insertion of MeCP2 cDNA (c2 isoform) after the first 31 amino acids of the Tau-coding sequence [a gift from Dr Rudolf Jaenisch] [Luikenhuis et al. 2004] was modified in the following way: To introduce the 3’ loxP site, a neomycin resistance cassette driven by a hybrid PKG-EM7 promoter with an upstream loxP site from PL452 [Liu et al. 2003] replaced the previous neomycin resistance cassette by recombining. The 5’ loxP site was inserted by standard cloning of a loxP-containing oligo into the Bpfl site upstream of Tau exon 2. The 5’ homology arm was extended by integrating a KpnI fragment (mm9: chromosome 11: 104,142,690–104,143,022) obtained by PCR from E14 Tg2a embryonic stem cell DNA. To integrate point mutations within Mecp2 [T158M, R133C, and R306C], the QuikChange II XL site-directed mutagenesis kit [Strategene, 200521] was used [primers: R306C-S [TCCCCCATCAAGAGTGCAAGCCTTAGG GA], R306C-AS [CTCCCTGGTCTCAGTCCTTGTGAG GA], R133C-S [CCAGGGAAAAAGCTTTTGGCTTGA TA GAATTC], R133C-AS [CAATTCATCTTGAAGCAAAAACGC TTTCCTCTGCG], T158M-S [GGCGCAATGAATTGTACCT CATGGTAATGCGGAGAAG], and T158M-AS [CTCTCCCATGAAGCTTAAATTATGCCTGTC]]. The targeting vector was linearized using Saci before electroporation. Electroporation of the targeting vectors and neomycin selection were performed using standard conditions in E14 Tg2a [129P2/OlaHsd] embryonic stem cells. Embryonic stem cells were grown in Glassm MEM [Gibco] supplemented with 10% FBS [batch tested] (Gibco), 1% nonessential amino acids (Gibco), 1% sodium pyruvate (Gibco), 0.1% β-mercaptoethanol (Gibco), and 1000 U/mL LIF (ESGRO). Positive embryonic stem cell clones were identified by Southern blotting and PCR fragment sequencing confirmation. They were injected into C57BL/6/Jola blastocysts and transferred into pseudo-pregnant recipient mice. Transgenic offspring were backcrossed onto C57BL/6/Jola or crossed with MeCP2-null animals on a C57BL/6/Jola x CBA F1 background for further experiments. Tau knockout mice were generated by mating Tau-Mecp2 mice with CMV-CRE mice [JAX, 006054] to delete the sequence in between the two loxP sites.

All mice used in this study were bred and maintained at the University of Edinburgh animal facility under standard conditions, and procedures were carried out by staff licensed by the UK Home Office and in accordance with the Animal and Scientific Procedures Act 1986. Transgenic mice were caged with their wild-type littermates.

Biochemical analysis

Brains (usually at 6–8 wk of age) were harvested by snap-freezing in liquid nitrogen. DNA isolation was performed by overnight incubation in 1× TEN (50 mM Tris at pH 9.0, 20 mM EDTA at pH 8.0, 40 mM NaCl, 1% SDS, 0.5 mg/mL proteinase K) at 55°C followed by high-salt extraction and precipitation using isopropanol. For Southern blots, 10- to 20-µg aliquots of genomic DNA were digested overnight with 2 U/µg suitable restriction enzyme and separated by gel electrophoresis on 0.8% agarose gels and 1× TBE at 5.3 V/cm. The gel was denatured twice for 30 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and blotted for at least 18 h onto Hybond-XL nylon membranes (GE Healthcare) in denaturing solution. After neutralization of the membrane in 20 mM Na2HPO4, the membrane was prehybridized for 18 h in Church wash (1% SDS, 20 mM Na2HPO4, 7% SDS, 1 mM EDTA) at pH 8.0. After hybridization with radioactive probes in Church buffer for 18 h at 65°C, the membranes were washed twice in 0.1× SSC, 0.1% SDS, and 1× SSC. An autoradiograph was taken after 18 h of exposure to phosphorimaging screens.

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Brain nuclear extracts were prepared as described previously [Lyst et al. 2013]. HDAC3-containing complexes were isolated
using a biotin-tagged MeCP2-derived peptide (residues 285–313) immobilized on streptavidin sepharose (GE Healthcare) as described previously [Lyst et al. 2013]. Bound proteins were analyzed by SDS-PAGE followed by Western blotting or by using the HDAC-Glo(TM) I/II assay and screening system (Promega) according to the manufacturer’s instructions.

Flow cytometry analysis was performed as described previously [Tillotson et al. 2017] using a NeuN antibody conjugated to AF488 (Millipore, MAB377X) and an MeCP2 antibody (Sigma, M6818) conjugated to AF647 (APEX antibody labeling kit, Invitrogen, A10475). For the immunofluorescent stainings, mouse brains were hemisected and rapidly frozen by immersion in isopentane chilled on dry ice. A Leica CM1900 cryostat was used to cut 10-µm parasagittal sections, which were mounted on SuperFrost Plus slides (VWR) and stored at −80°C prior to staining. Slides for staining were allowed to dry for 10 min at room temperature, fixed in 1:1 methanol:acetone mixture at −20°C for 20 min, and then dried for 5 min at room temperature. After two 10-min washes in PBS, sections were blocked for 1 h in 1.5% normal goat serum (Sigma) in PBS (blocking buffer) at room temperature in a humidified chamber. The following primary antibodies were incubated overnight at 4°C: anti-MeCP2 rabbit monoclonal D4F3 (Cell Signalling Technology) and anti-neuN Cy3-labeled mouse monoclonal A60 (EMD Millipore Corp.), both diluted 1:200 in blocking buffer. Sections were washed as described previously and then incubated with a biotinylated anti-rabbit secondary antibody (Vector Laboratories) diluted 1:200 in blocking buffer for 1.5 h at room temperature. After washing again, sections were incubated with avidin-FITC (Vector Laboratories) diluted 1:200 in 10 mM HEPES and 150 mM NaCl for 20 min at room temperature. Sections were then washed three times for 10 min in PBS, with the first wash including 1 µg/mL DAPI. They were then mounted in ProLong Gold (Life Technologies), left to cure in the dark overnight at room temperature, fixed in a 1:1 methanol:acetone mixture at −20°C, and then dried for 5 min at room temperature. After washing again, sections were incubated with avidin-FITC (Vector Laboratories) diluted 1:200 in 10 mM HEPES and 150 mM NaCl for 20 min, and then dried for 5 min at room temperature. Sections were then washed three times for 10 min in PBS, with the first wash including 1 µg/mL DAPI. They were then mounted in ProLong Gold (Life Technologies), left to cure in the dark overnight at room temperature, and then stored at 4°C. Stained sections were imaged on a Leica SP5 confocal microscope using a 63× objective, keeping all settings the same between samples (apart from R133C and T158M). Images were acquired using the HDAC-Glo(TM) I/II assay and screening system (Promega) according to the manufacturer’s instructions.

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References

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