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Crh and *Oprm1* mediate anxiety-related behavior and social approach in a mouse model of *MECP2* duplication syndrome

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Abstract

Genomic duplications spanning Xq28 are associated with a spectrum of phenotypes including anxiety and autism. The minimal region shared among affected individuals includes *MECP2* and *IRAK1*, however, it is unclear which gene, when overexpressed, causes anxiety and social behavior deficits. We report that doubling MeCP2 levels causes heightened anxiety and autism-like features in mice, and alters the expression of genes that influence anxiety and social behavior, such as *Crh* and *Oprm1*. To test the hypothesis that alterations in these two genes contribute to the heightened anxiety and social behavior deficits, we analyzed *MECP2* duplication mice (*MECP2*-TG1) with reduced *Crh* and *Oprm1* levels. In *MECP2*-TG1 animals, reducing *Crh*, or its receptor, *Crhr1*, suppresses anxiety-like behavior; in contrast, reducing *Oprm1* improves abnormal social behavior. These data demonstrate that increased MeCP2 levels impact molecular pathways

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Conflict of interest

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underlying anxiety and social behavior, and provide novel insight into potential therapies for *MECP2*-related disorders.

The discovery that loss-of-function mutations in *MECP2*, the gene encoding Methyl-CpG-Binding Protein 2, cause the neurological disorder Rett syndrome (MIM 312750) led to the identification of other neuropsychiatric phenotypes caused by *MECP2* mutations[1–6]. The most recently identified *MECP2*-related disorder is a genomic disorder that results from large non-recurrent duplications of chromosome Xq28[7–12] (MIM 300260). The shared region of overlap among affected individuals spans *Interleukin-1 Receptor-Associated Kinase 1* and *MECP2*[12–14], suggesting that the overexpression of either one or both of these genes contributes to the features of the disorder. Autism is common in boys with duplications spanning *MECP2*[12], and anxiety is a co-morbid condition[12]. Individuals with triplications spanning *MECP2* typically manifest a more severe phenotype[7,11].

We previously demonstrated that mice overexpressing MeCP2 at twice the normal levels (*MECP2*-TG1) on an FVB/N background have motor defects, stereotypies and seizures[15]. However, whether *MECP2*-TG1 animals display heightened anxiety remained unanswered because mice on a pure FVB/N background develop premature retinal degeneration, a potential confounder for the interpretation of anxiety-like behavior[16]. In addition, it is unknown whether social behavior abnormalities are present in *MECP2*-TG1 mice. We therefore tested the hypothesis that the overexpression of *MECP2* alone is sufficient to cause heightened anxiety and abnormal social behavior in mice.

Using F1 hybrid animals to overcome issues associated with pure inbred strains[17,18], we found that F1 hybrid *MECP2*-TG1 animals expressing two-fold the normal levels of MeCP2 and F1 hybrid *MECP2*-TG3 animals expressing in excess of three-fold the normal levels of MeCP2 displayed anxiety-like behavior in the elevated plus maze and light dark box (Fig. 1a–d). The new data here unequivocally demonstrate that doubling MeCP2 levels indeed causes heightened anxiety-like behavior given the robust phenotype on two different F1 hybrid backgrounds. Thus, the absence of detectable anxiety-like behavior in previously studied *MECP2*-TG1 animals of a pure FVB/N background in the light-dark box task was likely due to impaired vision in that background[15]. To investigate social behavior abnormalities, we tested F1 hybrid *MECP2*-TG1 and -TG3 mice in the partition test for social interest and recognition[19–21]. We discovered that *MECP2*-TG1 and -TG3 animals displayed less interest in familiar and novel partner animals (Fig. 2a, b). Although it is possible that the social interaction deficit in *MECP2*-TG3 animals may be confounded by decreased activity, as evident in an open field (Fig. 1e, f), the findings in the *MECP2*-TG1 animals, which do not show any motor deficits at the ages tested, are most relevant to the genomic disorder. We therefore chose to further understand the social behavior abnormalities of *MECP2*-TG1 mice by subjecting these animals to the three chamber test for sociability[22,23]. *MECP2*-TG1 animals displayed a deficit in social approach behavior towards novel partner mice, without a deficit in interest towards a novel object (Fig. 2c, d), or a deficit in activity or preference for either chamber (Fig. 2e–h). It is noteworthy that *MECP2*-TG3 mice had a more severe phenotype in some tests (indicated with plus signs,

Fig. 1, 2), reminiscent of the human data in which more severe clinical phenotypes are observed in individuals with triplications spanning *MECP2*[8,13,14].

Because either the loss or gain of MeCP2 is known to impact gene expression levels[24–26], we next sought to identify gene expression alterations that might contribute to the anxiety and social behavior phenotypes of *MECP2*-TG mice. We performed microarray experiments using RNA from the amygdala, an anatomical region important for anxiety and social behavior[27], of *MECP2*-TG animals, animals that lack MeCP2[28] (*Mecp2*$^{null/y}$), and the respective wild-type littermates. We focused on gene expression changes altered in opposite directions in the *MECP2*-TG animals compared with the *Mecp2*$^{null/y}$ animals, which are likely sensitive to MeCP2 dosage[24,25]. A total of 1,060 genes were altered in opposite directions in both MeCP2 mouse models compared with wild-type littermates (FDR-corrected *p* value (*q*) value < 0.05). Of these, 625 (~60%) genes were up-regulated and 435 (~40%) genes were down-regulated in the presence of excess MeCP2 (Fig. 3a, Supplementary Tables 1, 2). The gene ontology terms associated with these altered genes are listed in Supplementary Table 3. We then compared these 1,060 genes with phenotypic terms relevant to anxiety and social behavior in the Mouse Genome Informatics database and found a significant enrichment of genes whose mutations in mice caused anxiety-related behaviors and/or altered social behaviors (n=32, odds-ratio 1.88, p=0.0016). We selected these 32 genes and an additional 85 genes that have not been associated with anxiety- and/or social behavior-related defects for quantitative real-time reverse transcription-PCR (qRT-PCR) validation studies, and confirmed that 21 of the 32 anxiety- and/or social behavior-related genes (66% validation rate, *p* value < 0.05), and 58 of the 85 genes not implicated in anxiety and social behavior deficits (68% validation rate, *p* value < 0.05) are significantly altered in the *MECP2*-TG animals (Fig. 3b, c; Supplementary Tables 4, 5).

The identification of several anxiety and/or social behavior genes that are sensitive to MeCP2 levels suggests that altered dosage of some of these genes could modulate the behavioral phenotypes of *MECP2*-TG animals. Studies suggest that a 50% increase or decrease in gene dosage, as observed in humans with copy number variations, is sufficient to cause disease phenotypes[29]. Nine of the 21 anxiety- and/or social behavior-related genes that were significantly altered based on qRT-PCR analysis had at least a 50% fold change in the *MECP2*-TG animals (genes outlined in boxes in Supplementary Table 5). We therefore identified these genes as promising candidates to mediate either the heightened anxiety and/or social behavior deficits in this disease model.

To test the hypothesis that an expression change of 50% is sufficient to contribute to the anxiety and social behavior phenotypes of *MECP2*-TG mice, we focused on *Corticotropin-releasing hormone* (*Crh*), which encodes for the neuropeptide CRH, given evidence of increased anxiety-like behavior in mice that overexpress *Crh*[30,31], and on the *Opioid receptor, mu* (*Oprm1*), which encodes the G-protein coupled mu-opioid receptor MOR, one subtype of opioid receptor that has been shown to play a role in aspects of emotional and social behavior[32–34]. Similar to previous studies[35–38], we demonstrated that MeCP2 was bound to the promoters of these genes (Supplementary Fig. 1). Because *Crh* and *Oprm1* are up-regulated in the amygdala in *MECP2*-TG animals, we bred female *MECP2*-TG1 animals to either male *Crh*$^{+/-}$ animals[39] or male *Oprm1*$^{+/-}$ animals[40] to genetically reduce the levels

of these genes in *MECP2*-TG1 mice. We confirmed that *Crh* and *Oprm1* expression levels were indeed reduced in *MECP2*-TG1; *Crh*$^{+/-}$ and *MECP2*-TG1; *Oprm1*$^{+/-}$ double mutant animals, respectively (Fig. 3d–h), then tested the behavioral consequences of these genetic reductions.

We found that anxiety-like behavior was subdued in *MECP2*-TG1 mice lacking one copy of *Crh* (Fig. 4a, b). In contrast, *MECP2*-TG1 mice lacking one copy of *Oprm1* did not show any significant differences in anxiety-like behavior (Supplementary Fig. 2a, b). Both double mutant animals compared with their respective littermates showed normal exploratory activity in an open field (Fig. 4c, Supplementary Fig. 2c). Furthermore, we found that a 50% reduction in *Crh* decreased the stress-induced serum corticosterone levels in *MECP2*-TG1 animals (Fig. 4d). Because *Avp* levels can modulate anxiety[41], we tested *Avp* expression and found that *Avp* levels were not significantly altered in the amygdalae of either *Crh*$^{+/-}$, *MECP2*-TG1 or double mutant animals (Fig. 4e, f). Thus, these data suggest that the reduction in anxiety and stress-induced corticosterone levels is specific to the genetic reduction of *Crh* levels in the *MECP2*-TG1 animals. To ensure that the suppression of anxiety-like behavior was a direct consequence of modulating *Crh* levels, we explored this pathway further. CRH mediates its effects on anxiety-related behavior primarily through its predominant receptor in the brain, CRH receptor subtype 1, encoded by the *Crhr1* gene[42]. Based on the anxiolytic effects of CRHR1 antagonists[43,44], we reasoned that genetic reduction of *Crhr1* or pharmacologic blockade with the CRHR1 antagonist, antalarmin, should also improve the anxiety-like behavior in *MECP2*-TG1 animals if the anxiety phenotype is mediated by the increase in *Crh* levels. We bred female *MECP2*-TG1 mice to male *Crhr1*$^{+/-}$ mice[45] and found that the *MECP2*-TG1; *Crhr1*$^{+/-}$double mutants were less anxious (Fig. 5a, b), and displayed normal exploratory activity in an open field (Fig. 5c). In addition, pharmacologic intervention with antalarmin, a CRH receptor antagonist, demonstrated that an acute dose of 60 mg/kg antalarmin prior to social defeat stress[43] significantly reduced anxiety of wild-type animals in the elevated plus maze (Fig. 5d), and improved the anxiety-like behavior of *MECP2*-TG1 animals in the elevated plus maze and light-dark box (Fig. 5e, f).

Next, we evaluated social behavior in the double mutants and their littermates. Neither the genetic reduction of *Crh*, nor of *Crhr1*, affected the social deficit of *MECP2*-TG1 animals in the partition test (Supplementary Fig. 3a, b). However, *MECP2*-TG1; *Oprm1*$^{+/-}$ double mutant animals compared with *MECP2*-TG1 littermates spent significantly more time investigating familiar and novel partners (Fig. 6a). Furthermore, in the three chamber test, we discovered that *MECP2*-TG1; *Oprm1*$^{+/-}$ double mutant mice compared with *MECP2*-TG1 littermates spent more time investigating novel mice (Fig. 6b). Exploratory activity in the test apparatus, investigation of a novel object, and olfaction ability was comparable among the different test groups (Fig. 6c–e). Together, these data demonstrate that reducing the levels of *Oprm1* ameliorates the social behavior phenotype caused by increased MeCP2 dosage.

Based on the smallest region of overlap in individuals with Xq28 duplications, we hypothesized that *MECP2* duplication would be sufficient to cause anxiety- and autism-like phenotypes in mice. We discovered that mice of two F1 hybrid backgrounds with either

twice or three times the levels of endogenous MeCP2 are anxious and socially impaired. These findings provide strong evidence that the overexpression of MeCP2 contributes to autism and anxiety in this particular genomic disorder. We also demonstrate that the MeCP2-dependent increase in expression of two anxiety- and social behavior-related genes contribute to disease phenotypes. In the *MECP2*-TG mice, we found that an increase in *Crh* levels contributed to anxiety-like behavior, whereas an increase in *Oprm1* levels only impacted social behavior. Reducing the levels of either of these two genes specifically corrected the respective phenotypes. Thus, these data imply that MeCP2 regulates anxiety and social behavior through distinct pathways.

Of note, we previously showed that *Crh* is a *bona fide* MeCP2 target gene[35], yet it is intriguing to find its expression up-regulated in both the *Mecp2*$^{308/y}$ and *MECP2*-TG mice. The *Mecp2*^{308} allele is originally described as a loss-of-function allele[46]; however, it may also have features of a *hypermorphic* allele given that it lacks key phospho-serine sites (S421 and S424) that regulate DNA binding[47]. This is supported by a recent study demonstrating that mice harboring *Mecp2* mutations at these sites have neurophysiological, behavioral, and transcriptional changes similar to those observed in *MECP2*-TG1 mice[48]. Importantly, *Crh* levels which are increased in the *MECP2*-TG and *Mecp2*^{308} mice[35] yet decreased in *Mecp2*-null mice[24], correlate with heightened and reduced anxiety-like behavior[49], respectively. In *MECP2*-TG animals, heightened anxiety-like behavior can be decreased by modulating the CRH signaling pathway.

Similar to other studies[36–38], we show that MeCP2 binds to the promoter of *Oprm1*; however, we find that *Oprm1* expression is up-regulated in the context of increased MeCP2 gene dosage. Furthermore, we demonstrate that increased *Oprm1* levels likely underlie the social approach deficits in *MECP2-TG1* mice as these deficits are improved by genetically reducing *Oprm1* expression almost to wild-type levels. This result is interesting as pharmacological activation of MOR is associated with increased sociability[34], underscoring a difference between the effects of direct MOR activation versus up-regulation of *Oprm1* expression. Indeed, there are no reports regarding the effects of *Oprm1* overexpression in rodents, thus it is challenging to compare our findings with existing studies related to MOR activity on social behavior. Our data highlight the complexity of the regulation of social behavior by *Oprm1*, and call for further exploration of the distinct effects of *Oprm1* overexpression and MOR activation on such behavior.

In sum, our data highlight the importance of MeCP2 in governing genetic pathways related to normal anxiety and social behavior. It is conceivable that the phenotypes that arise due to an excess or loss of MeCP2 in mice and humans primarily result from misregulation of only a subset of genes that are altered by at least 50%, and that focusing on such genes could identify therapies that ameliorate the respective symptoms. These data provide a potential framework for investigating treatments of anxiety and social behavior in individuals carrying *MECP2* duplications.

Methods

Animal husbandry

Mice were maintained on a 12 h light:12 h dark cycle with standard mouse chow and water ad libitum. For experiments related to the phenotypic analysis of anxiety and social behavior in the *MECP2*-TG lines, we generated F1 hybrid animals by mating female *MECP2*-TG1 and -TG3 mice of a pure FVB/N background to either wild-type male 129S6/SvEv (Taconic Farms, Inc., USA) or C57BL/6 mice (Jackson Laboratories, USA). *MECP2*-TG mice harbored a ~99 kb P1 artificial chromosome (PAC 671D9) containing only the human *MECP2* genomic locus[15]. *MECP2*-TG1 and -TG3 adult male mice on both F1 hybrid genetic backgrounds, (FVB/N × 129S6/SvEv)F1 and (FVB/N × C57BL/6)F1, were viable through at least 20 weeks of age; therefore, it was feasible to test the behavior of these animals during adulthood. We tested only male mice, as our clinical study identified autism and heightened anxiety in boys with *MECP2* duplication syndrome[12]. For studies related to the genetic interaction of MeCP2 with either *Crh* or *Crhr1*, we generated F1 hybrid mice by mating female *MECP2*-TG1 mice of a pure FVB/N background to either male $Crh^{+/-}$ or male $Crhr1^{+/-}$ animals[39,45]. $Crh^{+/-}$ animals, originally generated by targeted disruption of the pre-pro-*Crh* coding region[39], were purchased from Jackson Laboratories on a pure C57BL/6 background. $Crhr1^{+/-}$ animals, originally generated by replacing exons 5 through 8 of *Crhr1* locus with a PGK-neo cassette[45], were also purchased from Jackson Laboratories on a pure C57BL/6 background. Both *Crh* and *Crhr1* lines were backcrossed to 129S6/SvEv for seven generations prior to breeding with *MECP2*-TG1 females to obtain F1 hybrid progeny for the genetic interaction experiments. For studies related to the genetic interaction of MeCP2 with *Oprm1*, we generated F1 hybrid mice by mating female *MECP2*-TG1 mice of a pure FVB/N background to male $Oprm1^{+/-}$ animals that were maintained on a pure C57BL/6 background[40]. $Oprm1^{+/-}$ animals, originally generated by insertion of a PGK-neo cassette into exon 2[40], were purchased from Jackson Laboratories on a pure C57BL/6 background. For studies related to the pharmacological blockade of CRHR1 with antalarmin hydrochloride, we used (FVB/N × 129S6/SvEv)F1 animals that were generated by mating *MECP2*-TG1 female mice of a pure FVB/N background with wild-type 129S6/SvEv male mice (Taconic Farms Inc., USA). The subsequent wild-type male F1 progeny from this mating scheme were used in testing the effect of acute antalarmin treatment after social defeat stress[43], whereas *MECP2*-TG1 F1 hybrid male progeny from this mating scheme were used in testing the effect of acute antalarmin treatment. In most cases, animals were housed 4 animals per cage with an equal balance of genotypes per cage. Animals used for the pharmacological studies were housed 4–5 animals per cage, and *MECP2*-TG1 and wild-type littermate F1 hybrid animals in these studies were housed in separate cages. All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Test colonies

Four (FVB/N × 129S6/SvEv)F1 *MECP2*-TG1 test colonies were generated for behavioral testing. Test colonies included both *MECP2*-TG1 animals and their wild-type littermates. The first *MECP2*-TG1 test colony was used to measure activity in an open field (8–9 weeks of life), to test anxiety-like behavior in the light-dark box (9–10 weeks of life), and to test

social interaction in the partition test (12–13 weeks of life). The second *MECP2*-TG1 test colony was used to test anxiety-like behavior in the elevated plus maze (9–10 weeks of life). The third *MECP2*-TG1 test colony was used to test social interaction in the three chamber test (12 weeks of life). The fourth *MECP2*-TG1 test colony (16 – 20 weeks of life) was used for the pharmacological studies related to antalarmin. One (FVB/N × C57BL/6)F1 *MECP2*-TG1 test colony was generated to confirm the anxiety-like behavior and social behavior deficits; anxiety and social behavior tests were performed at equivalent timepoints listed for (FVB/N × 129S6/SvEv)F1 *MECP2*-TG1 test cohorts.

Two (FVB/N × 129S6/SvEv)F1 *MECP2*-TG3 test colonies were generated for behavioral testing. Test colonies included both *MECP2*-TG3 animals and their wild-type littermates. The first *MECP2*-TG3 test colony was used to measure activity in an open field (8 weeks of life), to test anxiety-like behavior in the light-dark box (8–9 weeks of life), and to test social interaction in the partition test (10–12 weeks of life). The second *MECP2*-TG3 test colony was used to test anxiety-like behavior in the elevated plus maze (8–9 weeks of life). One F1 hybrid FVB/N × C57BL/6 *MECP2*-TG3 test colony was generated to confirm the anxiety-like behavior and social behavior deficits; anxiety and social behavior tests were performed at equivalent timepoints listed for (FVB/N × 129S6/SvEv)F1 *MECP2*-TG3 test cohorts.

One test colony each was generated to test the effect of either *Crh* or *Crhr1* haploinsufficiency in *MECP2*-TG1 animals in an open field, elevated plus maze, light dark box and partition test (~4–5 months of life). Test colonies included all possible animal genotypes; the parental genotypes (*MECP2*-TG1 and either $Crh^{+/-}$ or $Crhr1^{+/-}$), wild-type littermates, and double mutant (*MECP2*-TG1; $Crh^{+/-}$ or *MECP2*-TG1; $Crhr1^{+/-}$) animals.

One test colony was generated to test the effect of *Oprm1* haploinsufficiency in *MECP2*-TG1 animals in an open field, elevated plus maze, light dark box, partition test, and three chamber test (~2–3 months of life). Test colonies included all possible animal genotypes; the parental genotypes (*MECP2*-TG1 and $Oprm1^{+/-}$), wild-type littermates, and double mutant (*MECP2*-TG1; $Oprm1^{+/-}$) animals.

Behavioral tests and statistical analysis of behavioral data

Methods for behavioral tests and statistical analysis of behavioral data are presented in the Supplementary Note.

Microarray experiments and statistical analysis of microarray data

Total RNA was extracted from the amygdalae of $Mecp2^{null/y}$ animals[28] and their wild-type littermates (6 weeks of life, n=4 per genotype), and from the amygdalae of male *MECP2*-TG3 and their wild-type littermates (6 weeks of life, n=5 per genotype) using Trizol (Invitrogen, USA). Microarray experiments were performed as previously described using the Affymetrix Mouse Exon 1.0 ST microarray[24,25]. Probe level data were normalized using the RMA method and the exonmap package. The resulting probe set expression summaries were then annotated to each gene and exon using the information from the Affymetrix using the na27 build of annotation for the MoEx 1 array (see MoEx-1_0-st-v1.na27.AFFX_README.NetAffx-CSV-Files.txt). Exon coordinate annotation for each

gene were then obtained from the UCSC genome browser using the mm9 build of the mouse genome; probe sets were assigned to exons using the combined information from the Affymetrix annotation file and data from the UCSC browser; data on a total of 24,277 distinct gene symbols were considered. Once the normalized data were assigned to exons, a linear model was constructed extending our previous work[24,25]. This model considered RMA expression summaries at the level of probe sets with model terms for probe set, gene, genotype, genotype-exon interaction, and individual animal effects. The model was fit using R and the base method for analysis of variance. The error estimate and coefficient parameters from this model were then used to construct three linear contrasts: one contrast for the difference between WT and NULL mice using only the appropriate matched WT control; another contrast for the difference between the TG mice and their WT control; and a final contrast considered the sum of these two differences. The 2-sided *p* values for these linear contrasts were then converted to q-values using the p. adjust method in the base R installation. Values where the mean gene-level fold change difference exceeded 0.2 with an FDR q-value less than 0.05 were selected as differentially expressed, as well as genes where the total difference exceeded 0.4 with a q-value less than 0.05. Content analyses for the GeneOntology were performed as previously described[24].

Mouse genome informatics (MGI) database phenotype analysis

Methods for MGI database phenotype analysis are presented in the Supplementary Note.

Chromatin immunoprecipitation-PCR (ChIP-PCR)

Methods for ChIP-PCR are presented in the Supplementary Note.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

For validation of expression profiling data, total RNA was extracted from the amygdalae of *MECP2*-TG3 animals and their wild-type littermates (n=4 of each genotype) using Trizol (Invitrogen, USA). For testing the effect of reducing either *Crh* or *Oprm1* levels in *MECP2*-TG1 mice, and to check *Avp* expression in *MECP2*-TG1 × $Crh^{+/-}$ animals, total RNA was extracted from the hypothalami and amygdalae of the double mutant animals and their respective littermates (n=3–6 of each genotype). Three μg of RNA was used to synthesize cDNA according to the manufacturer's protocol (Invitrogen, USA). QRT-PCR was performed as previously described[20] using PerfeCTa qPCR FastMix (Quanta Biosciences, Inc., USA). Primers were designed to amplify a single gene product using an online primer design tool (Primer-BLAST, www.ncbi.nlm.nih.gov) or were obtained from an online public resource (PrimerBank[50]). Sequences are available upon request. Expression levels were normalized to S16 and data are represented as fold change relative to wild-type levels. Significant differences were determined using paired T tests.

Non-radioactive in situ hybridization (ISH)

Crh ISH probe was PCR-amplified from wild-type mouse brain cDNA, followed by digoxigenin labeling as previously described[35]. ISH was performed on brain tissue obtained from wild-type, $Crh^{+/-}$, *MECP2*-TG1 and *MECP2*-TG1; $Crh^{+/-}$ animals (~4–5 months of life), and ISH signal intensity was quantified as previously described[21,35,51].

Corticosterone studies

Serum corticosterone levels (n=3–6 animals of each genotype for each measurement) were determined as previously described[35]. Briefly, basal corticosterone levels were obtained from animals that were undisturbed for at least 12 hours. Stress-induced corticosterone levels were obtained from animals restrained in 50 mL conical tubes for 30 minutes. For both test measurements, animals were rapidly decapitated after the indicated time period. Trunk blood was collected and placed in 1.5 mL conical tubes on ice for at least 30 minutes. Blood was centrifuged at max speed for 10 minutes. Serum was collected and analyzed using an enzyme-linked immunoassay (IDS Inc., USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

RS, CMB and CMM performed experiments; RS and CMM analyzed the data. CS performed statistical analyses of microarray data. BM provided intellectual contribution to and initiated CRH genetic interaction studies. RS and HZ designed experiments, reviewed the data and wrote the manuscript. All authors reviewed the manuscript in its preparation. We thank Drs. Melissa Ramocki and Jeffrey Neul for critical reading of the manuscript, Drs. Corinne Spencer, Richard Paylor, and Paolo Moretti for advice on neurobehavioral tasks, the BCM Microarray core and the BCM Intellectual and Developmental Disabilities Research Center (IDDRC) *In situ* Hybridization and Neurobehavioral Cores for use of facilities. This work was funded by the U.S. National Institutes of Health Grants NS043124 (RS), NS073317 (CMM), NS057819 (HZ), and HD24064 (HZ, BCM IDDRC), Autism Speaks (Predoctoral Fellowship to RS), the Carl C. Anderson, Sr. and Marie Jo Anderson Charitable Foundation, the Simons Foundation, and the Rett Syndrome Research Trust (HZ). Huda Zoghbi is a Howard Hughes Medical Institute investigator.

References

1. Amir RE, et al. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet. 1999; 23:185–188. [PubMed: 10508514]
2. Lam CW. Spectrum of mutations in the MECP2 gene in patients with infantile autism and Rett syndrome. Journal of Medical Genetics. 2000; 37:41e–41. [PubMed: 10633133]
3. Watson P, et al. Angelman syndrome phenotype associated with mutations in MECP2, a gene encoding a methyl CpG binding protein. J Med Genet. 2001; 38:224–228. [PubMed: 11283202]
4. Klauck SM, et al. A mutation hot spot for nonspecific X-linked mental retardation in the MECP2 gene causes the PPM-X syndrome. Am J Hum Genet. 2002; 70:1034–1037. [PubMed: 11885030]
5. Carney RM, et al. Identification of MeCP2 mutations in a series of females with autistic disorder. Pediatr Neurol. 2003; 28:205–211. [PubMed: 12770674]
6. Milani D, Pantaleoni C, D'Arrigo S, Selicorni A, Riva D. Another patient with MECP2 mutation without classic Rett syndrome phenotype. Pediatr Neurol. 2005; 32:355–357. [PubMed: 15866439]
7. del Gaudio D, et al. Increased MECP2 gene copy number as the result of genomic duplication in neurodevelopmentally delayed males. Genet Med. 2006; 8:784–792. [PubMed: 17172942]
8. Van Esch H, et al. Duplication of the MECP2 region is a frequent cause of severe mental retardation and progressive neurological symptoms in males. Am J Hum Genet. 2005; 77:442–453. [PubMed: 16080119]
9. Meins M, et al. Submicroscopic duplication in Xq28 causes increased expression of the MECP2 gene in a boy with severe mental retardation and features of Rett syndrome. J Med Genet. 2005; 42:e12. [PubMed: 15689435]
10. Friez MJ, et al. Recurrent infections, hypotonia, and mental retardation caused by duplication of MECP2 and adjacent region in Xq28. Pediatrics. 2006; 118:e1687–1695. [PubMed: 17088400]

11. Carvalho CMB, et al. Complex rearrangements in patients with duplications of MECP2 can occur by fork stalling and template switching. Hum Mol Genet. 2009; 18:2188–2203. [PubMed: 19324899]
12. Ramocki MB, et al. Autism and other neuropsychiatric symptoms are prevalent in individuals with MeCP2 duplication syndrome. Ann Neurol. 2009; 66:771–782. [PubMed: 20035514]
13. Velinov M, et al. De-novo 2.15 Mb terminal Xq duplication involving MECP2 but not L1CAM gene in a male patient with mental retardation. Clin Dysmorphol. 2009; 18:9–12. [PubMed: 19090026]
14. Prescott TE, Rødningen OK, Bjørnstad A, Stray-Pedersen A. Two brothers with a microduplication including the MECP2 gene: rapid head growth in infancy and resolution of susceptibility to infection. Clin Dysmorphol. 2009; 18:78–82. [PubMed: 19057379]
15. Collins AL, et al. Mild overexpression of MeCP2 causes a progressive neurological disorder in mice. Hum Mol Genet. 2004; 13:2679–2689. [PubMed: 15351775]
16. Cook MN, Williams RW, Flaherty L. Anxiety-related behaviors in the elevated zero-maze are affected by genetic factors and retinal degeneration. Behav Neurosci. 2001; 115:468–476. [PubMed: 11345971]
17. Wolfer DP, Lipp HP. Dissecting the behaviour of transgenic mice: is it the mutation, the genetic background, or the environment? Exp Physiol. 2000; 85:627–634. [PubMed: 11187958]
18. Mutant mice and neuroscience: recommendations concerning genetic background. Banbury Conference on genetic background in mice. Neuron. 1997; 19:755–759. [PubMed: 9354323]
19. Moretti P, Bouwknecht JA, Teague R, Paylor R, Zoghbi HY. Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. Hum Mol Genet. 2005; 14:205–220. [PubMed: 15548546]
20. Samaco RC, et al. A partial loss of function allele of methyl-CpG-binding protein 2 predicts a human neurodevelopmental syndrome. Hum Mol Genet. 2008; 17:1718–1727. [PubMed: 18321864]
21. Samaco RC, et al. Loss of MeCP2 in aminergic neurons causes cell-autonomous defects in neurotransmitter synthesis and specific behavioral abnormalities. Proc Natl Acad Sci USA. 2009; 106:21966–21971. [PubMed: 20007372]
22. Moy SS, et al. Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav. 2004; 3:287–302. [PubMed: 15344922]
23. Chao HT, et al. Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature. 2010; 468:263–269. [PubMed: 21068835]
24. Chahrour M, et al. MeCP2, a key contributor to neurological disease, activates and represses transcription. Science. 2008; 320:1224–1229. [PubMed: 18511691]
25. Ben-Shachar S, Chahrour M, Thaller C, Shaw CA, Zoghbi HY. Mouse models of MeCP2 disorders share gene expression changes in the cerebellum and hypothalamus. Hum Mol Genet. 2009; 18:2431–2442. [PubMed: 19369296]
26. Nan X, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature. 1998; 393:386–389. [PubMed: 9620804]
27. Yilmazer-Hanke DM. Morphological correlates of emotional and cognitive behaviour: insights from studies on inbred and outbred rodent strains and their crosses. Behav Pharmacol. 2008; 19:403–434. [PubMed: 18690101]
28. Guy J, Hendrich B, Holmes M, Martin JE, Bird A. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet. 2001; 27:322–326. [PubMed: 11242117]
29. Cook EH, Scherer SW. Copy-number variations associated with neuropsychiatric conditions. Nature. 2008; 455:919–923. [PubMed: 18923514]
30. Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci. 1994; 14:2579–2584. [PubMed: 8182429]
31. van Gaalen MM, Stenzel-Poore MP, Holsboer F, Steckler T. Effects of transgenic overproduction of CRH on anxiety-like behaviour. Eur J Neurosci. 2002; 15:2007–2015. [PubMed: 12099906]

32. Filliol D, et al. Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. Nat Genet. 2000; 25:195–200. [PubMed: 10835636]
33. Moles A, Kieffer BL, D'Amato FR. Deficit in attachment behavior in mice lacking the mu-opioid receptor gene. Science. 2004; 304:1983–1986. [PubMed: 15218152]
34. Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ. Nucleus accumbens μ-opioid receptors mediate social reward. J Neurosci. 2011; 31:6362–6370. [PubMed: 21525276]
35. McGill BE, et al. Enhanced anxiety and stress-induced corticosterone release are associated with increased Crh expression in a mouse model of Rett syndrome. Proc Natl Acad Sci USA. 2006; 103:18267–18272. [PubMed: 17108082]
36. Hwang CK, et al. Up-regulation of the mu-opioid receptor gene is mediated through chromatin remodeling and transcriptional factors in differentiated neuronal cells. Mol Pharmacol. 2010; 78:58–68. [PubMed: 20385708]
37. Hwang CK, et al. Evidence of endogenous mu opioid receptor regulation by epigenetic control of the promoters. Mol Cell Biol. 2007; 27:4720–4736. [PubMed: 17452465]
38. Hwang CK, et al. Epigenetic programming of mu-opioid receptor gene in mouse brain is regulated by MeCP2 and Brg1 chromatin remodelling factor. J Cell Mol Med. 2009; 13:3591–3615. [PubMed: 19602036]
39. Muglia L, Jacobson L, Dikkes P, Majzoub JA. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature. 1995; 373:427–432. [PubMed: 7830793]
40. Matthes HW, et al. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature. 1996; 383:819–823. [PubMed: 8893006]
41. Landgraf R, et al. Candidate genes of anxiety-related behavior in HAB/LAB rats and mice: focus on vasopressin and glyoxalase-I. Neurosci Biobehav Rev. 2007; 31:89–102. [PubMed: 16934871]
42. Keck ME, Holsboer F, MüLler MB. Mouse Mutants for the Study of Corticotropin-Releasing Hormone Receptor Function: Development of Novel Treatment Strategies for Mood Disorders. Annals of the New York Academy of Sciences. 2004; 1018:445–457. [PubMed: 15240401]
43. Griebel G, et al. 4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-N-(2-propynyl)-1, 3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor(1) receptor antagonist. II Characterization in rodent models of stress-related disorders. J Pharmacol Exp Ther. 2002; 301:333–345. [PubMed: 11907191]
44. Habib KE, et al. Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. Proc Natl Acad Sci USA. 2000; 97:6079–6084. [PubMed: 10823952]
45. Smith GW, et al. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron. 1998; 20:1093–1102. [PubMed: 9655498]
46. Shahbazian M, et al. Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron. 2002; 35:243–254. [PubMed: 12160743]
47. Tao J, et al. Phosphorylation of MeCP2 at Serine 80 regulates its chromatin association and neurological function. Proc Natl Acad Sci USA. 2009; 106:4882–4887. [PubMed: 19225110]
48. Li H, Zhong X, Chau KF, Williams EC, Chang Q. Loss of activity-induced phosphorylation of MeCP2 enhances synaptogenesis, LTP and spatial memory. Nat Neurosci. 2011; 14:1001–1008. [PubMed: 21765426]
49. Pelka GJ, et al. Mecp2 deficiency is associated with learning and cognitive deficits and altered gene activity in the hippocampal region of mice. Brain. 2006; 129:887–898. [PubMed: 16467389]
50. Spandidos A, Wang X, Wang H, Seed B. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res. 2010; 38:D792–799. [PubMed: 19906719]
51. Carson JP, Eichele G, Chiu W. A method for automated detection of gene expression required for the establishment of a digital transcriptome-wide gene expression atlas. J Microsc. 2005; 217:275–281. [PubMed: 15725131]

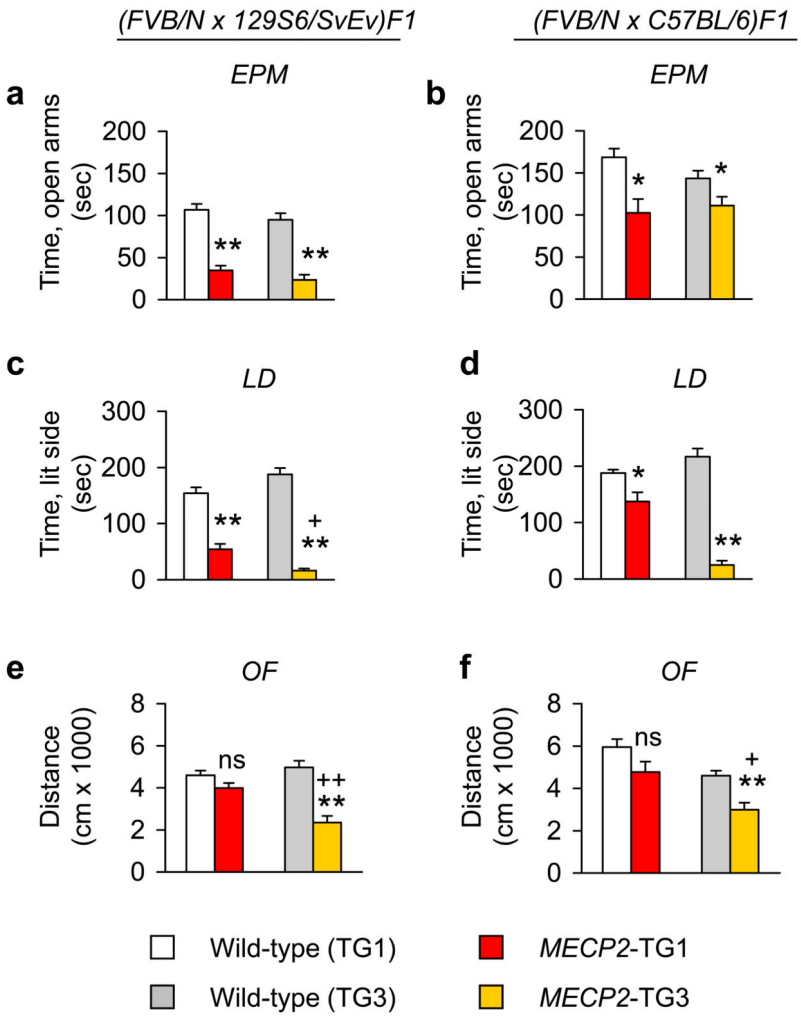

Figure 1. Increasing the endogenous levels of MeCP2 causes heightened anxiety-like behavior in mice

(a–d) *MECP2*-TG1 and -TG3 mice compared to respective wild-type littermate animals spend less time in the open arms of an elevated plus maze **(a, b)**, and spend less time in the lit compartment of the light-dark box **(c, d). (e, f)** *MECP2*-TG1 mice compared to respective wild-type littermate animals travel a similar distance in an open field; in contrast *MECP2*-TG3 mice travel less compared with their wild-type littermates. Data from **a, c, e** correspond to (FVB/N × 129S6/SvEv)F1 animals; data from **b, d, f** correspond to (FVB/N × C57BL/6)F1 animals. White bars represent wild-type littermates for the *MECP2*-TG1 line (n=14–22), red bars represent *MECP2*-TG1 animals (n=10–22), grey bars represent wild-type littermates for the *MECP2*-TG3 line (n=20–27), orange bars represent *MECP2*-TG3 animals n=17–21). Values represent mean ± s.e.m., asterisks indicate significant differences between either *MECP2*-TG1 or -TG3 animals compared with their respective wild-type littermates, plus signs indicate significant differences between *MECP2*-TG1 and *MECP2*-TG3 animals. * and +, p value < 0.05; ** and ++, p value < 0.001; ns, not significant; EPM, elevated plus maze, LD, light dark box exploration; OF, open field. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

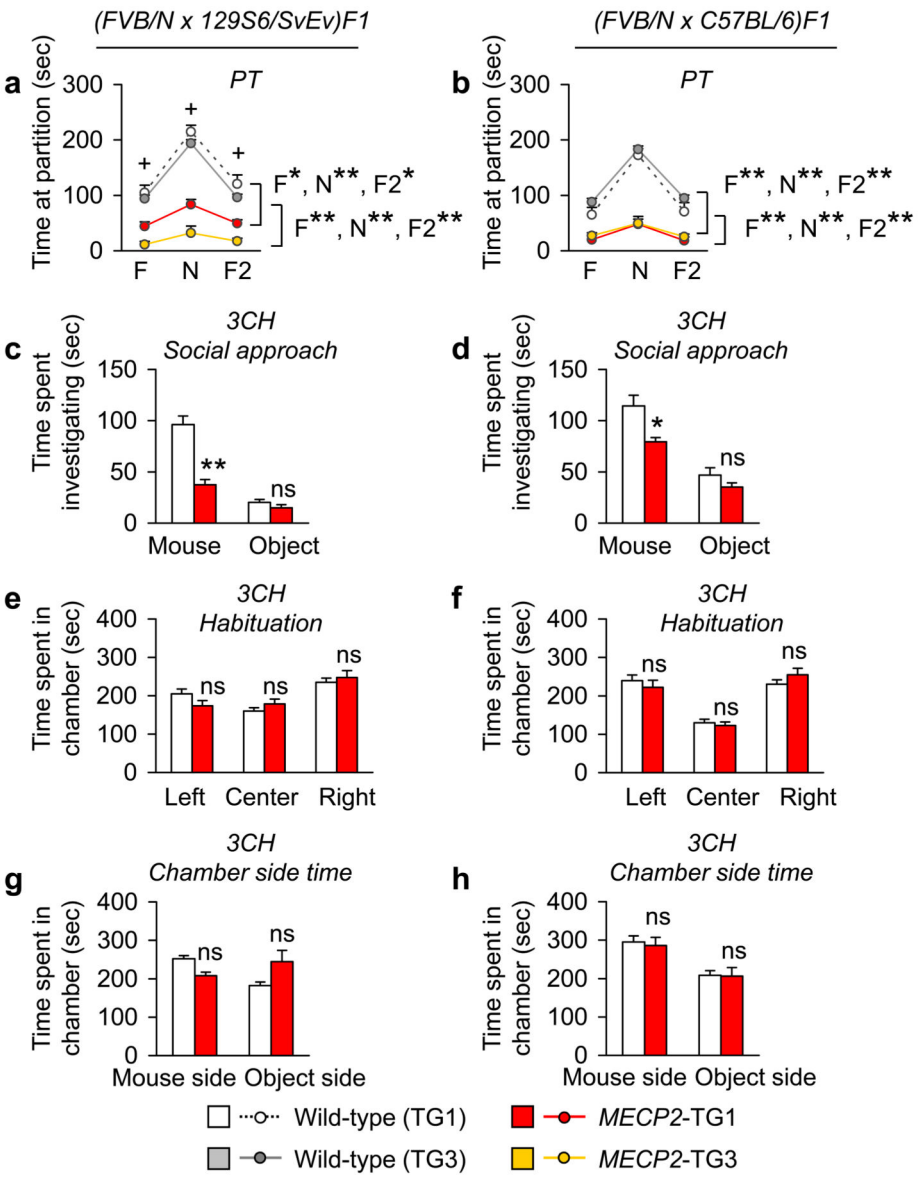

Figure 2. Increasing the endogenous levels of MeCP2 causes social behavior deficits in mice

(a, b) *MECP2*-TG1 and -TG3 animals are less interested in their familiar or novel partner mice in the partition test for social interaction and recognition. Significant differences are denoted for familiar partners during the first encounter (F), novel partners (N), and familiar partners during a second encounter (F2). **(c–h)** In the three-chamber test for social approach, *MECP2*-TG1 mice compared to wild-type littermates spend the same amount of time investigating a novel object but are less interested in a novel mouse **(c, d)**. *MECP2*-TG1 mice compared with wild-type littermates show no side preferences during either the habituation phase **(e, f)** or test phase **(g, h)**. Data from **a, c, e, g** correspond to (FVB/N × 129S6/SvEv)F1 animals; data from **b, d, f, h** correspond to (FVB/N × C57BL/6)F1 animals. White bars and dashed lines represent wild-type littermates for the *MECP2*-TG1 line (n=12), red bars and lines represent *MECP2*-TG1 animals (n=10–12), grey lines represent wild-type

littermates for the *MECP2*-TG3 lines (n=12–15), orange lines represent *MECP2*-TG3 animals (n=10–11). Values represent mean ± s.e.m., asterisks indicate significant differences between either *MECP2*-TG1 or -TG3 animals compared with their respective wild-type littermates, plus signs indicate significant differences between *MECP2*-TG1 and *MECP2*-TG3 animals. * and +, p value < 0.05; ** and ++, p value < 0.001; ns, not significant; PT, partition test for social interest and recognition; 3CH, three chamber test for sociability. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

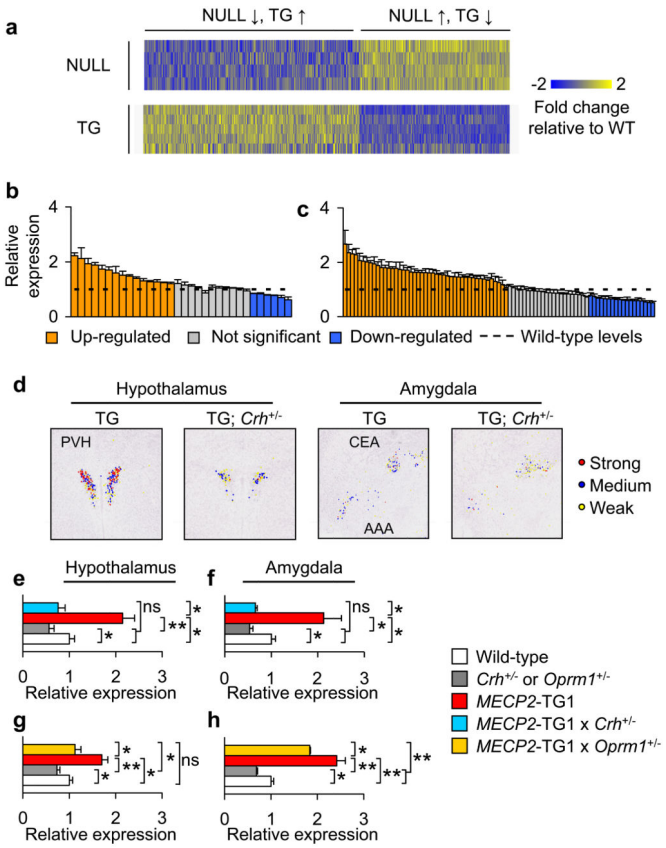

Figure 3. Gene expression analysis of the amygdala identifies a subset of altered genes implicated in anxiety and/or social behavior

(a) Transcriptional profiling heat map displaying fold changes of the 1,060 genes oppositely altered genes with a 0.2 to 2 $\log_2$-fold change in the two MeCP2 mouse models compared with wild-type littermates (q value < 0.05). **(b, c)** Quantitative real-time reverse transcription-PCR (qRT-PCR) results displaying fold changes of 32 anxiety- and/or social behavior-related genes **(b)** and 85 genes not implicated in anxiety and/or social behavior **(c)**. Expression levels were normalized to wild-type levels (dashed line). For both **b** and **c**, orange bars represent statistically significant up-regulated genes, blue bars represent statistically significant down-regulated genes, and grey bars represent non-significant gene alterations. **(d)** *In situ* hybridization (ISH) shows that *MECP2*-TG1 animals have increased levels of *Crh* in the paraventricular nucleus of the hypothalamus and amygdala; in contrast *MECP2*-TG1;*Crh*$^{+/-}$ animals have reduced *Crh* levels. Representative images are pseudo-colored to indicate signal intensity levels (red=strong expression, blue=medium expression, yellow=weak expression). **(e, f)** Quantification of the signal intensity from multiple sections shows levels of *Crh* are decreased in *MECP2*-TG1; *Crh*$^{+/-}$ mice compared with *MECP2*-TG1 animals in both the hypothalamus **(e)** and amygdala **(f). (g, h)** QRT-PCR of *Oprm1* levels demonstrate a reduction in expression levels in *MECP2*-TG1; *Oprm1*$^{+/-}$ double mutant animals compared with *MECP2*-TG1 littermates in both the hypothalamus **(g)** and the amygdala **(h)**. For **e–h**, white bars represent wild-type littermates, dark grey bars represent *Crh*$^{+/-}$ littermates or *Oprm1*$^{+/-}$ littermates, red bars represent *MECP2*-TG1 littermates, blue bars represent *MECP2*-TG1; *Crh*$^{+/-}$ animals, and orange bars represent

MECP2-TG1; *Oprm1*$^{+/-}$animals. PVH, paraventricular nucleus; AAA, anterior amygdalar area; CEA, central amygdalar nucleus. Values represent mean ± s.e.m.; asterisks indicate significant differences between indicated genotypes. *, p value < 0.05; **, p value < 0.001; ns, not significant.

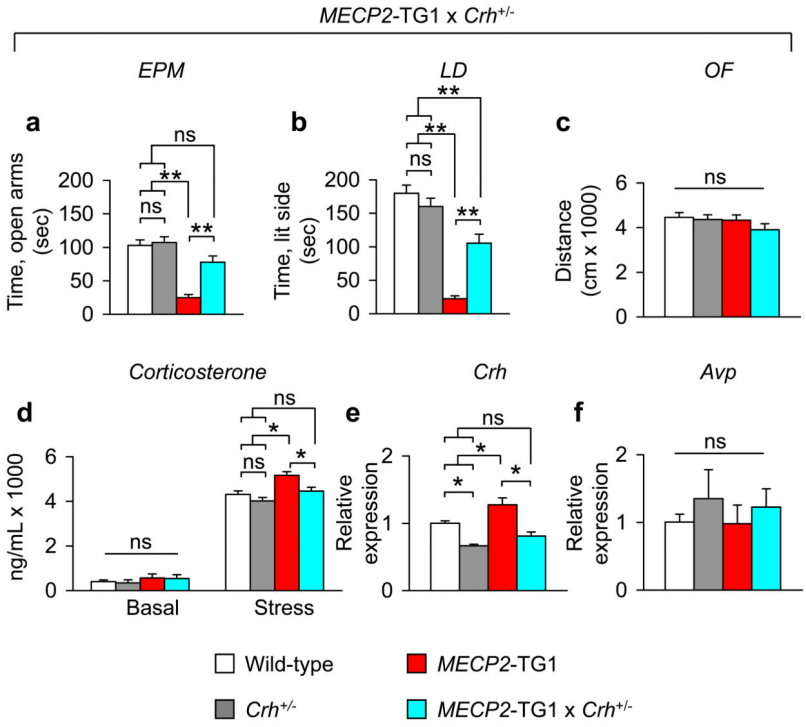

Figure 4. Genetic reduction of *Crh* improves anxiety-like behavior in *MECP2* duplication mice

(a–c) *MECP2*-TG1; $Crh^{+/-}$ animals are less anxious in the elevated plus maze **(a)** and light-dark box **(b)**. The total distance traveled in the open field is normal among all groups **(c). (d)** Basal corticosterone levels are normal in *MECP2*-TG1; $Crh^{+/-}$ double mutant animals compared with their respective littermates (n=3–6 animals of each genotype). Stress-induced corticosterone levels are significantly higher in *MECP2*-TG1 animals; this is suppressed in *MECP2*-TG1; $Crh^{+/-}$ animals (n=4 animals of each genotype). **(e, f)** *Crh* expression level differences in the amygdalae of an independent set of animals was confirmed by QPCR **(e)**. *Avp* expression level differences were not observed across all genotypes (n=3–6 animals of each genotype) **(f)**. For behavioral studies, white bars represent wild-type littermates (n=10–23,), dark grey bars represent $Crh^{+/-}$ littermates (n=17), red bars represent *MECP2*-TG1 littermates (n=13–21), and blue bars represent *MECP2*-TG1; $Crh^{+/-}$ animals (n=13–18). Values represent mean ± s.e.m.; asterisks indicate significant differences between indicated genotypes, *, p value < 0.05; **, p value < 0.001; ns, not significant; EPM, elevated plus maze, LD, light dark box exploration; OF, open field. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

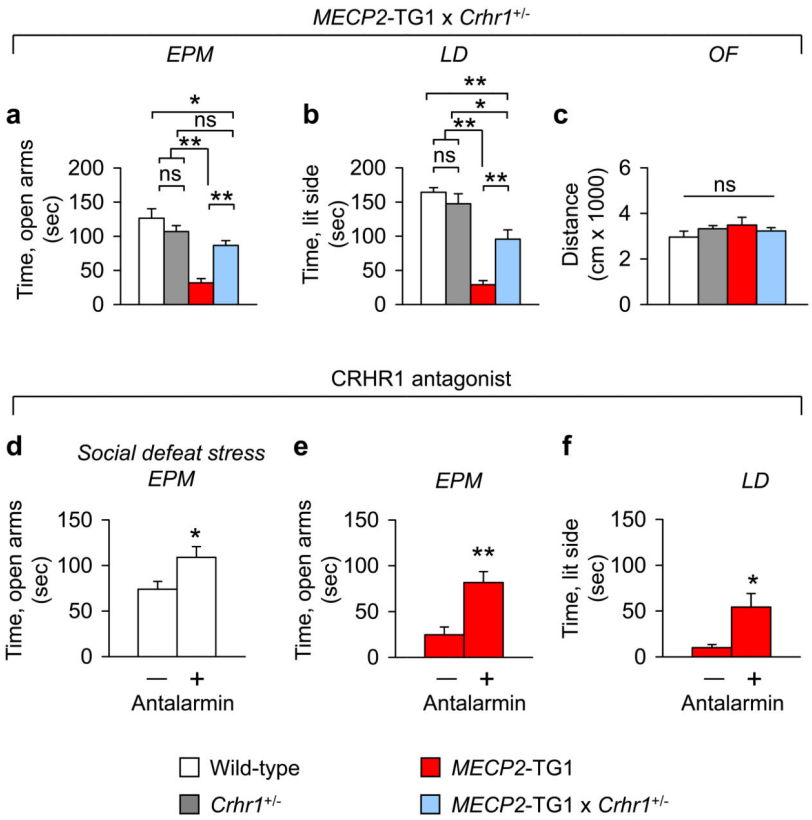

Figure 5. Genetic reduction of the CRH receptor, CRHR1, and pharmacologic intervention using a CRHR1 antagonist improves anxiety-like behavior in *MECP2* duplication mice

(a–c) The levels of anxiety-like behavior in the elevated plus maze **(a)** and light-dark box **(b)** were suppressed in *MECP2*-TG1; *Crhr1*$^{+/-}$ animals. No difference was observed in the total distance traveled in an open field **(c). (d)** Pre-treatment with 60 mg/kg antalarmin had an anxiolytic effect in (FVB/N × 129S6/SvEv)F1 wild-type littermates of *MECP2*-TG1 mice in the elevated plus maze (vehicle group, n=20; antalarmin group, n=16). **(e, f)** Antalarmin-treated *MECP2*-TG1 animals spent more time in the open arms of the elevated plus maze **(e)** and spent more time in the lit side of the light dark box **(f)** (EPM vehicle group, n=14; antalarmin group, n=25; LD vehicle group, n=8; antalarmin group, n=17). For **a–c**, white bars represent wild-type littermates (n=12), dark grey bars represent *Crhr1*$^{+/-}$ littermates (n=15), red bars represent *MECP2*-TG1 littermates (n=9), light blue bars represent *MECP2*-TG1; *Crhr1*$^{+/-}$ animals (n=18). For **d–f**, dash or plus signs indicate either vehicle-treated animals (−) or 60 mg/kg antalarmin-treated animals (+). Values represent mean ± s.e.m.; asterisks indicate significant differences either among genotypes for genetic interaction data, or between drug-treated and vehicle-treated animals for antalarmin studies, *, p value < 0.05; **, p value < 0.001; ns, not significant; EPM, elevated plus maze, LD, light dark box exploration; OF, open field. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

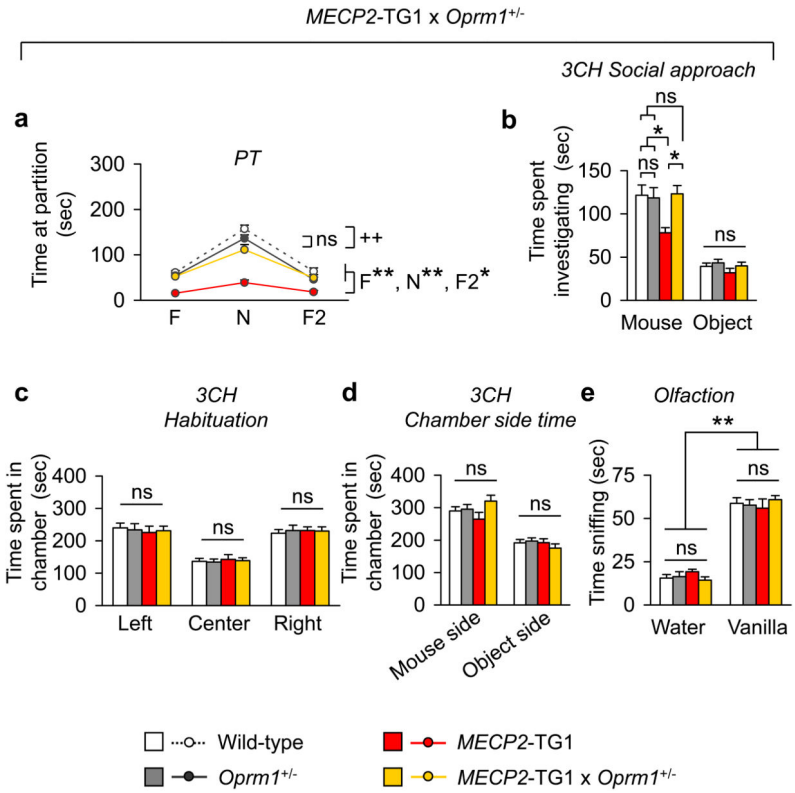

Figure 6. Genetic reduction of *Oprm1* improves the social behavior deficits of *MECP2* duplication mice

(a) Reducing the levels of *Oprm1* in *MECP2*-TG1 animals improved their social behavior deficits, as double mutant mice spent more time investigating both familiar and novel partners compared with *MECP2*-TG1 mice (asterisks). The level of interest was similar to wild-type and *Oprm1*$^{+/-}$ mice for the familiar encounters, but for novel partners, the level of interest for double mutants was similar to *Oprm1*$^{+/-}$ but not wild-type mice (significant difference indicated by plus signs). **(b)** In the three chamber test, *MECP2*-TG1; *Oprm1*$^{+/-}$ double mutants demonstrated normal social approach behavior towards a novel partner mouse compared with both wild-type and *Oprm1*$^{+/-}$ animals; *MECP2*-TG1 animals showed a decrease in social approach behavior towards a novel partner mouse. All genotypes spent an equal time investigating a novel object. **(c, d)** *MECP2*-TG1; *Oprm1*$^{+/-}$ double mutant animals compared with their littermates showed no side preferences during the habituation **(c)** and test **(d)** phases. **(e)** Animals of all genotypes spent more time sniffing a novel odor (vanilla) compared with a familiar odor (water); no differences were observed among all genotypes in the time spent sniffing the odors. White bars and dashed lines represent wild-type littermates (n=13), dark grey bars and lines represent *Oprm1*$^{+/-}$ littermates (n=12), red bars and lines represent *MECP2*-TG1 littermates (n=9), and orange bars and lines represent *MECP2*-TG1; *Oprm1*$^{+/-}$ animals (n=10–12). Values represent mean ± s.e.m.; significant differences between genotypes are denoted by asterisks, for partition test data, asterisks indicate significant differences between *MECP2*-TG1 and littermates, and plus signs indicate significant differences between double mutants and wild-type littermates, *, *p* value < 0.05; ** or ++, *p* value < 0.001; ns, not significant; PT, partition test for social interest and

recognition; 3CH, three chamber test for sociability. A complete statistical summary of behavioral data is provided in Supplementary Table 6.