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

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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, although 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 heightened anxiety and social behavior deficits, we analyzed MECP2 duplication mice (MECP2-TG1) that have reduced Crh and Oprm1 expression. In MECP2-TG1 animals, reducing the levels of Crh or its receptor, Crhr1, suppressed anxiety-like behavior; in contrast, reducing Oprm1 expression improved abnormal social behavior. These data indicate that increased MeCP2 levels affect molecular pathways underlying anxiety and social behavior and provide new 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 (OMIM 312750) led to the identification of other neuropsychiatric phenotypes caused by MECP2 mutations1–6. The most recently identified MECP2-related disorder is a genomic disorder that results from large nonrecurrent duplications of chromosome Xq28 (ref. 7). The shared region of overlap among affected individuals spans the IRAK1 (interleukin-1 receptor–associated kinase 1) and MECP2 (refs. 8–14) loci, suggesting that 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 (ref. 12), and anxiety is a comorbid condition15. Individuals with triplications spanning MECP2 typically manifest a more severe phenotype7,11.

We previously reported that transgenic mice overexpressing MeCP2 at twice the normal levels (MECP2-TG1) on an FVB/N background have motor defects, stereotypes and seizures15. 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 behavior16. 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 strains17,18, we found that F1 hybrid MECP2-TG1 animals, expressing twice the levels of MeCP2 seen in wild-type mice, and F1 hybrid MECP2-TG3 animals, expressing in excess of threefold the normal levels of MeCP2, displayed anxiety-like behavior in the elevated plus maze and the light/dark box (Fig. 1a–d). The new data here unequivocally show that doubling MeCP2 levels indeed causes heightened anxiety-like behavior, as robust phenotypes were observed in two different F1 hybrid backgrounds (Fig. 1). Thus, the absence of detectable anxiety-like behavior in previously studied MECP2-TG1 animals on a pure FVB/N background in the light/dark box task was likely caused by impaired vision in that background15. To investigate social behavior abnormalities, we tested F1 hybrid MECP2-TG1 and MECP2-TG3 mice in the partition test for sociability and recognition19–21. We discovered that MECP2-TG1 and MECP2-TG3 animals compared with their wild-type littermates showed less interest in familiar and novel, unfamiliar partner animals (Fig. 2a,b). As it is possible that the social interaction deficit in MECP2-TG3 animals is 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 characterize the social behavior abnormalities of MECP2-TG1 mice by subjecting these animals to the three-chamber test for sociability22,23. MECP2-TG1 animals showed a deficit in social approach behavior toward novel partner mice, without a deficit in interest toward 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 deficit than MECP2-TG1 mice (Fig. 2c,d).
phenotype than MECP2-TG1 mice in some tests (indicated by plus signs in Figs. 1 and 2), reminiscent of the human disorders in which the most severe clinical phenotypes are observed in individuals with triplications spanning MECP2 (refs. 8, 13, 14).

Because either the loss or gain of MeCP2 is known to affect gene expression levels24–26, we next sought to identify alterations in gene expression 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 behavior27, of MECP2-TG3 mice, mice that lack MeCP2 (MeCP2−/−)28 and the respective wild-type littermates. We focused on gene expression changes altered in opposite directions in the MECP2-TG3 animals compared with the MeCP2−/− animals, which are likely to be sensitive to MeCP2 dosage.

Figure 1 Increasing the endogenous levels of MeCP2 causes heightened anxiety-like behavior in mice. (a, b) MECP2-TG1 and MECP2-TG3 mice compared to respective wild-type littermate animals spend less time in the open arms of an elevated plus maze. (c, d) MECP2-TG1 and MECP2-TG3 animals spend less time in the light compartment of the light/dark box compared to their wild-type littermates. (e, f) MECP2-TG1 mice, compared to respective wild-type littermate controls, travel a similar distance in an open field; in contrast, MECP2-TG3 mice travel less compared with their wild-type littermates. White, wild-type littermates for the MECP2-TG1 line (N = 14–22); red, MECP2-TG1 animals (N = 10–22); gray, wild-type littermates for the MECP2-TG3 line (N = 20–27); yellow, MECP2-TG3 animals (N = 17–21). Mice in a, c and e were in the FVB/N × 129S6/SvEv F1 hybrid background, and those in b, d and f were in the FVB/N × C57BL/6 F1 hybrid background. Values represent mean ± s.e.m. Asterisks indicate significant differences between either MECP2-TG1 or MECP2-TG3 animals and their respective wild-type littermates; plus signs indicate significant differences between MECP2-TG1 and MECP2-TG3 animals: * and +, P < 0.05; ** or ++, P < 0.001. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

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 mutation 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 reverse transcription PCR (qRT-PCR) validation studies and confirmed that 21 of the 32 anxiety- and/or social behavior-related genes (66%) and 58 of the 85 genes not implicated in anxiety and social behavior deficits (68%) were significantly (P < 0.05) altered in the MECP2-TG3 animals relative to controls (Fig. 3b, c and Supplementary Tables 4 and 5).

The identification of several genes with functions in anxiety and/ or social behavior that are sensitive to MeCP2 levels suggests that

Figure 2 Increasing the endogenous levels of MeCP2 causes social behavior deficits in mice. (a, b) MECP2-TG1 and MECP2-TG3 animals are less interested in their familiar or novel partner mice in the partition test for social interaction and recognition. F, familiar partners during the first encounter; N, novel partners; F2, familiar partners during a second encounter. (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, showed no side preferences during either the habituation (e, f) or test (g, h) phase of the three-chamber test. White bars and dashed lines, wild-type littermates for the MECP2-TG1 line (N = 12); red bars and lines, MECP2-TG1 animals (N = 10–12); gray lines, wild-type littermates for the MECP2-TG3 lines (N = 12–15); yellow lines, MECP2-TG3 animals (N = 10–11). Mice in a, c, e and g were in the FVB/N × 129S6/SvEv F1 hybrid background, and those in b, d, f and h were in the FVB/N × C57BL/6 F1 hybrid background. Values represent mean ± s.e.m. Asterisks indicate significant differences between either MECP2-TG1 or MECP2-TG3 animals compared with their respective wild-type littermates; plus signs indicate significant differences between MECP2-TG1 and MECP2-TG3 animals: * and +, P < 0.05; **, P < 0.001. A complete statistical summary of behavioral data is provided in Supplementary Table 6.
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. Nine of the 21 anxiety- and/or social behavior–related genes that had significantly altered expression in qRT-PCR analysis had at least a 50% increase or decrease in mRNA levels in the MECP2-TG3 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-TG1 mice, which are most relevant to the genomic disorder, we focused on Crh, which encodes the neuropeptide corticotropin-releasing hormone (CRH), given evidence of increased anxiety-like behavior in mice that overexpress Crh, and on Oprml, which encodes the G protein–coupled µ-opioid receptor MOR, one subtype of opioid receptor that has been shown to have a role in aspects of emotional and social behavior. We upregulated in the amygdala in MECP2-TG1 animals (Fig. 3d–h), we bred female MECP2-TG1 mice to either male Crh–/– mice or male Oprml–/– mice to genetically reduce the levels of these genes in MECP2-TG1 animals. We confirmed that Crh and Oprml expression levels were indeed upregulated in MECP2-TG1 mice and reduced in MECP2-TG1; Crh–/– and MECP2-TG1; Oprml–/– double mutant animals, respectively, and then tested the behavioral consequences of these genetic reductions (Fig. 3d–h).

We found that anxiety-like behavior was reduced in MECP2-TG1 mice lacking one copy of Crh (Fig. 4a,b). In contrast, MECP2-TG1 mice lacking one copy of Oprml 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 and 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

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) relative to their respective littermates. The total distance traveled in the open field is normal among all groups (c). For a–c, white bars, represent wild-type littermates (N = 10–23); dark gray bars, Crh–/– littermates (N = 17); red bars, MECP2-TG1 littermates (N = 13–21); blue bars, MECP2-TG1; Crh–/– animals (N = 13–18). (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 response is suppressed in MECP2-TG1; Crh–/– animals (N = 4 animals of each genotype). (e,f) Crh expression differences in the amygdalae of an independent set of animals were confirmed by qRT-PCR (e). Aasp expression differences were not observed across all genotypes (N = 3–6 animals of each genotype) (f). Values represent mean ± s.e.m. Asterisks indicate significant differences between the indicated genotypes: *P < 0.05; **P < 0.001. 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 showing fold changes of the 1,060 genes oppositely altered with a 0.2 to 2- log2-fold change in the two MECP2 mouse models compared with wild-type littermates (Q-value < 0.05). Null, MECP2–/– mice; TG, MECP2-TG3 mice; WT, wild-type littermate controls. (b,c) qRT-PCR results showing fold change in mRNA levels 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). Orange and blue bars represent statistically significant upregulation and downregulation of genes, respectively. Gray bars represent gene alterations that are not statistically significant. (d) In situ hybridization (ISH) shows that MECP2-TG1 animals have increased levels of Crh in the paraventricular nucleus (PVH) of the hypothalamus and amygdala; in contrast, MECP2-TG1; Crh–/– animals have reduced Crh levels. Representative images are pseudo-colored to indicate signal intensity, AAA, anterior amygdalar area; CEA, central amygdalar nucleus. Scale bar, 400 µm. (e,f) Quantification of the signal intensity from multiple sections shows Crh levels 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 Oprml levels shows a reduction in its expression in MECP2-TG1; Oprml–/– double mutant animals compared with MECP2-TG1 littermates in both the hypothalamus (g) and the amygdala (h). For a–h, values represent mean ± s.e.m. Asterisks indicate significant differences between the indicated genotypes: *P < 0.05; **P < 0.001.
anxiety⁴¹, we examined Avp expression and found that Avp levels were not significantly altered in the amygdalae of Crh⁹⁻/⁻, 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 (CRHR1), encoded by the Crhr1 gene in the mouse⁴². Based on the anxiolytic effects of CRHR1 antagonists⁴³,⁴⁴, 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 was mediated by the increase in Crh levels. We bred female MECP2-TG1 mice to male Crhr1⁹⁻/⁻ mice⁴⁵ and found that the MECP2-TG1; Crhr1⁹⁻/⁻ double mutants were less anxious (Fig. 5a,b) and displayed normal exploratory activity in an open field (Fig. 5c). In additional pharmacologic intervention experiments, an acute dose of antalarmin (60 mg/kg) before social defeat stress significantly reduced the anxiety of wild-type animals in the elevated plus maze (EPM) (e) and in the lit side of the light/dark (LD) box (f). EPM vehicle group, N = 14; antalarmin group, N = 25; LD vehicle group, N = 8; antalarmin group, N = 17. Values represent mean ± s.e.m. Asterisks indicate significant differences either between genotypes in genetic interaction data or between drug- and vehicle-treated animals in antalarmin studies. *P < 0.05; **P < 0.001. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

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; Oprml¹⁰⁻/⁻ 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; Oprml¹⁰⁻/⁻ 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 were comparable among the different test groups (Fig. 6c–e). Together, these data indicate that reducing the levels of Oprml ameliorates the social behavior phenotype caused by increased MeCP2 dosage.

On the basis of 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 in 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 show that the MeCP2-dependent increase in expression of two anxiety- and

Figure 5 Genetic reduction of the CRH receptor, Crhr1, and pharmacologic intervention using a CRHR1 antagonist improve 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; Crhr1⁹⁻/⁻ animals relative to MECP2-TG1 littermates. No difference was observed in the total distance traveled in an open field (c). For a–c, white bars, wild-type littermates (N = 12); dark gray bars, Crhr1⁹⁻/⁻ littermates (N = 15); red bars, MECP2-TG1 littermates (N = 9); light blue bars, MECP2-TG1; Crhr1⁹⁻/⁻ animals (N = 18). (d) Pre-treatment with 60 mg/kg of 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 (EPM) (e) and in the lit side of the light/dark (LD) box (f). EPM vehicle group, N = 14; antalarmin group, N = 25; LD vehicle group, N = 8; antalarmin group, N = 17. Values represent mean ± s.e.m. Asterisks indicate significant differences either between genotypes in genetic interaction data or between drug- and vehicle-treated animals in antalarmin studies: *P < 0.05; **P < 0.001. A complete statistical summary of behavioral data is provided in Supplementary Table 6.

Figure 6 Genetic reduction of Oprml improves the social behavior deficits of MECP2 duplication mice. (a) Reducing Oprml 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. Interest level was similar to wild-type and Oprml¹⁰⁻/⁻ mice for the familiar encounters, but with novel partners, the level of interest for double mutants was similar to that of Oprml¹⁰⁻/⁻ but not wild-type mice. F, familiar partners during the first encounter; N, novel partners; F2, familiar partners during a second encounter. (b) In the three-chamber test, MECP2-TG1; Oprml¹⁰⁻/⁻ double mutants showed normal social approach behavior toward a novel partner mouse compared with both wild-type and Oprml¹⁰⁻/⁻ animals; MECP2-TG1 animals showed a decrease in social approach behavior toward a novel partner mouse. All genotypes spent an equal amount of time investigating a novel object. (c,d) MECP2-TG1; Oprml¹⁰⁻/⁻ double mutant animals, compared to littermates, showed no side preference during the habituation (c) and test (d) phases. (e) All genotypes spent more time sniffing a novel odor (vanilla) versus a familiar odor (water); no differences were observed in time spent sniffing the odors. White bars and dashed lines, wild-type (N = 13); dark gray bars and lines, Oprml¹⁰⁻/⁻ (N = 12); red bars and lines, MECP2-TG1 (N = 9); yellow bars and lines, MECP2-TG1; Oprml¹⁰⁻/⁻ (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 < 0.05; ** or ++, P < 0.001.

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social behavior–related genes contributes 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 corresponding phenotype. 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 gene45, yet we find its expression upregulated in both the Mecep2308y and MECP2-TG mice. The Mecep2308 allele was originally described as a loss-of-function allele46, however, it may also have features of a hypermorphic allele, given that it lacks key serine residues (Ser421 and Ser424) that are sites of phosphorylation important in the regulation of DNA binding47. This idea is supported by a recent study showing that mice harboring Mecep2 mutations at these sites have neurophysiological, behavioral and transcriptional changes similar to those observed in MECP2-TG1 mice48. Of note, Crh levels, which are increased in the MECP2-TG and Mecep2308 mice45 but decreased in Mecep2–null mice34, correlate with heightened and reduced anxiety-like behavior, respectively49. In MECP2-TG animals, heightened anxiety-like behavior can be decreased by modulating the CRH signaling pathway.

In results similar to those of other studies50–52, we show that MeCP2 binds to the promoter of Oprm1; however, we find that Oprm1 expression is upregulated in the context of increased MeCP2 gene dosage. Furthermore, we show 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 levels in wild-type animals. Pharmacological activation of MOR is associated with increased sociability53, underscoring a difference between the effects of direct MOR activation and upregulation 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 in 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 as a result of an excess or loss of MeCP2 in mice and humans primarily result from misregulation of only a subset of genes whose expression 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.

URLs. Affymetrix MoEx 1 array annotation, http://www.affymetrix.com/support/technical/byproduct.affx?product=mogene-1_0-st-v1.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/

Accession numbers. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (GSE33457).

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

R.C.S., C.M.-B. and C.M.M. performed experiments. R.C.S. and C.M.M. analyzed the data. C.A.S. performed statistical analyses of microarray data. B.E.M. provided intellectual contribution to and initiated CRH genetic interaction studies. R.C.S., and H.Y.Z. designed experiments, reviewed the data and wrote the manuscript. All authors reviewed the manuscript in its preparation.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE 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 MECP2-TG3 behavioral testing. Test colonies included both MECP2-TG3 mice on a pure FVB/N background to either wild-type male 129S6/SvEv (Taconic Farms) or C57BL/6 (Jackson Laboratories) mice. MECP2-TG mice harbored a ~99-kb PI artificial chromosome (PAC671D9) containing only the human MECP2 genomic locus. MECP2-TG1 and MECP2-TG3 adults were designated (PAC671D9) containing only the human MECP2 genomic locus. MECP2-TG1 and MECP2-TG3 adults were designated by targeted disruption of the pre-pro-Crh coding region, were purchased from Jackson Laboratories on a pure C57BL/6 background. Both Crh and Crh1 lines were backcrossed to 129S6/SvEv mice for seven generations before being bred with MECP2-TG1 females to obtain F2 hybrid progeny for the genetic interaction experiments. For studies related to the genetic interaction of MeCP2 with either Crh or Crh1, we generated F1 hybrid mice by mating female MECP2-TG1 mice on a pure FVB/N background to either wild-type male Crh+/- or male Crh1+/- animals. Original hybrid animals, originally generated by replacing exons 5–8 of the Crh1 locus with a phosphoglycerine kinase (PGK)-neo cassette, were also purchased from Jackson Laboratories, which were maintained on a pure C57BL/6 background. Original hybrid animals, originally generated by a point of the genetic interaction treatment after social defeat stress, whereas MECP2-TG1 F1 hybrid male progeny from this mating scheme were used in testing the effect of acute antalarmin treatment following social defeat stress, whereas MECP2-TG1 F1 hybrid male progeny from this mating scheme were used in testing the effect of acute antalarmin treatment following social defeat stress. In most cases, animals were housed with four animals per cage with an equal balance of genotypes per cage. Animals used for the pharmacological studies were housed with four to five 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 and MECP2-TG2 mice and their wild-type littermates. The first MECP2-TG1 test colony was used to measure activity in an open field test (8–9 weeks of age), to test anxiety-like behavior in the light/dark box (9–10 weeks) and to test social interaction in the partition test (10–12 weeks). The second MECP2-TG1 test colony was used to test anxiety-like behavior in the elevated plus maze (8–9 weeks of age). One F1 hybrid FVB/N × C57BL/6 MECP2-TG2 test colony was generated to confirm the anxiety-like behavior and social behavior deficits; anxiety and social behavior tests were performed at equivalent time points 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 Crh1 haploinsufficiency in MECP2-TG1 animals in an open field, elevated plus maze, light/dark box and partition test (~4–5 months of age). Test colonies included all possible animal genotypes; the parental genotypes (MECP2-TG1 and either Crh+/+ or Crh1+/+), wild-type littermates and double mutant (MECP2-TG1; Crh1+/- or MECP2-TG1; Crh+/+) animals.

One test colony was generated to test the effect of Oprml 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 age). Test colonies included all possible animal genotypes: the parental genotypes (MECP2-TG1 and Oprml+/-), wild-type littermates and double mutant (MECP2-TG1; Oprml+/-) animals.

Behavioral tests and statistical analysis of behavioral data. A detailed description of these methods is presented in the Supplementary Note.

Microarray experiments and statistical analysis of microarray data. Total RNA was extracted from the amygdala of MeCP2+/- animals and their wild-type littermates (6 weeks of age; N = 4 per genotype) and from the amygdala of male MECP2-TG3 and their wild-type littermates (6 weeks of age; N = 5 per genotype) using TRIzol (Invitrogen). Microarray experiments were performed as previously described using the Affymetrix Mouse Exon 1.0 ST microarray. 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 na27 build of annotation for the Moe1 array (see URLs). Exon coordinate annotation for each gene was then obtained from the UCSC genome browser using the Mus musculus 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. This model considered RNA 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 wild-type and null mice using only the appropriate matched wild-type control; another contrast for the difference between the MECP2-TG mice and their wild-type controls; and a final contrast considered the sum of these two differences. The two-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 of <0.05 were selected as differentially expressed, as well as genes where the total difference exceeded 0.4 with a Q value of <0.05. Content analyses for Gene Ontology were performed as previously described.

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 Supplemental Note.

Quantitative reverse transcription PCR (qRT-PCR). For validation of expression profiling data, total RNA was extracted from the amygdala of MECP2-TG3 animals and their wild-type littermates ( N = 4 of each genotype) using TRIzol. To test the effect of reducing either Crh or Oprml levels in MECP2-TG1 mice and to check Avp expression in MECP2-TG1 × Crh1+/- animals, total RNA was extracted from the hypothalami and amygdala of the double mutant animals and their respective littermates ( N = 3–6 of each genotype). RNA (3 µg) was used to synthesize cDNA according to the manufacturer’s protocol (Invitrogen). qRT-PCR was performed as previously described using PerfeCta qPCR FastMix (Quanta Biosciences). Primers were designed to amplify a single gene product using an online primer design tool.
expression levels were normalized to 16S ribosomal RNA, 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.** The Crh ISH probe was PCR amplified from wild-type mouse brain cDNA and underwent digoxigenin labeling as previously described. ISH was performed on brain tissue obtained from wild-type, Crh+/−, MECP2-TG1 and MECP2-TG1; Crh+/− animals (~4–5 months of age), and ISH signal intensity was quantified as previously described.

**Corticosterone studies.** Serum corticosterone levels (N = 3–6 animals of each genotype for each measurement) were determined as previously described. Briefly, basal corticosterone levels were obtained from animals that were undisturbed for at least 12 h. Stress-induced corticosterone levels were obtained from animals restrained in 50-ml conical tubes for 30 min. 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 min. Blood was centrifuged at maximum speed for 10 min. Serum was collected and analyzed using an enzyme-linked immunosassay (IDS).

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