Sex-specific gene–environment interactions underlying ASD-like behaviors

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The male bias in the incidence of autism spectrum disorders (ASDs) is one of the most notable characteristics of this group of neurodevelopmental disorders. The etiology of this sex bias is far from known, but pivotal for understanding the etiology of ASDs in general. Here we investigate whether a "three-hit" (genetic load × environmental factor × sex) theory of autism may help explain the male predominance. We found that LPS-induced maternal immune activation caused male-specific deficits in certain social responses in the contactin-associated protein-like 2 (*Cntnap2*) mouse model for ASD. The three "hits" had cumulative effects on ultrasonic vocalizations at postnatal day 3. Hits synergistically affected social recognition in adulthood: only mice exposed to all three hits showed deficits in this aspect of social behavior. In brains of the same mice we found a significant three-way interaction on corticotropin-releasing hormone receptor-1 (*Crhr1*) gene expression, in the left hippocampus specifically, which co-occurred with epigenetic alterations in histone H3 N-terminal lysine 4 trimethylation (H3K4me3) over the *Crhr1* promoter. Although it is highly likely that multiple (synergistic) interactions may be at work, change in the expression of genes in the hypothalamic–pituitary–adrenal/stress system (e.g., *Crhr1*) is one of them. The data provide proof-of-principle that genetic and environmental factors interact to cause sex-specific effects that may help explain the male bias in ASD incidence.

maternal immune activation | prenatal stress | sex differences | *Cntnap2* | autism

Autism spectrum disorders (ASDs) comprise a heterogeneous group of neurodevelopmental disorders. The core symptoms of ASD are deficits in social communication and social interactions (*Diagnostic and Statistical Manual of Mental Disorders V*) and one of the most noticeable biological constants in this group of disorders is the sex difference: ∼80% of the children diagnosed with an ASD are boys (1). Many genes have been implicated in the etiologies underlying ASD in humans and ASD-like behavior in animal models. Some of these genes are located on the sex chromosomes and many of these genes may be able to affect other sex-chromosomal genes or may otherwise indirectly lead to sex-specific effects (reviewed in ref. 2). However, it seems unlikely that these genes can account for the full sex bias in incidence and it is becoming increasingly clear that environmental factors play a very important role as well (3), and that these factors can interact with one another (2, 4). Moreover, prenatal testosterone (an indirect genetic factor) affects human behavior (5, 6) and may increase the risk to develop an ASD (7).

Although today it is commonly proposed that many neurodevelopmental disorders result from interactions between "nature" and "nurture," studies investigating the gene–environment interaction in the development of ASD are scarce.

In this study we tested whether an interaction between an ASD-related genetic mutation and an environmental factor may be able to explain some of the sex-specific phenotypes of ASD in a mouse model. We used the contactin-associated protein-like 2 (*Cntnap2*) knockout mouse model for ASD. A homozygous mutation of CNTNAP2 in humans leads to an ASD in approximately two-thirds of the cases (8), and CNTNAP2 is reported to be androgen-sensitive, at least in breast and prostate cancer tissue (9, 10). One of the most well-known environmental factors that may play a role in the etiology of ASD is early stress through maternal immune activation (MIA) (11, 12), especially early during pregnancy (13). Animal models support the notion that MIA plays a pivotal role in ASD's etiology because experimentally induced MIA leads to deficits that are the key features in ASD: social communication and interactions (14, 15).

Thus, we tested whether MIA may be able to account for some of the sex-specific phenotypes of ASD in a genetic ASD mouse model. To study this "three hit" hypothesis of autism (16), we used the *Cntnap2* mouse model (17) in which we induced MIA in half of the subjects and tested *Cntnap2*$^{-/-}$ and *Cntnap2*$^{+/+}$ male and female littermates on social behavior [ultrasonic vocalizations (USVs) during a maternal separation paradigm and social recognition in adulthood], one of the hallmarks of ASD. Additionally, we investigated motor activity and anxiety-like behavior, as hyperactivity and anxiety are common comorbidities in individuals diagnosed with an ASD (18, 19).

In the same animals, we measured mRNAs related to the stress response-regulating neuropeptide corticotropin-releasing hormone (20, 21) (CRH). At least in primates, stress-induced maternal hypothalamic–pituitary–adrenal (HPA)-axis activation increases CRH levels in the placenta, which leads to increased circulating CRH in the fetus, which can affect the developing

Significance

Autism spectrum disorders (ASDs) comprise a heterogeneous set of neurodevelopmental disorders. Although hundreds of genes have now been identified to be associated with ASD, genetic factors cannot fully explain ASD's incidence. The early environment is now known to be pivotal in ASD's etiology too. In the face of this complexity, one aspect of ASD has stood out constantly as a causative biological factor: the sex difference. Approximately 80% of the children diagnosed are boys. This current set of experiments tests, in an animal model, the "three-hit theory of autism," which states that interactions among (*i*) being male, (*ii*) suffering early (especially, prenatal/immunological) stress, and (*iii*) having certain genetic mutations will predispose to an ASD diagnosis.

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hippocampus, likely by activating CRH receptors (22). In rodents, placental CRH mRNA has also been reported (23, 24). It has been shown that neurogenesis, neuron functioning, and cell survival in the hippocampus are affected by MIA (25–27). There are two subtypes of CRH receptors, CRH receptor 1 and 2 (CRHR1 and CRHR2), the first one being the predominant subtype in the mouse hippocampus. Furthermore, increased expression of *Crhr1* (but not *Crhr2*) in response to peripubertal stress in rats was shown to be associated with social deficits, and these effects were prevented by treatment with a CRHR1 antagonist (28). Interestingly, in light of ASD's strong male bias in incidence, prenatal stress affects the expression of *Crhr1* in the paraventricular nucleus in a sex-specific way; only males show *Crhr1* up-regulation (29). Prolonged activation of CRHR1 in the hippocampus as a result of early stress affects the structure, synaptic function, and cognition (30, 31), but we are not aware of any studies that have investigated the sex-specific effect of prenatal (immunological) stress on CRH or its receptors in the hippocampus. Because ASD is associated with changes in lateralization [behavioral study (32), MRI studies (33, 34), magnetoencephalography study (35), and references in these studies], and many genes are asymmetrically expressed in the rodent hippocampus (36), we performed *Crh*, *Crhr1*, *Crhr2*, and *Crhbp* (corticotropin-releasing factor-binding protein) mRNA gene-expression assays on the two hemispheres separately. Because we found the strongest effects on *Crhr1* mRNA expression in the left hippocampus, we then continued to investigate histone modifications on the promoter area of the *Crhr1* gene in the left hippocampus. The promoter area chosen is thought to bind SRY (re: sex) and NF-κB (re: stress). Because tissue for gene-expression assays originated from the mice used in the behavioral tests, we could investigate the correlates between the behavioral and molecular measures.

Results

Because the three-hit hypothesis revolves around the synergistic effects of several factors, whereby factors that have low or no effect independently can have significant effects when combined, we present the data in a categorical fashion (four categories: zero hits to three hits). The results of the statistical models can be found in Dataset S1. Because the individual hits might give us additional information, we also present the data on the individual hits and their interactions; their statistics and figures are presented in *SI Methods*.

Ultrasonic Vocalizations. We investigated social communication by means of the number of ultrasonic vocalizations during a 5-min maternal-separation paradigm. Both male and female pups typically emit these calls, which elicit maternal retrieval of the pups. We found a significant effect of number of hits the animals were exposed to on the number of vocalizations on postnatal day (PD) 3, the day featuring the highest amount of calling (Fig. 1). The zero-hit animals (no-MIA female WTs) vocalized significantly more than the three-hit mice (MIA male KOs). In the monotonic increase, animals exposed to one-hit (MIA female WTs or no-MIA male WTs or no-MIA female KOs) vocalized significantly more than animals exposed to two or three hits. Finally, animals exposed to two hits vocalized significantly more than animals exposed to three hits.

Of the different hits, MIA had the largest effect and genotype, although still significant, had the lowest effect on number of vocalizations (Fig. S1).

Social Recognition. We tested the effects of the three hits on social recognition, which can be defined by reduced time spent investigating a familiar conspecific as a result of social habituation, and subsequent reinstatement of investigation when a novel intruder is introduced (dishabituation). When considering all five

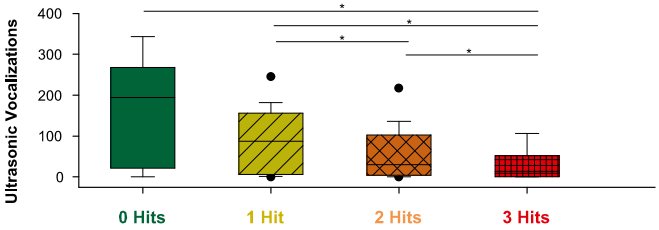

Fig. 1. Box-plot of number of USV emitted by pups at PD3. Number of USV significantly differed between the categories ($\chi^2 = 21.585$, df = 3, $P < 0.001$). Zero-hit mice vocalized more than the three-hit mice ($P = 0.009$). One-hit mice vocalized more than both the two-hit ($P = 0.038$) and the three-hit mice ($P < 0.001$). Two-hit mice vocalized more than three-hit mice ($P = 0.017$). Outliers are depicted as dots. Boxes represent 25th and 75th percentiles, whiskers are 5th and 95th percentiles. Horizontal line is the median. *$P < 0.05$.

trials, we found a significant effect of the number of hits on social recognition (Fig. 2 and Dataset S1). Because habituation may simply reflect loss of the interest in the testing environment, the dishabituation phase (difference in social interest between the fourth and the fifth trial) may be a more important measure for true social recognition. We found significant differences between the categories because the three-hit mice show deficits in both habituation (tests 1–4) and dishabituation (tests 4–5). Strikingly, the three-hit mice were the only experimental group that showed no changes in social behaviors over any of the trials (Fig. 2). The two-hit mice tended to show somewhat increased social recognition: they showed a nonsignificant tendency to show increased habituation compared with the zero-hit mice and a nonsignificant increased dishabituation compared with the zero-hit mice. The two-hit mice were the only category of mice that performed significantly better than the three-hit category ($P = 0.045$).

Looking at the individual hits, we found that genotype had an overall negative effect on social recognition, caused by deficits in dishabituation. Additionally, a significant sex × MIA interaction effect was present, as MIA caused deficits in social interaction in males only. This interaction effect was only present in the KOs, not in the WTs, and was again mainly caused by differences in dishabituation (Fig. S2).

Open Field. Because ASD symptoms often co-occur with hyperactivity and anxiety, and to investigate whether increased activity or anxiety could account for the differences observed in the social-recognition test, we measured hyperactivity and anxiety in an open-field apparatus. We found that there are significant differences between groups in the distance traveled during the 10 min of free exploration in the open-field arena (Fig. 3*A*). The three-hit mice covered greater distance than the zero-hit and the one-hit mice. The two-hit mice covered more distance than the one-hit mice. No significant differences were found in time spent in the middle of the apparatus, a measure for anxiety (Fig. 3*B*). Looking at the individual hits, we found that only genotype resulted in significant differences, with WT mice covering less distance and spending less time in the middle of the apparatus than KO mice (Fig. S3).

Gene Expression. Next, in the same animals, we investigated the *Crh* system in the hippocampus. In the left hippocampus we found a trend for the effect of the number of hits on *Crh* expression (Fig. 4). The two-hit category expressed significantly more *Crh* mRNA than the zero-hit category. To investigate whether *Crh* expression was related to social recognition, we checked for a correlation between the total time spent sniffing the stimulus during the habituation phase (trials 1–4) and *Crh* expression. We found a positive correlation between *Crh* mRNA and habituation (Pearson's $r = 0.335$, $P = 0.040$). The correlation

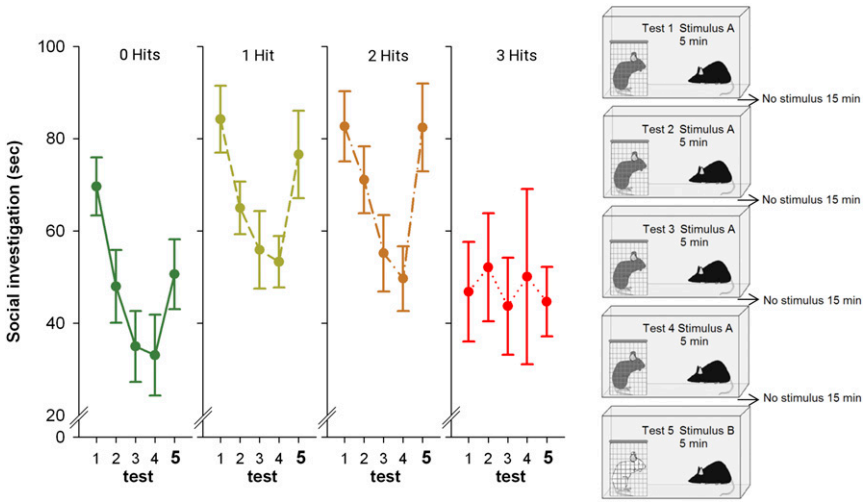

Fig. 2. Social recognition. Seconds socially investigating a conspecific [same conspecific in tests 1–4; novel conspecific in test 5 (marked bold to emphasize that a new stimulus mouse was introduced)]. Social recognition significantly differed between the three-hit categories [$F(3, 58) = 2.820$, $P = 0.047$]. Post hoc planned comparisons showed that habituation to the same stimulus conspecific (tests 1–4) was significant in the zero-hit [$F(3, 24) = 9.611$, $P < 0.001$], one-hit [$F(3, 75) = 7.995$, $P < 0.001$], and two-hit [$F(3, 60) = 7.509$, $P < 0.001$], but not in the three-hit category. Dishabituation was significant in the zero-hit category [$F(1, 8) = 7.598$, $P = 0.025$], borderline significant in the one-hit category [$F(1, 25) = 3.630$, $P = 0.068$], significant in the two-hit category [$F(1, 21) = 21.504$, $P < 0.001$], and not significant in the three-hit category. Mean and SEMs are shown.

is absent during the last trial (trial 5) of the social-recognition test. No correlations between *Crh* mRNA expression in the hippocampus and the number of USVs emitted or anxiety and mobility measures were found.

We also found a trend for the effect of the number of hits on *Crhr1* expression in the left hippocampus (Fig. 4). Both the two-hit and the three-hit category expressed significantly less *Crhr1* mRNA than the zero-hit category. Looking at individual hits, we found that in the left hippocampus *Crhr1* shows a significant three-way interaction. Only in the KOs (not the WTs) there is a trend for a sex × MIA interaction effect: MIA tends to affect males, but does not affect females (Fig. S4). Additionally, we found a positive and significant correlation between *Crhr1* expression and social behavior over the five trials (Pearson's $r = 0.347$, $P = 0.035$). During the habituation phase (trials 1–4), this correlation with *Crhr1* mRNA is significant (Pearson's $r = 0.376$, $P = 0.022$). The correlation is absent during the last trial (trial 5) of the social-recognition test. No correlation between *Crhr1* mRNA expression in the hippocampus and the number of USVs emitted or anxiety and mobility measures were found.

No effects were found on *Crhbp* or *Crhr2* expression, nor did we find effects in the right hippocampus.

Histone N-Terminus Modifications. Histone modifications can be induced by environmental factors, such as stress (37). For potential explanations of the mRNA data, we studied alterations of lysine modifications on the histone H3 N terminus, as well as global acetylation levels of H3 over the *Crhr1* promoter. We chose a primer pair that amplifies a genomic region −560 to −461 bp relative to *Crhr1*'s transcriptional start site, which contains the promoter area that, according to TFSEARCH prediction (www.cbrc.jp/), is very likely to include transcription factor binding sites for testis-determining SRY, as well as for the cellular stress-related signal NF-κB1, and may therefore be especially important in the underlying mechanisms of sex-specific effects of gene–environment interactions.

The number of hits significantly affected H3 trimethylation on lysine 4 (H3K4me3) at the promoter area of *Crhr1* in the left hippocampus (Fig. 5). Post hoc tests showed that the three-hit category had the lowest levels of H3K4me3. H3K4me3 is associated with transcriptionally active genes. The low levels of H3K4me3 in the three-hit category can therefore (partly) explain the low levels of *Crhr1* mRNA in the same group. H3 acetylation (H3Ac, an activation mark) or H3 trimethylation on lysine 27 (H3K27me3, a repressive mark) were not affected.

Looking at the individual hits, we found a significant three-way interaction between genotype, MIA, and sex on H3K4me3 levels in the left hippocampus. Further investigation revealed that this three-way interaction was caused by a two-way interaction between MIA and genotype in males only: only in KO males did MIA cause a decrease in H3K4me3 levels. There was no significant three-way interaction between the factors on H3Ac or H3K27me3 (Fig. S5).

Discussion

ASD, one of the most severe of the childhood psychiatric disorders, is likely to often be a result of the interplay between genetic and environmental factors. However, because of the complexity of the potentially relevant studies, this interplay is often neglected in the literature. Here, we have demonstrated that a genetic and an environmental factor show a complex interplay with each other as

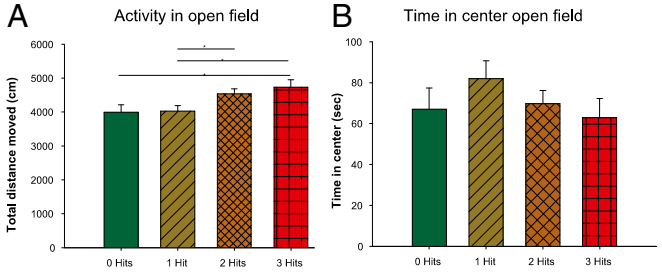

Fig. 3. Open field. (*A*) Bar graph of the total distance (centimeters) moved during a 10-min open field. Total distance moved significantly differed between the categories [$F(3, 64) = 3.262$, $P = 0.027$]. Zero-hit mice moved less than three-hit mice ($P = 0.049$), one-hit mice moved less than two-hit mice ($P = 0.020$) and three-hit mice ($P = 0.026$). (*B*) Bar graph of the time spent in the middle of the open field (seconds). No significant differences between the categories were found. Mean and SEMs are shown. *$P < 0.05$.

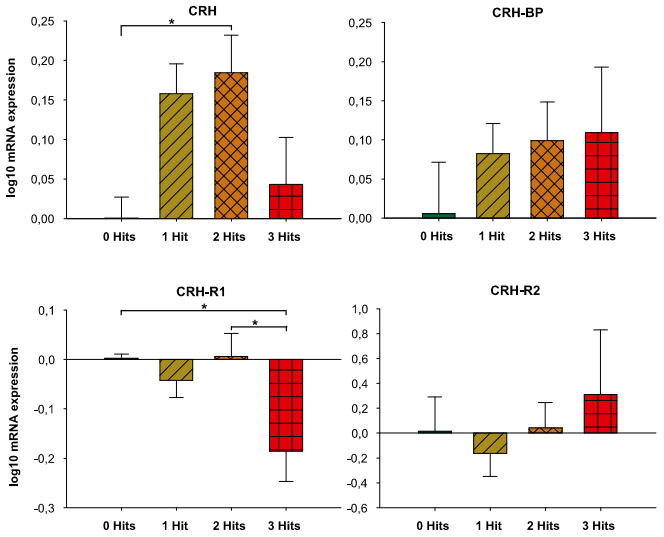

Fig. 4. Bar graphs of log-transformed mRNA expression in adult mice. There was a trend for an overall effect of number of hits on *Crh* expression [$F(3, 55) = 2.371$, $P = 0.080$] and on *Crhr1* expression [$F(3, 53) = 2.532$, $P = 0.067$]. Post hoc tests showed that the two-hit category expressed significantly more *Crh* mRNA than the zero-hit category ($P = 0.028$) and that three-hit category expressed significantly less *Crhr1* mRNA than the zero-hit group ($P = 0.043$) and the two-hit group ($P = 0.012$). No significant effects of number of hits on *Crhbp* and *Crhr2* were found. *$P < 0.05$.

well as with the sex of the organism in a subset of the autism behavioral domains.

Besides our study's relevance in the ASD field, the current study may be of interest to the general field of neurodevelopmental disorders. First, CNTNAP2 functioning is associated to a wide variety of developmental disorders, such as childhood apraxia of speech and language impairments (38), epilepsy, and schizophrenia (39). Second, our finding that MIA can have male-specific effects (social recognition and histone modifications) may also be of interest to the general field of neurodevelopmental disorders because of the growing body of evidence showing a male-specific vulnerability to develop a wide variety of neurodevelopmental disorders, such as attention deficit and hyperactivity disorder, conduct disorder, dyslexia, specific language impairment, and Tourette syndrome (reviewed in ref. 40).

Our data corroborate earlier studies that have shown that the number of USVs is reduced because of the *Cntnap2* mutation (17), following MIA (14, 41), and because of being male (42). Our study suggests that these factors do not synergistically interact with each other to affect the number of USVs at PD3; instead, we showed a cumulative effect, such that the more hits a subject was exposed to, the fewer USVs it emitted.

Similarly, studies have shown that, in agreement with the present results, social recognition is affected by the *Cntnap2* mutation (43). We did not replicate the finding that social recognition in males is higher compared with females (44). Although studies have investigated the effect of MIA on social behavior in rodents (e.g., refs. 45 and 46), we are not aware of studies that have investigated the effect of MIA on social recognition. Our study exposed a sex-specific effect of MIA; social recognition was affected in males only. The Connors et al. (45) study in rats also found a male-specific effect of MIA, but they observed an overall decrease in social interactions, which we only see in the MIA-exposed male KO mice, not in the WT mice, possibly accounted for by a species-specific effect of MIA.

Our data suggest that the three hits have some synergistic effects on social recognition. Surprisingly, mice that were exposed to two hits performed somewhat better (trend level) than the zero-hit category. Mice exposed to three hits performed significantly worse than mice exposed to two-hits. In contrast to all other groups, the three-hit mice showed no habituation to the same animal in the four trials of the test and they showed no dishabituation to a new stimulus mouse in trial 5 of the test (with the exception of only a borderline dishabituation of the two-hit mice). This result suggests that the *Cntnap2* mutation leaves males vulnerable to the effect of MIA. Whether the lack of apparent social recognition in the three-hit mice can be entirely attributed to a lack of true social recognition, or whether these mice just show a total lack of social interest, cannot be distinguished. However, it is clear that these mice do show clear deficits in social behavior in the social-recognition test.

The decreased social behavior and social recognition of the three-hit mice is not likely to be mediated by a decrease in overall locomotor activity or increased anxiety, as the open-field data showed increased locomotor activity in the two-hit and three-hit mice compared with the zero-hit mice and no differences in anxiety. The analyses on the individual hits showed that the *Cntnap2* mutation was responsible for differences between the groups in locomotor activity, in agreement with previous work (17).

Furthermore, the effects of MIA are not likely to be mediated by differences in maternal behavior, as we found no effect of MIA on the amount of nesting material (a measure for maternal behavior) used.

MIA can result in increased levels of inflammatory cytokines in the fetal environment and can activate the fetal HPA axis (47). Upon activation of the HPA axis, the paraventricular nucleus of the hypothalamus releases CRH, which leads to release of adrenocorticotropic hormone (ACTH) by the anterior pituitary, which in turn leads again to release of cortisol by the cortex of the adrenal glands. Cortisol inhibits both CRH and ACTH release, which results in a negative feedback mechanism. Prenatal HPA axis activation has long-lasting effects on brain and behavior (48). Several studies suggest that the HPA axis may be altered in ASD (see ref. 49 and references therein). The HPA axis alterations can have wide effects on behaviors and has been attributed as being one of the main correlates of social deficits in patients with fragile X syndrome (50). Prenatal LPS exposure, which activates the fetal HPA axis (47), causes structural, neurophysiological, and functional changes in the hippocampus (e.g., refs. 51–53) and the hippocampus has been repeatedly shown to be affected in ASD (refs. 54–56; because many studies do not consider lateralization, some studies may find no or smaller effects if they only consider one side of the brain, or combine the results from the left and right hemisphere). We investigated the CRH system in the hippocampus by means of gene-expression assays and found trend-level differences

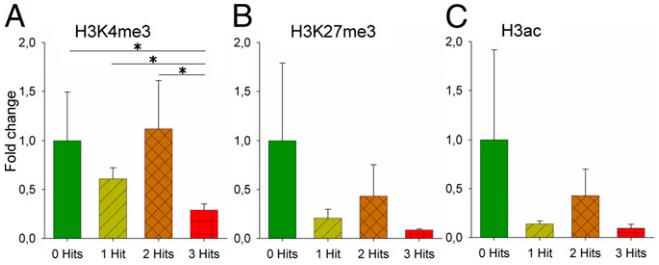

Fig. 5. Epigenetic alterations in the left hippocampus of adult animals. (*A*) H3K4me3. Number of hits significantly affected H3K4me3 ($\chi^2 = 11.122$, $df = 3$, $P = 0.011$). The three-hit group had significantly lower levels of H3K4me3 than all other groups (zero-hit $P = 0.033$; one-hit: $P = 0.007$; two-hit: $P = 0.001$) on Prom3. (*B*) H3K27me3. Number of hits did not affect H3K27me3. (*C*) Pan-acetylation on H3 (H3Ac). Number of hits did not affect H3Ac. Levels are relative to the zero-hit group. Mean and SEMs are shown. *$P < 0.05$.

between the three-hit categories, both for *Crh* and *Crhr1*. Surprisingly, post hoc tests showed that *Crh* mRNA levels were increased in the two-hit mice. Notably, the two-hit mice tended to perform better in the social-recognition task. Because we also found a trend for a positive correlation between *Crh* mRNA expression in the hippocampus and social behavior in the social-recognition data, it may be worthwhile to look further into this link in the future.

Post hoc tests further showed that *Crhr1* mRNA expression was lower in the hippocampi of mice exposed to all three hits. The analyses on the separate hits showed a significant three-way interaction, whereas the individual hits did not show a significant effect, indicating a possible synergetic effect of the MIA, the *Cntnap2* mutation, and being male on *Crhr1* mRNA expression. These changes in mRNA expression may underlie the effects seen in the social-recognition test, as we found a significant positive correlation between *Crhr1* mRNA expression and social behavior in the social-recognition task. These correlations corroborate studies that show that HPA-axis disturbances can cause deficits in social behavior later in life (reviewed in ref. 57) and that social behavior is (co)regulated by the hippocampus (58).

Subsequently, we showed that the down-regulated *Crhr1* gene expression in the hippocampus of the three-hit category may possibly be mediated by a decrease in the protranscriptional H3K4 trimethylation of the promoter area of the *Crhr1* gene. However, we do note that the down-regulation of *Crh* and *Crhr1* gene expression was only changed at a trend level. The possible changes in *Crh* and *Crhr1* are therefore not likely to be the only factors underlying the changes observed in social-recognition behavior. Furthermore, they do not seem to underlie the changes observed in USVs during the maternal separation paradigm and in hyperactivity.

Limitations. Our data highlight one line of evidence showing that an environmental and genetic factor can interact to cause sex-specific effects; a line of evidence that leads from *Crhr1* promoter function to *Crhr1* mRNA levels, arguably leading to behavioral changes in this mouse model of ASD. However, the exact mechanism underlying the effect of the *Cntnap2* mutation (in concert with MIA) on *Crhr1* functioning remains elusive. The sample sizes in this study—especially concerning the histone modification (because of the fact that samples from individuals had to be pooled to have adequate material)—are low and thus limit interpretation until a new, larger study is completed.

The MIA and the *Cntnap2* data held up despite potential subtleties. That is, to just give one of many examples, even within a species the effect of MIA can be widespread as different mouse strains react differently to MIA (59). Furthermore, the extent of the contribution of the interaction with the *Cntnap2* gene may be limited, as heterozygous single-nucleotide variants in the CNTNAP2 gene do not seem to be associated with ASD to a high level (60).

We realize that this line of research is just one of many possible pathways of how the environment can interact with the genetic background of an individual. Our data show a proof-of-principle that such a pathway can cause sex-specific effects, but it is more than likely that many of such synergistic interactions will be at work.

Methods

Subjects. *Cntnap2* heterozygous females on a C57BL/6J background were obtained from the Peles laboratory (Weizmann Institute of Science, Rehovot, Israel) via the Abrahams laboratory (Albert Einstein College of Medicine, New York). *Cntnap2* KO and WT mice were obtained from heterozygous crossings. The discovery of a vaginal plug was marked as gestational day 1 (GD1). At GD7, the pregnant females were randomly assigned to a MIA and a control group. The MIA group received 0.30 mg/kg LPS in saline by subcutaneous injections, whereas the control females received saline only. The date of birth was set as PD0. At PD21 the pups were weaned and housed in same sex groups (maximum five per cage). To avoid litter effects, we never used more than two mice per experimental group from each litter. At PD50 ± 1, the mice were killed and hippocampi isolated for gene expression or ChIP assays.

All experimental protocols were conducted according to US National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at The Rockefeller University (protocol #11456).

Behavioral Tests. At PD3, pups were removed from the dam and USVs were recorded for each individual separately for 5 min using the Avisoft-UltraSoundGate 116 Hb with high-quality condenser microphone CM16/CMPA. At PD40 ± 1, locomotor activity in a novel environment was tested using an open-field set-up. The measures obtained were time spent in the middle of the apparatus (14 cm × 14 cm middle area) and total distance traveled. At PD45 ± 2 social recognition was tested. Each focal mouse was tested five times (tests 1–5) in their home cage in which a container with an age- and sex-matched stimulus mouse was introduced. Each test lasted 5 min and the tests were repeated with a 15-min interval. In the first four tests the same stimulus mouse was used, whereas for the fifth test the stimulus mouse was replaced with another unfamiliar sex- and age-matched conspecific. During the tests the mice were left undisturbed and their behavior was videotaped and subsequently scored using the software program JWatcher (www.JWatcher.UCLA.edu).

Gene-Expression Assays. Total RNA was isolated from whole left hippocampus using TriZol reagent (Life Technologies) in accordance with the manufacturer's instructions. Equal amount (100 ng) of total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies) and the resultant cDNA was used as template for quantitative PCR (qPCR). TaqMan Gene Expression Assays (Life Technologies) were used to amplify mRNA for the *Crhr1* gene, ID: Mm00432670_m1. Eukaryotic 18S rRNA (4352930E) was used as internal control. The relative ratio of the expression of the gene was calculated using the $2^{-\Delta\Delta Ct}$ method (59).

ChIP and qPCR. ChIP assays were performed using EZ-Magna ChIP kit (Millipore) in three batches following the manufacturer's instructions. To have enough material, the hippocampi of two mice were combined. qPCR was used to measure the amount of immunoprecipitated DNA on the promoter area of the mouse *Crhr1* gene (accession no. NM_007762). Primers were designed using PrimerQuest (Integrated DNA Technologies).

General Statistical Methods. Data organized to investigate the three-hit hypothesis (four categories: zero hits to three hits) are analyzed either with an ANOVA (on raw or on transformed data) or with a generalized linear model depending on the distribution of the data and the best fit to the model (Dataset S1). Data organized to investigate the individual hits (genotype, MIA, and sex) were analyzed using the same models as used for the three-hit hypothesis. For all measures (Dataset S1) we performed at least three tests: (*i*) one analysis to investigate the main effects, (*ii*) one analysis to investigate the two-way interactions (all main effects were included in the model), and (*iii*) one analysis to investigate the three-way interaction (all lower level interactions and the main effect were included in the model). All analyses were performed in SPSS 17.0 (SPSS Inc.).

Additional details are available in *SI Methods*.

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