Complex Interactions between Genes and Social Environment Cause Phenotypes Associated with Autism Spectrum Disorders in Mice

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Abstract

The etiology of autism spectrum disorders (ASDs) is a complex combination of genetic and environmental factors. Neuroligin3, a synaptic adhesion protein, and cytoplasmic FMR1 interacting protein 1 (CYFIP1), a regulator of protein translation and actin polymerization, are two proteins associated with ASDs that interact in neurons in vivo. Here, we investigated the role of the Neuroligin3/CYFIP1 pathway in behavioral functioning and synapse formation in mice and found that it contributes to motor learning and synapse formation in males. Similar investigation in female mice revealed an absence of such phenotypes, suggesting that females are protected against mutations affecting this pathway. Previously, we showed that the social environment influences the behavior of male mice. We extended this finding and found that the transcriptome of wild-type mice housed with their mutant littermates, lacking Neuroligin3, differed from the transcriptome of wild-type mice housed together. Altogether, these results identify the role of the Neuroligin3/CYFIP1 pathway in male mouse behavior and highlight its sensitivity to social environment.

Key words: autism spectrum disorders; motor learning; mouse behavior; social environment

Significance Statement

The causes of autism spectrum disorders (ASDs) remain elusive. In this study, we investigate the combined effect of mutations in two genes associated with ASDs, Nlgn3 and Cyfip1, and the effect of the social environment on phenotypes relevant for ASDs. We show that when both mutations are present the behavior can be restored, emphasizing the importance of considering gene interactions. We also show sex differences in behavior, suggesting that female subjects should be included in the studies of ASDs. We show that wild-type animals can exhibit phenotypes associated with ASDs as a result of being housed with their mutant littermates, highlighting the necessity to re-evaluate the use of wild-type animals as controls to define phenotypic traits of mouse models.

Introduction

Autism spectrum disorders (ASDs) are characterized by behavioral manifestations, primarily social communication deficits and stereotyped behavior, frequently accompanied by a wide range of secondary comorbid clinical symptoms (American Psychiatric Association, 2012). The etiology of ASDs is far from being fully understood but is likely to be a complex interaction between specific genetic mutations and environmental factors triggering the emergence of a constellation of behavioral symptoms. Understanding the relationship between the different causes of the disorder and the behavioral manifestation is key for the development of targeted treatments.

Among the genes associated with ASDs is the X-linked gene NLGN3 (Jamain et al., 2003; Talebizadeh et al., 2006; Giessner et al., 2009; Yasuda et al., 2011; Sanders...
The complex genotype–phenotype relationships are further shaped by factors such as sex of the subjects and their social environment. For example, only females with the complete deletion of Nlgn3 showed deficits in behavior (Kalbassi et al., 2017). The social environment might also be an environmental factor triggering ASD-related phenotypes. Male mice lacking Nlgn3 and wild-type (WT) mice influence each other’s behavior, an effect attributed to the role of Nlgn3 in controlling social dominance. Housing mice carrying the 16p11.2 microdeletion, another model of ASD, with littermates of a different genotype was also shown to influence the vocalization during courtship (Yang et al., 2015). In addition to investigating the role of interaction between Nlgn3 and Cyfip1 in controlling behavioral phenotypes, the secondary aims of this study were to determine the role of sex in the phenotypic outcome of mutations in Nlgn3 and Cyfip1 and the effect of social environment on mRNA expression in these mice.

Materials and Methods

Animals

All procedures were conducted in accordance with the Animals (Scientific Procedure) Act 1986 (amended in 2012). Mice were kept on a 12 h light/dark cycle with free access to food and water, in groups of two to five in a cage. All behaviors were assessed during the light phase of the light/dark cycle. All mice were tested as adults, P60–P70 at the start of testing. Mice were handled for at least 2 days before any procedure. The mice were habituated to the room where the behavioral assessment was taking place for 30 min before commencing any procedure.

Nlgn3+/−, Nlgn3+/−/Cyfip1+/− (Tanaka et al., 2010) and Cyfip1+/− (EUCOMM) mice were crossed with mice containing Thy1-EGFP transgene (stock # 007788, The Jackson Laboratory; Feng et al., 2000) to obtain the following male mice: Nlgn3+/−/Thy1-EGFP, Cyfip1+/−/Thy1-EGFP, Nlgn3+/−/Cyfip1+/−/Thy1-EGFP, and Thy1-EGFP; and the following female mice: Nlgn3+/−/Thy1-EGFP, Cyfip1+/−/Thy1-EGFP, Nlgn3+/−/Cyfip1+/−/Thy1-EGFP, and Thy1-EGFP. Thus, all of the mice used in the behavioral experiments and used to investigate the dendritic spine density were littermates. A proportion of the mice used in the behavioral experiments had the Thy-EGFP transgene. The lack of an effect of the transgene on the behavioral outcomes was confirmed by repeating every statistical analysis with the presence of the transgene as a variable. There were no significant differences in any of the behavioral outcomes between the mice with and without the transgene.

In the first RNA experiment, an additional cohort of wild-type animals was bred and included in the analysis. In the second RNA experiment, a cohort of wild-type animals was bred and included in the analysis. This research was supported by the Sêr Cymru program of the Welsh Government; and Cardiff University Grant 50110000866 and Welcome Trust Grant 100010269 to M.S. and S.J.B. Acknowledgments: We thank Angela Marchbank at the Cardiff University Genome Hub for performing the RNA sequencing and Daniel Pass for help with the analysis.

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20 cm) and were allowed to explore. The test was conducted in the dark; however, the bottom of the arena was illuminated by an infrared lamp to allow tracking of the mice. The movement of the mice was recorded using a video camera above the arena. The traces were recorded and quantified in EthoVision XT (Noldus).

Rotarod

Motor learning of the mice was assessed using a rotarod (Jones and Roberts, 1968). Latency to fall off the rod was assessed for 3 days in a row, with 10 subsequent 5 min trials each day. During a trial, mice were placed on the rod. The rotarod was then switched on and accelerated from 4 to 40 rpm over the course of 5 min. The mice were allowed to walk on the rod until they fell off, gripped to the rod and the rod made a full revolution, or 5 min have passed. Falling off or gripping the rod was interpreted as an inability to cope with the task and signaled the end of the trial. Latency to fall was measured using a stopwatch (Casio). After each trial, the mice were allowed to rest for 5 min at the bottom of the apparatus.

Social odor interest

Social odors originated from a cage of three to four WT mice that were maintained with the same home cage bedding for a week to allow for the concentration of odorants present in the urine. For some of the trials, the cage also contained a maximum of one Nlgn3y/Cyfip1+/− or Nlgn3y/Cyfip1−/− mouse. Before the trial, a cotton bud was wiped across the bottom of the home cage in a zig-zag fashion to obtain the social odor cue. Mice were placed in the experimental arena and were allowed to habituate for 2 min. Mice were exposed to a clean cotton bud for 2 min, which was then swapped for a new clean cotton bud, and mice were allowed to interact with it for another 2 min. Next, the mice were exposed to the cotton bud with the odor cue for 2 min, which was finally swapped for a new cotton bud with an olfactory cue for another 2 min. Male mice were exposed to olfactory cues originating from a cage of male mice, while female mice were exposed to olfactory cues originating from a cage of female mice. The trials were recorded with a video camera placed above the experimental arena. Time spent sniffing the cotton bud was scored manually, blinded to the genotype.

Courtship vocalization

Female mice in estrus were identified using vaginal lavage, followed by cytological staining (Giemsa solution, Polyscience) and visual assessment. Male mice were habituated to the experimental arena (40 × 20 cm) for 3 min. Next, an unfamiliar female mouse in estrus was added to the arena, and the mice were allowed to interact freely for 3 min. Ultrasonic vocalizations (USVs) between 40 and 250 Hz produced by the male mice were recorded using a preamplifier (UltraSoundGate 416 H, Avisoft Bioacoustics) connected to a microphone (UltraSoundGate CM16, Avisoft Bioacoustics). The total number of USVs and their duration was analyzed using SASLabPro (Avisoft Bioacoustics).

Histology

Dendritic spine quantification was conducted in the motor and visual cortices of Nlgn3+/−-Cyfip1−/−, Nlgn3+/−-Cyfip1+/− males and their WT littermates, as well as of Nlgn3+/−-Cyfip1−/−, Nlgn3+/−, and Cyfip1−/− females and their WT littermates, all of which also expressed EGFP under the Thy-1 promoter. Mice were anesthetized with Euthatal and perfused with 4% paraformaldehyde in 0.1 M PBS. The entire brain was dissected out and postfixed overnight in the 4% paraformaldehyde in 0.1 M PBS, kept in 30% sucrose solution until saturated and stored at −80°C. The brains were cut coronally into 50 µm sections on a cryostat (Leica Biosystems) and immediately mounted on glass slides. The regions of interest were identified using a mouse brain atlas (Paxinos and Franklin, 2004), and Z-stack images spaced 0.5 mm apart were acquired on a Zeiss LSM700 upright confocal microscope (Carl Zeiss), using a 40 water-immersion lens. The images were reconstructed into two dimensions using Z-stack maximum intensity projection in ImageJ (NIH). The images were analyzed by an experimenter blinded to the genotype of the animals. Spines were identified manually and counted on a 20- to 250-µm-long stretch of a dendrite, with a minimum of 24 dendrites from four mice, per condition. Spine density was calculated as the number of spines per 10 µm of a dendrite.

RNA sequencing

All procedures were conducted in RNAase-free conditions. Mice were culled by cervical dislocation, their brains were extracted, and relevant brain regions were dissected following a mouse brain atlas (Paxinos and Franklin, 2004). The samples were immediately snap frozen in liquid nitrogen and stored at −80°C. Invitrogen TRIzol Reagent (Thermo Fisher Scientific) was added to the samples, at 1 ml per 50–100 mg of tissue. Tissue was homogenized and incubated at room temperature for 5 min. Samples were transferred to Invitrogen Phrasemaker Tubes (Thermo Fisher Scientific), and 0.2 ml of chloroform was added. The samples were shaken for 15 s, incubated at room temperature for 15 min, and centrifuged for 5 min at 14,000 × g, at 4°C. The RNA-containing upper phase was mixed with 0.5 ml of isopropanol, and the samples were incubated for 1 h at −80°C. Following the incubation, the samples were centrifuged for 10 min at 10,000 × g. The supernatant was removed, and the pellet was washed using 75% EtOH. The pellet was dissolved in RNA-free water and treated with DNase (QIAGEN) as per manufacturer instructions.

For reverse transcription, 1250 ng of RNA was incubated with 1 μl of random primers (Promega) and 1 μl of deoxyribonucleotide triphosphates (dNTPs; 10 mm, Promega) for 5 min, at 65°C and for 1 min on ice. Then 4 μl of buffer (Thermo Fisher Scientific), 1 μl dithiothreitol (0.1 M, Thermo Fisher Scientific), 1 μl Rnasin plus (Promega), and 1 μl of superscript III reverse transcriptase (Thermo Fisher Scientific) were added, and the mix was incubated for 5 min at room temperature and then for 2 h at 50°C. The samples were then incubated for 10 min at 70°C.

Quality control of the RNA samples was confirmed by Tape Station and Qubit. The library was prepared...
according to manufacturer instruction (Illumina TruSeq). Total RNA was purified to remove ribosomal and non-mRNA with magnetic beads. mRNA was then transferred for first-strand cDNA synthesis with superscript II. Next, the second strand of the cDNA was synthesized, and the template was eradicated. Adapters were ligated to the cDNA. The cDNA was then amplified to enrich the libraries template was eradicated. Adapters were ligated to the second strand of the cDNA was synthesized, and the

Statistical analysis

The data analysis was conducted using R software, version 3.6.2. (R Core Team, 2019) or when an appropriate package was not available using Graph Pad Prism version 8.3.1. The normality of raw data or residuals was checked using the Shapiro–Wilk test as well as visual inspection of Q-Q plots and histograms. The homogeneity of variance was checked using Levene’s test or visual inspection of a plot of residuals versus plotted values, depending on the data type. If the data were deemed to violate the assumption of normality or homogeneity of variance, an appropriate nonparametric test was used. The nonparametric mixed ANOVA was conducted according to Noguchi et al. (2012). The details of normality assessment, the tests used, and the number of samples per group can be found in the statistics table in Extended Data Figure 1-2.

Results

**Nlgn3**+/− Cyfip1−/− mice phenocopy the enhanced exploratory behavior of Nlgn3−/− males

We generated Nlgn3−/− Cyfip1−/− double-mutant mice to investigate the effect of the Nlgn3/Cyfip1 pathway on...
phenotypes associated with autism: changes in behavior and dendritic spine density. In the first instance, we investigated the behavior of the mutant mice.

As previously reported $\text{Nlgn3}^{+/+}$ males ($n = 24$; day 2: mean = 6307.63 cm, SE = 256.25 cm) were hyperactive in the open field compared with their WT littermates ($n = 18$; day 2: mean = 4962.81 cm, SE = 258.24 cm; one-way ANOVA, main effect of genotype: $F_{(3,62)} = 6.55$, $P < 0.001$; Tukey’s HSD: $t_{(1,62)} = 3.47$, $p < 0.01$; Fig. 1A). Similarly, $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ mice ($n = 12$; day 2: mean = 6597.82 cm, SE = 420.93 cm) traveled farther in the open field than their WT littermates (Tukey’s HSD: $t_{(1,62)} = 3.53$, $p < 0.01$). The $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ mice were also hyperactive in relation to their $\text{Cyfip1}^{+/+}$ littermates ($n = 12$; day 2: mean = 5227.60 cm, SE = 345.70 cm; $t_{(1,62)} = 2.7$, $p < 0.05$). However, there was no evidence of a more pronounced phenotype in the $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ mice as they did not significantly differ from the $\text{Nlgn3}^{+/+}$ single-mutant mice. The same pattern of results emerged when the effect of genotype on the distance traveled was considered across the two days of testing (mixed-model ANOVA, main effect of genotype: $F_{(3,62)} = 6.45$, $p < 0.001$; main effect of day: $F_{(1,62)} = 35.54$, $p < 0.001$; Tukey’s HSD: WT vs $\text{Nlgn3}^{+/+}$: $t_{(1,62)} = -2.70$, $p < 0.05$, WT vs $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$: $t_{(1,62)} = -3.53$, $p < 0.01$, $\text{Cyfip1}^{+/+}$ vs $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$: $t_{(1,62)} = -2.82$, $p < 0.05$, $\text{Cyfip1}^{+/+}$ vs $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$: $t_{(1,62)} = -3.61$, $p < 0.01$). The hyperactivity in these mice may arise from a more pronounced response to a novel environment. To investigate this possibility, we considered the habituation pattern between the first and second day of testing. A significant decrease in the distance traveled between the 2 days was only observed in the WT males (Tukey’s HSD: $t_{(2,62)} = -5.30$, $p < 0.001$; Fig. 1B), suggesting a habituation deficit in the $\text{Nlgn3}^{+/+}$, $\text{Cyfip1}^{+/+}$, as well as $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ mice. The phenotype was unlikely to occur because of differences in anxiety levels as there were no differences between genotypes in the time spent in the center of the open field (Extended Data Fig. 1-1A).

Sex is known to impact on the rate of diagnosis of ASDs (Werling and Geschwind, 2013); thus, we aimed to investigate potential sex differences in exploratory behavior. We tested $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ ($n = 17$) double-mutant female mice alongside their $\text{Nlgn3}^{+/+}$ ($n = 20$), $\text{Cyfip1}^{+/+}$ ($n = 10$), and WT ($n = 16$) littermates. The $\text{Nlgn3}^{+/+}$ mice as well as the $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ mice were no different to their WT littermates in their exploratory behavior in the open field. However, the $\text{Nlgn3}^{+/+}$ females (day 2: mean = 6129.85 cm, SE = 301.30 cm) and $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$ females (day 2: mean = 5059.01 cm, SE = 274.50 cm) were hyperactive in relation to their $\text{Cyfip1}^{+/+}$ littermates (day 2: mean = 4550.05 cm, SE = 309.99 cm; one-way ANOVA, main effect of genotype: $F_{(3,59)} = 4.64$, $p < 0.001$; Tukey’s HSD: $t_{(1,59)} = 3.34$, $p < 0.01$, and $t_{(1,59)} = 2.66$, $p < 0.05$, respectively; Fig. 1C). This finding was corroborated when the exploratory behavior across the two days of testing was considered (mixed-model ANOVA, main effect of genotype: $F_{(3,59)} = 4.51$, $p < 0.001$; Tukey’s HSD $\text{Nlgn3}^{+/+}$ vs $\text{Cyfip1}^{+/+}$: $t_{(1,59)} = 3.42$, $p < 0.01$). All of the female mice showed a significant decrease in the distance traveled between the first and second day of testing, characteristic of habituation (WT: $t_{(1,59)} = 5.61$, $p < 0.001$; $\text{Nlgn3}^{+/+}$: $t_{(1,59)} = 4.42$, $p < 0.01$; $\text{Cyfip1}^{+/+}$: $t_{(1,59)} = 3.83$, $p < 0.01$; $\text{Nlgn3}^{+/+}\text{Cyfip1}^{+/+}$: $t_{(1,59)} = 5.30$, $p < 0.001$; Fig. 1D). The subtly altered behavior in the female mice cannot be attributed to differences in anxiety levels, as the time spent in the center of the open field was comparable between the different groups (Extended Data Fig. 1-1B). Finally, to

**Figure 2.** The effect of $\text{Nlgn3}$ deletion and $\text{Cyfip1}$ haploinsufficiency on motor learning. **A.** Latency to fall off the rotarod in the $\text{Nlgn3}^{+/-}\text{Cyfip1}^{+/-}$ male mice and their littermates. **B.** Latency to fall off the rotarod in the $\text{Nlgn3}^{+/-}\text{Cyfip1}^{+/-}$ female mice and their littermates. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data for the comparison of individual trials and sex are available in Extended Data Figure 2-1.
investigate whether there is a sex difference in the exploratory behavior of these mice, we directly compared the WT and Cyfip1+/−/− males and females; however, we found no significant differences in the distance traveled or the time spent in the center of the open field between males and females (Extended Data Fig. 1-1C,D). There were no significant differences between Nlgn3+/−/− males and Nlgn3+/−/− females neither in their exploratory behavior in the open field (Extended Data Fig. 1-1E,F).

While Nlgn3+/−/− Cyfip1+/−/− males phenocopied Nlgn3+/−/− males in their hyperactivity, the Nlgn3+/−/− Cyfip1+/−/− females were no different from their WT littermates. There was no evidence, however, that reducing the levels of Cyfip1 affected the level of activity in the males, suggesting that only Nlgn3 influences this behavior.

Reduction of Cyfip1 level restores motor learning in Nlgn3+/−/− Cyfip1+/−/− male mice

The ability to learn new motor routines was evaluated by training mice to stay on an accelerating rotating rod, with multiple trials within a day (Extended Data Fig. 2-1) and over 3 consecutive days (Fig. 2). There was no overall effect of genotype on the latency to fall off the rotarod, averaged across days and trials. However, the performance of mice across the days of testing depended on their genotype, indicating that there might be differences in their learning curves (nonparametric mixed-model ANOVA, genotype × day interaction: \( F_{(3,1577)} = 4.61, p < 0.01; \) Extended Data Fig. 2-1A). In line with this observation, an increased ability to stay on the rod across multiple days was observed in WT male mice \((n = 17);\) day 1 vs day 2 simple effect: \( t_{(1,16)} = 2.12, p < 0.05, \) day 1 vs day 3 simple effect: \( t_{(1,16)} = 6.28, p < 0.001;\) day 2 vs day 3 simple effect: \( t_{(1,16)} = 2.86, p < 0.05, \) in the Nlgn3+/−/− male mice \((n = 16);\) day 1 vs day 2 simple effect: \( t_{(1,16)} = 6.16, p < 0.001;\) day 1 vs day 3 simple effect: \( t_{(1,16)} = 9.14, p < 0.001;\) day 2 vs day 3 simple effect: \( t_{(1,16)} = 3.02, p < 0.01, \) and in the Nlgn3−/− Cyfip1+/−/− male mice \((n = 12);\) day 1 vs day 2 simple effect: \( t_{(1,12)} = 5.05, p < 0.01;\) day 1 vs day 3 simple effect: \( t_{(1,12)} = 4.69, p < 0.01; \) (Fig. 2A). As previously reported, the Cyfip1+/−/− mice \((n = 12)\) did not improve in their ability to stay on the rod across the days of training, suggesting that they are unable to learn new motor routines within this protocol (Bachmann et al., 2019). These results indicate that the deficit in motor learning seen in Cyfip1+/−/− mice is restored by deleting Nlgn3 in Nlgn3+/−/− Cyfip1+/−/− double-mutant mice.

Next, we investigated whether a sex difference was affected the level of activity in the males, suggesting that only Nlgn3 influences this behavior.

Lack of Nlgn3 and Cyfip1 haploinsufficiency have little effect on social behavior

The social behavior of double-mutant mice and their littermates was evaluated by investigating their interest in social odors and, for the males only, their courtship behavior and ability to discriminate between control and social odors in WT mice \((n = 14);\) C2: mean = 14.61 s, SE = 1.79 s; S1: mean = 28.46 s; SE = 4.23; simple effect: \( t_{(1,14)} = 4.60, p < 0.05, \) Nlgn3−/− mice \((n = 16);\) C2: mean = 14.61 s, SE = 1.79 s; S1: mean = 28.46 s, SE = 4.23 s; simple effect: \( t_{(1,14)} = 5.91, p < 0.01, \) Cyfip1+/−/− mice \((n = 12);\) C2: mean = 12.43 s, SE = 1.72 s; S1: mean = 23.57 s, SE = 1.65 s; simple effect: \( t_{(1,12)} = 8.74, p < 0.001, \) and Nlgn3−/− Cyfip1+/−/− mice \((n = 12);\) C2: mean = 10.88 s, SE = 2.29 s; S1: mean = 23.62 s, SE = 3.54 s; simple effect: \( t_{(1,12)} = 4.40, p < 0.05; \) (Fig. 3A). However, habituation to the social odor was observed only in the Cyfip1+/−/− mice \((S1: \text{mean} = 23.57 \text{s, } SE = 1.65 \text{s}; S2: \text{mean} = 18.40 \text{s, } SE = 1.84 \text{s; simple effect: } t_{(1,12)} = 4.70, p < 0.05, \) and the Nlgn3−/− Cyfip1+/−/− mice \((S1: \text{mean} = 23.62 \text{s, } SE = 3.54 \text{s; S2: mean} = 16.53 \text{s, } SE = 1.85 \text{s; simple effect: } t_{(1,12)} = 4.71, p < 0.05)). The level of interest in the social odor was also the same between the different genotypes (Fig. 3B).

The genotype of the female mice had no impact on their ability to discriminate between control and social odors. An increase in the time spent with the cue was observed between the control cue and the first presentation of the social odor in WT mice \((n = 13);\) C2: mean = 6.37 s, SE = 1.15 s; S1: mean = 14.57 s, SE = 0.70 s; simple effect: \( t_{(1,13)} = 10.80, p < 0.001, \) Nlgn3−/− mice \((n = 13);\) C2: mean = 6.71 s, SE = 1.13 s; S1: mean = 12.50 s, SE = 0.89 s; simple effect: \( t_{(1,13)} = 5.50, p < 0.01, \) Cyfip1+/−/− mice \((n = 14);\) C2: mean = 5.96 s, SE = 0.69 s; S1: mean = 10.57 s, SE = 1.33 s; simple effect: \( t_{(1,14)} = 4.82, p < 0.05, \) and Nlgn3−/− Cyfip1+/−/− mice \((n = 14);\) C2: mean = 7.18 s, SE = 1.23 s; S1: mean = 14.01 s, SE = 1.12 s;
simple effect: $t_{(1,14)} = 5.44, p < 0.01$; Fig. 3C). In the female mice, habituation was only observed in Nlgn3$^{+/−}$ mice (S1: mean = 12.50 s, SE = 0.89 s; S2: mean = 8.34 s, SE = 1.08 s; simple effect: $t_{(1,13)} = 4.80, p < 0.05$) and Nlgn3$^{+/−}$/Cyfip1$^{+/−}$ mice (S1: mean = 14.01 s, SE = 1.12 s; S2: mean = 10.10 s, SE = 1.40 s; simple effect: $t_{(1,14)} = 6.39, p < 0.01$). The interest in the social odor was reduced in the Cyfip1$^{+/−}$ females compared with the WT females (one way ANOVA: main effect of genotype: $F_{(3,50)} = 2.93, p < 0.05$; Tukey’s HSD: $t_{(1,50)} = −2.68, p < 0.05$; Fig. 3D).

The courtship behavior of the males exposed to a female in estrus was equivalent in all the mutants (Nlgn3$^{−/−}, n = 16$; Cyfip1$^{−/−}, n = 12$; Nlgn3$^{−/−}$/Cyfip1$^{−/−}, n = 12$) and their WT littermates ($n = 17$). Specifically, the number and the duration of ultrasonic vocalizations emitted in response to the female as well as the time spent in direct interaction with the female was not different between the WT littermates and the other males (Fig. 3E–G). Deficits in courtship behavior in Nlgn3$^{−/−}$ males were reported multiple times (Radyushkin et al., 2009; Fischer and Hammerschmidt, 2011; Kalbassi et al., 2017). In contrast to these findings, there is no evidence for a deficit in courtship vocalization in the Nlgn3$^{−/−}$ males here. However, it is important to note that while the number and duration of vocalization in the Nlgn3$^{−/−}$ males are comparable here to the previous literature, the level of vocalization in the WT littermates is lower than expected. The reduced level of vocalization during courtship in the WT males might be a result of being housed with mutant animals, similar to a previously reported effect of the social environment (Kalbassi et al., 2017).

In opposition to previous literature, neither Nlgn3 nor Cyfip1 had a substantial impact on social behavior in male mice. This could be because of the effect of the social environment modulating the behavior of the WT controls as well as potentially the mutant animals.

**Nlgn3 and Cyfip1 collectively impact on dendritic spine density in the motor cortex**

Altered dendritic spine regulation is another phenotype associated with ASDs (Phillips and Pozzo-Miller, 2015).
We investigated the impact of deleting Nlgn3 and Cyfip1 haploinsufficiency on dendritic spine density. For this purpose, we obtained Nlgn3<sup>−/−</sup>/Cyfip1<sup>+/−</sup> double-mutant mice as well as their Nlgn3<sup>−/−</sup> and Cyfip1<sup>+/−</sup> littermates, where the EGFP transgene was expressed in a subset of neurons, allowing the visualization of dendritic spines. Changes in dendritic spine density, as well as turnover, have previously been reported in the motor cortex of Cyfip1<sup>+/−</sup> male mice (Bachmann et al., 2019); thus, this brain region was included in the analysis. No changes in the visual cortex were reported in these mice, hence we included this region as a control. In the motor cortex, Nlgn3<sup>−/−</sup>/Cyfip1<sup>+/−</sup> males had a significantly greater density of spines per 10 µm of dendrite (mean = 2.27, SD = 0.12) than Nlgn3<sup>−/−</sup> males (mean = 1.69, SD = 0.09; Kruskal–Wallis test: χ²<sub>(3153) = 15.64, p < 0.01</sub>, z<sub>(1153) = −3.27, p < 0.01</sub>) and WT males (mean = 1.66, SD = 0.13; z<sub>(1153) = −3.41, p < 0.01</sub>; Fig. 4A). No significant differences in spine density were observed in the visual cortex, emphasizing the regional specificity of the effect. In contrast to the males, no significant differences in spine density in the motor or visual cortex were present in the females (Fig. 4B). To confirm the sex difference, we obtained numbers of dendritic spines in the male and female mice directly. The WT and Cyfip1<sup>+/−</sup> females had on average more dendritic spines in the cortex than the WT and Cyfip1<sup>+/−</sup> males (Scheirer–Ray–Hare test, main effect of sex: H<sub>(1326) = 98.97, p < 0.001</sub>; Extended Data Fig. 4-1). The selectively reduced numbers of dendritic spines in male mice raised the possibility that the social environment might impact on this phenotype, whereby being raised with their mutant littermates, WT males showed lower than expected density of dendritic spines.

Figure 4. The effect of Nlgn3 deletion and Cyfip1 haploinsufficiency on spine density in the cortex. A, The dendritic spine density in the M1 area of motor cortex and the V1 area of visual cortex in Nlgn3<sup>−/−</sup>/Cyfip1<sup>+/−</sup> males and their littermates. B, The dendritic spine density in the M1 area of motor cortex and the V1 area of visual cortex in Nlgn3<sup>−/−</sup>/Cyfip1<sup>+/−</sup> females and their littermates. Scale bars, 10 µm. *p < 0.05, **p < 0.01, ***p < 0.001. The sex comparison is available in Extended Data Figure 4-1.

Lack of Nlgn3 and Cyfip1 haploinsufficiency shapes the transcriptome profiles in male mice

We investigated the effect of lack of Nlgn3 and Cyfip1 haploinsufficiency as well as the combined effect of both on RNA expression. Previous results raised the possibility that the social environment might affect the behavior and the spine density of the WT mice, potentially impacting on the interpretation of these findings (Kalbassi et al., 2017). To investigate the possibility that the social environment might impact on the transcriptome, we included a control group of WT males that were housed with mutant animals [single genotype housing (SGH)] in addition to WT males that were housed with mutant animals [mixed genotype housing (MGH)]. We performed RNA sequencing of the brain tissue, specifically the hippocampus, in Nlgn3<sup>−/−</sup>/Cyfip1<sup>+/−</sup>, Nlgn3<sup>−/−</sup>, and Cyfip1<sup>+/−</sup> males as well as in SGH and MGH WT mice. We have selected the hippocampus as a region of interest, as it is well recognized for being sensitive to environmental changes. Moreover,
hippocampal functions have been associated with the emergence of individuality in genetically identical wild-type mice (Freund et al., 2013; Kempermann, 2019). Thus, we hypothesized that differences in the social environment of wild-type mice could affect the RNA profile in the hippocampus.

Initially, we compared the number of differentially expressed genes in the hippocampi of males from different conditions. First, we compared the mutant mice to their WT littermates (MGH WT). There were very few differentially expressed genes between the mutant mice and the MGH WT controls (\textit{Cyfip1}^{+/+}/Cyfip1^{+/+} vs MGH WT: two upregulated, zero downregulated; \textit{Nlgn3}^{+/+}/Nlgn3^{+/+} vs MGH WT: three upregulated, zero downregulated; \textit{Nlgn3}^{+/−}/ \textit{Nlgn3}^{+/−} vs MGH WT: four upregulated, 1 downregulated; Fig. 5A). The differences between the MGH and SGH WT males were, however, more substantial, with 15 upregulated and 3 downregulated genes. This difference suggests that housing conditions have the capacity to shape transcription profiles. Thus, next, we compared the mutant animals to the SGH WT controls. The differences between the SGH WT animals and the \textit{Cyfip1}^{+/−} animals were still small (SGH WT vs \textit{Cyfip1}^{+/−}/\textit{Cyfip1}^{+/−}: 2 upregulated, 1 downregulated), while the differences between the WT males and \textit{Nlgn3}^{+/−} animals were more substantial (SGH WT vs \textit{Nlgn3}^{+/−}: 12 upregulated, 5 downregulated). There were also some differences between SGH WT controls and the \textit{Nlgn3}^{+/−}/\textit{Cyfip1}^{+/−} double mutants (SGH WT and \textit{Nlgn3}^{+/−}/\textit{Cyfip1}^{+/−}: 21 upregulated, 2 downregulated), suggesting that while \textit{Cyfip1} haploinsufficiency has little impact on the transcription profile, the lack of \textit{Nlgn3} has a role in shaping it. Despite the presence of differentially expressed genes, a clear separation based on housing or the genotype of the mice was not evident in the hierarchical clustering (Fig. 5B). However, a degree of separation was present following principal component analysis, with SGH WT and MGH WT samples in particular occupying nonoverlapping space (Fig. 5C).

In the course of the analysis of a differentially expressed gene, a wealth of information about the correlation between different genes is lost because of using an arbitrary \textit{p} value level. To investigate the similarities between the transcription profiles in more depth, we used a weighted gene correlation network analysis (WGCNA). We constructed a coexpression network that contained 20 modules. Of these, the lack of \textit{Nlgn3} was significantly associated with module 10 ($R^2 = −0.42$, false discovery rate (FDR) = 0.09), while the reduced level of \textit{Cyfip1} was not linked to any of the modules. However, when the two mutations were combined in the \textit{Nlgn3}^{+/−}/\textit{Cyfip1}^{+/−} mutant mice, module 11 ($R^2 = 0.66$, FDR = 0.003), module 13 ($R^2 = −0.42$, FDR = 0.08), and module 15 ($R^2 = −0.43$, FDR = 0.08) were all significantly associated with this trait, suggesting an additive effect of the two mutations (Fig. 5D). When the housing condition was considered, module 8 was found to be upregulated in the SGH animals compared with the MGH animals ($R^2 = 0.75$, FDR < 0.01), while module 17 was downregulated ($R^2 = −0.58$, FDR = 0.01; Fig. 5E). The differential expression of the modules of genes depending on housing and genotype of the males suggests that both the social environment and the presence of \textit{Nlgn3} and \textit{Cyfip1} impact on the transcription profile. The genes in module 8 were associated with development, in particular with the development of the loop of Henle as well as synaptic transmission and cerebellar cell proliferation and signalling, while the genes in module 17 were associated with metabolic processes (Fig. 5E).

While there were some differences between the transcription profile arising from mice with different genetic mutations, the effect of housing was more pronounced.

Social environment impacts on transcriptome profiles in male mice

We have found that WT males housed with their WT littermates have a distinctly different transcription profile from WT animals housed with mutant animals as well as the mutant animals themselves. However, the previous experiment used a cohort of WT animals from a different breeding line than the mutant animals, potentially artificially increasing the differences between the SGH and MGH WT mice. Thus, we attempted to replicate the effect of the social environment on the transcriptome in SGH and MGH WT males that came from the same breeding line and thus had the same mothers as each other as well as mutant males. Additionally, we included the single and mixed genotype housing condition for the \textit{Nlgn3}^{+/−} males (SGH \textit{Nlgn3}^{+/−} and MGH \textit{Nlgn3}^{+/−}) to investigate the potential impact of the social environment on the mutant mice. As previously, there was a fair number of genes that were differentially expressed between the MGH and SGH WT males (23 upregulated, 34 downregulated), suggesting that housing conditions shaped the transcription profiles in this group (Fig. 6A). The effect of housing was also evident to a smaller extent in the \textit{Nlgn3}^{+/−} males, where there were 2 upregulated and 18 downregulated genes between SGH \textit{Nlgn3}^{+/−} and MGH \textit{Nlgn3}^{+/−} males. While between the SGH WT and SGH \textit{Nlgn3}^{+/−} males that were never housed with mice of the same genotype, there were many differentially expressed genes (39 upregulated and 18 downregulated), and there were very few differences between the MGH WT and MGH \textit{Nlgn3}^{+/−} mice that were housed together (8 upregulated, 0 downregulated). These findings suggest that the social environment impacts the transcriptome profile not only in the WT littermates but also in the mutant \textit{Nlgn3}^{+/−} mice. Despite the presence of a number of differentially expressed genes, a clear separation based on housing was not evident in the hierarchical clustering (Fig. 6B). A degree of separation was present following principal component analysis with SGH WT and MGH WT samples in particular occupying nonoverlapping space (Fig. 6C). WGCNA resulted in a network that contained 20 modules. In SGH WT animals, modules 9 and 10 were significantly upregulated ($R^2 = 0.5$, FDR = 0.09), and modules 14 ($R^2 = −0.53$, FDR = 0.08), 16 ($R^2 = −0.61$, FDR = 0.04), and 18 ($R^2 = 0.68$, FDR = 0.02) were significantly downregulated (Fig. 6D). In SGH \textit{Nlgn3}^{+/−} males, module 14 ($R^2 = 0.60$, FDR = 0.04) was significantly upregulated and module 2 was significantly downregulated ($R^2 = −0.75$, FDR = 0.005; Fig. 6D). Meanwhile in the MGH WT animals, modules 18 and 20 were significantly upregulated ($R^2 = 0.55$, FDR = 0.06; and
Figure 5. Nlgn3 and Cyfip1 mutations affect the transcriptome profile of male mice. A, Differentially expressed genes between the SGH WT, MGH WT, Cyfip1−/−, Nlgn3−/−, and Nlgn3−/− Cyfip1−/− male mice. B, Hierarchical clustering of differentially expressed genes in the different housing and genotype conditions. C, Position of the individual samples from different housing and genotype conditions in the principal component space based on the 100 genes with the greatest fold change. D, WGCNA modules correlation with the different housing and genotype combinations. E, GO pathways for modules correlated with SGH WT condition.
Figure 6. Social environment affects the transcriptome profile of WT and Nlgn3<sup>−/−</sup> mice. A, Differentially expressed genes in the SGH WT, MGH WT, SGH Nlgn3<sup>−/−</sup>, and MGH Nlgn3<sup>−/−</sup>. B, Hierarchical clustering of differentially expressed genes in the different housing and genotype conditions. C, Position of the individual samples from different housing and genotype conditions in the principal component space based on the 100 genes with the greatest fold change. D, WGCNA module correlation with the different housing and genotype combinations. E, GO pathways for modules correlated with SGH WT condition.
the RNA expression. A complex genetic the transcription profile. Together, these findings suggest that the phenotypes associated with ASDs. We uncovered the combined effect of genetic and environmental factors on some of the genes in the relevant modules were different depending on the cohort.

Here we show that lack of $Nlgn3$ has an impact on the RNA expression in the hippocampus, while $Cyfip1$ haploinsufficiency does not. Additionally, we extend the previous finding that the presence of $Nlgn3^+$ littermates influences the social behavior of their WT littermates (Kalbassi et al., 2017) to potentially include an effect on the transcription profile. Together, these findings suggest a complex genetic–environment interaction that shapes the RNA expression.

**Discussion**

In the first attempt of this kind, we investigated the combined effect of genetic and environmental factors on the phenotypes associated with ASDs. We uncovered that the $Nlgn3$/$Cyfip1$ pathway plays a role in mouse behavior, dendritic spine density, and RNA expression. We found that sex can be a protective factor, where the females carrying the risk allele do not show the same deficits as the males. The behavior and transcriptome of WT mice is further influenced by the social group they originate from, suggesting that the social environment is an important factor modulating the phenotypes in mouse models.

As in previous literature, we found that male mice lacking $Nlgn3$ engage in more exploratory behavior in the open field (Radyushkin et al., 2009; Kalbassi et al., 2017) and that male mice with a reduced level of $Cyfip1$ expression are unable to learn motor routines (Bachmann et al., 2019). We have extended these findings to show that, unexpectedly, the accumulation of mutations can lead to a correction of the motor learning deficit. The interaction between Neurologin3 and CYFIP1 at the synapse has been previously reported (Bachmann et al., 2019). The current findings support the idea that the consequences of this protein interaction extend beyond the molecular events, resulting in a change in the behavior of the animal. As the genetic architecture of ASDs is complex and far from being entirely understood, it is increasingly important to consider the interaction between the different proteins involved and how they shape the phenotypes seen in ASDs.

The behavioral results suggest that the nature of the functional relationship between Neurologin3 and CYFIP1 is inhibitory. In the males with $Cyfip1$ haploinsufficiency, the level of CYFIP1 was found to be reduced in several brain areas (Bachmann et al., 2019). We found that this decrease resulted in a deficit in motor learning that was accompanied by unaltered levels of activity and dendritic spine density in the cortex (Fig. 7). This suggests that in the males heterozygous for $Cyfip1$, the Neurologin3 might be binding some of the available CYFIP1 in the cellular population important for motor learning, preventing it from performing its physiological function and resulting in a deficit. In the males with $Nlgn3$ deletion, on the other hand, physiological levels of CYFIP1 result in the mice being able to learn the motor routines. However, the lack of Neurologin3 might result in increased levels of available CYFIP1, potentially leading to the increased activity levels seen in this model. The alternative explanation is that the increase in activity results from another pathway regulated by Neurologin3. In the double-mutant males, which lack $Nlgn3$ and are heterozygous for $Cyfip1$, the increase in activity and dendritic spine density is accompanied by normalized motor learning. In this model, the level of CYFIP1 is likely reduced, but there is no Neurologin3 to inhibit the remaining CYFIP1. As a result, there is enough CYFIP1 available to restore motor learning. Meanwhile, the lack of Neurologin3 also results in an increased level of activity. Interestingly, only the two mutations together result in an increase of dendritic spine density. These results suggest that in the WT animals, Neurologin3 inhibits the portion of available CYFIP1, regulating motor learning as well as potentially activity levels and dendritic spine density in the cortex.

We showed that while male mice with reduced levels of $Cyfip1$ have a clear phenotype, females with the same genotype are capable of learning motor routines as usual. Additionally, our data suggest that there might be a reduction in the number of dendritic spines in the males compared with the females in the cortex. While this finding needs to be confirmed in a cohort of wild-type animals, accounting for the social dominance status, it implies that only males are affected by carrying a risk allele. While a sex difference in ASDs is frequently reported, with the affected male to female ratio being 4:1, remarkably little is known about the source of this sex difference (Halladay et al., 2015). The female protective effect is one theory aiming to explain this discrepancy, whereby females require more substantial disruption of the genetic network and the associated biological pathways to show ASD symptoms (Ferri et al., 2018). Some pathways relevant for ASDs might be redundant in females, as a number of genes associated with ASDs are X linked. While $Cyfip1$ is present on chromosome 7, it interacts with two X-linked genes: $Nlgn3$ and $Fmr1$. $Cyfip1$ being part of the X-linked pathway results in unimpaired females likely because they carry two rather than one allele of the interacting genes.

In opposition to the previous report, $Cyfip1$ haploinsufficiency did not affect the spine density in the motor cortex of the male mice (Bagni and Greenough, 2005; Pathania et al., 2014; Bachmann et al., 2019). While in the previous report $Cyfip1$ haploinsufficiency resulted in a decrease in the dendritic density in the motor cortex, here the double-mutant mice showed an increase in dendritic spine density. The reliability of this finding is undermined by the low numbers of dendritic spines in the male mice of 1–5 spines per 10 μm, while the numerous previous studies reported a density of 5–15 spines per 10 μm both in the
males heterozygous for Cyfip1 and in WT males (De Rubeis et al., 2014; Pathania et al., 2014; Abekhoukh et al., 2017). The sample from the female mice showed a range of 2–10 spines per 10 μm, which is in line with the previous reports. The low number of dendritic spines is unlikely to be because of the technical difficulties, as the male and female mice used in the experiment were littermates, came from the same line, and were analyzed in parallel. Therefore, it is unlikely that the low numbers in the males but not in the females are a technical artifact. The alternative explanation is that there is another factor selectively impacting the spine density in the WT males. One such factor could be the social environment. The effect of the social environment in the mouse models of ASDs has not been extensively studied. However, there were some reports of mutant animals, including males lacking Nlgn3, impacting on the behavior of the WT littermates (Yang et al., 2011; Kalbassi et al., 2017). The biological processes underlying mouse behavior are complex; however, there is a possibility that the density of dendritic spines might be correlated with behavior. Therefore, the low number of dendritic spines seen in the WT mice housed with the mutant littermates might be the result of the social environment. To confirm that the social housing effect can extend to the dendritic spine density, it would be necessary to extend the analysis to a cohort of WT males that have never been housed with mutant littermates.

In addition to the possible effect of the social environment on behavior and dendritic spine density, social housing also impacts on the transcriptome in the hippocampus. While the effect of the social environment on the gene expression was replicated in two separate cohorts of animals, the identity of the genes associated with different housing conditions varied. In the first cohort, single-genotype housing of the WT animals was associated with synaptic transmission and metabolic processes, and in the second cohort the same trait was associated with cell cycle processes and RNA regulation. The differences might arise from the fact that one of the two cohorts of SGH WT arose from the same breeding line as the corresponding MGH WT animals while the other cohort did not. We found that wild-type individuals could potentially be differentiated based RNA expression, indicating that the different social environments have a distinct impact on wild-type mice. Although this experiment does not draw a direct link between the identity of the RNA profile and the phenotypes, we could postulate that wild-type behavior, shaped by its social environment, can be underlined by the activation of specific molecular pathways that remain to be determined.

Here we showed that not only genetic factors but also sex and social environment play a fundamental role in shaping the phenotypes of different mouse models of ASDs. Of these factors, the most complex to consider is arguably the role of the social environment. Together with a previous published study (Kalbassi et al., 2017), our
experiments suggest that the presence of mutant animals influence wild-type animals in that they adopt behavioral, cellular, and molecular phenotypic traits of mutant animals. The opposite effect, from the mutant to the wild type, seems to occur as well. The resulting effect might be that when the animals with different levels of sociability are placed in a common social context, a homogenization of their behavior, underlined by neuronal morphology and transcriptome profile changes, occurs to set an optimal level of functioning within the social group. This remains a hypothesis that needs to be validated and extended to social groups in general. But if our postulate were to be confirmed, these findings could have important implications regarding experimentation using laboratory animals and, in particular, could lead to re-evaluation of the use of wild-type animals as controls to define phenotypic traits of mouse models.

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