



Sex bias in social deficits, neural circuits and nutrient demand in *Cttnbp2* autism models

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Autism spectrum disorders caused by both genetic and environmental factors are strongly male-biased neuropsychiatric conditions. However, the mechanism underlying the sex bias of autism spectrum disorders remains elusive. Here, we use a mouse model in which the autism-linked gene *Cttnbp2* is mutated to explore the potential mechanism underlying the autism sex bias.

Autism-like features of *Cttnbp2* mutant mice were assessed via behavioural assays. C-FOS staining identified sex-biased brain regions critical to social interaction, with their roles and connectivity then validated by chemogenetic manipulation. Proteomic and bioinformatic analyses established sex-biased molecular deficits at synapses, prompting our hypothesis that male-biased nutrient demand magnifies *Cttnbp2* deficiency. Accordingly, intakes of branched-chain amino acids (BCAA) and zinc were experimentally altered to assess their effect on autism-like behaviours.

Both deletion and autism-linked mutation of *Cttnbp2* result in male-biased social deficits. Seven brain regions, including the infralimbic area of the medial prefrontal cortex (ILA), exhibit reduced neural activity in male mutant mice but not in females upon social stimulation. ILA activation by chemogenetic manipulation is sufficient to activate four of those brain regions susceptible to *Cttnbp2* deficiency and consequently to ameliorate social deficits in male mice, implying an ILA-regulated neural circuit is critical to male-biased social deficits. Proteomics analysis reveals male-specific downregulated proteins (including SHANK2 and PSD-95, two synaptic zinc-binding proteins) and female-specific upregulated proteins (including RRAGC) linked to neuropsychiatric disorders, which are likely relevant to male-biased deficits and a female protective effect observed in *Cttnbp2* mutant mice. Notably, RRAGC is an upstream regulator of mTOR that senses BCAA, suggesting that mTOR exerts a beneficial effect on females. Indeed, increased BCAA intake activates the mTOR pathway and rescues neuronal responses and social behaviours of male *Cttnbp2* mutant mice. Moreover, mutant males exhibit greatly increased zinc demand to display normal social behaviours.

Mice carrying an autism-linked *Cttnbp2* mutation exhibit male-biased social deficits linked to specific brain regions, differential synaptic proteomes and higher demand for BCAA and zinc. We postulate that lower demand for zinc and BCAA are relevant to the female protective effect. Our study reveals a mechanism underlying sex-biased social deficits and also suggests a potential therapeutic approach for autism spectrum disorders.

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Introduction

Autism spectrum disorders, a group of early-onset neurodevelopmental disorders, are ~4–5-fold more prevalent in males than females (<https://www.cdc.gov/ncbddd/autism/data.html>). Several hypotheses have been proposed to explain the male bias of autism spectrum disorders, including female protective effects, male risk factors, extreme male brain theory, enhanced plasticity hypotheses, sex chromosome theory, sex hormone theory and a neuroendocrine hypothesis.^{1–8} A multiple-hit hypothesis (encompassing genetic, environmental and sex interactions) has also been proposed.¹ Given that autism spectrum disorders are highly heterogeneous, each hypothesis may account for certain conditions and these hypotheses may not be mutually exclusive.

Autism spectrum disorders are characterized by impaired social interaction, communication deficits, repetitive sensory-motor behaviours and cognitive inflexibility (<https://www.psychiatry.org/patients-families/autism/what-is-autism-spectrum-disorder>). Recently, large-scale whole-exome sequencing of patients with autism spectrum disorders identified hundreds of disease-linked genes.^{4,9–12} Genes involved in transcriptional regulation and synaptic formation/signalling have been shown to be critical to autism spectrum disorders.^{4,9–12} These autism-linked genes may be subclassified into three groups, i.e. male-enriched, female-enriched and shared genes based on sex-specific incidence rates. For male-enriched genes, their mutations have been identified far more predominantly in male but not female patients. Moreover, there are more male-enriched genes than for either of the other two groups.^{13,14} Thus, sex likely differentially influences the outcome of genetic variations, but further validation is needed.

In this report, we investigate the mechanism underlying sex bias in autism spectrum disorders using the cortactin binding protein 2 (*Cttnbp2*) mouse genetic models. CTTNBP2, a brain-specific cytoskeleton-associated scaffold protein, is highly enriched at dendritic spines where it regulates dendritic spine formation and maintenance in mature neurons.^{15–17} Controlled by alternative splicing, at least three isoforms of *Cttnbp2* transcripts have been identified in mice.¹⁵ However, immunoblotting analyses have indicated that only the short form of CTTNBP2, a protein of 630 amino acid residues, is the dominant isoform in the brain.^{15,18} Thus, variations within the coding region of the short isoform are expected to be more relevant to autism spectrum disorders.¹⁹ Indeed, six variations in the short form of CTTNBP2, including A113T, M121I, G343R, P354A, R536* (stop codon) and D580Y, have been reported for patients with autism spectrum disorders but not case controls.¹² Moreover, all of the patients carrying those CTTNBP2 variants are male, indicating that CTTNBP2-dependent autism phenotypes are enriched among males.^{12,13}

Among those six CTTNBP2 variants, the impact of the M121I, R536* and D580Y mutations have already been investigated in mice. Similar to *Cttnbp2* deletion, the corresponding autism-linked mutations in the mouse *Cttnbp2* gene, i.e. M120I, R533* and D570Y, all result in reduced

dendritic spine density.^{18,19} Mouse genetic models carrying a null allele or the autism-linked mutations of *Cttnbp2* also exhibit autism-like behaviours, including social deficits, supporting that those CTTNBP2 mutations are disease-causative.^{18,19}

The short form of CTTNBP2 consists of an N-terminal coiled-coil region and a C-terminal intrinsically disordered region.²⁰ The intrinsically disordered region is required for condensate formation via phase separation.²⁰ It also associates with microtubule and F-actin to regulate dendritic arborization and dendritic spine formation, respectively.^{15–17} The N-terminal coiled-coil region is involved in homo- and hetero-oligomerization and zinc binding.²⁰ Its interaction with zinc induces higher-order CTTNBP2 multimerization and promotes synaptic retention of CTTNBP2.²⁰ In addition, since the intrinsically disordered region is very flexible, it can fold back to interact with the N-terminal coiled-coil region.^{19,20} Moreover, *Cttnbp2* deletion reduces total zinc levels in mouse brains.¹⁸ Together, these findings suggest that CTTNBP2 binds zinc, regulates zinc levels in neurons, and that its function is also controlled by zinc.

Although only male but not female autism patients have been found to harbour CTTNBP2 mutations,¹³ both male and female case control individuals carry variations in the CTTNBP2 gene.¹² Thus, one of the possibilities to explain male enrichment of CTTNBP2 mutations in patients is that females are resilient to those mutations. To explore this speculation, we investigated if *Cttnbp2* deletion and autism-linked mutation solely elicit social deficits in male but not female mice. Given that the M120I mutation in mice impairs almost all examined functions of CTTNBP2—including the N- and C-terminal interaction, cortactin binding, biological condensate formation, dendritic spine retention, dendritic spine formation, and social interactions^{18–20}—the *Cttnbp2* M120I mutant line represents an interesting model for studying autism. Since monoallelic CTTNBP2 variation has been documented among patients, accordingly we explored the mechanism underlying sex bias in autism-like phenotypes using *Cttnbp2*^{+/M120I} mice.¹²

Materials and methods

Antibodies, reagents and plasmids

The following antibodies were used in this study: C-FOS (Cell Signaling, #2250, Clone#9F6, rabbit, 1:500); GFP (Abcam, ab13970, chicken, 1:1000); RAGC (Cell Signaling, #5466, rabbit, 1:500); TIPRL (Abcam, #ab70795, rabbit, 1:500); SHANK2 (Cell Signaling, #12218, rabbit, 1:500); SYNPO (Acris, #BM5086P, rabbit, 0.1 µg/ml); PSD95 (Cell Signaling, #2507, rabbit, 1:1000); HSP90 serum (gift from Dr Chung Wang, IMB, Taiwan, rabbit, 1:2000)²¹; p-mTOR (Cell Signaling, #2448, rabbit, 1:500); mTOR (Cell Signaling, #2972S, rabbit, 1:500); S6K (Cell Signaling, #2708S, rabbit, 1:500); p-S6K70 T389 (Cell Signaling, #9205S, rabbit, 1:500); 4EBP (Cell Signaling, #9644S, rabbit, 1:500). The following reagents were obtained commercially: ProHance (Gadolinium contrast agent, Bracco Diagnostics, #111181); Vectastain Elite ABC kit (Vector Labs, #PK-6102); C21

(Tocris, #6422); OCT (Tissue-TeK, 4583); ZnSO₄ (Sigma-Aldrich, #Z0251); 30 ppm zinc diet (Research Diets, D19410B). AAV vectors used in this study were: pAAV-CamKII-hM3D(Gq)-mCherry (Addgene, #50476); pAAV-CamKII-hM4D(Gi)-mCherry (Addgene, #50477)²²; pAAV-CAG-tdTomato (Addgene, #59462).

Animals

Cttnbp2^{-/-} and *Cttnbp2*^{+/^{M1201}} mice have been described previously.^{18,19} All mouse lines were maintained by backcrossing to C57BL/6JNarl purchased from the National Laboratory Animal Center, Taiwan. Mice were housed in the animal facility of the Institute of Molecular Biology, Academia Sinica, under controlled temperature (20–23°C) and humidity (48–55%) and a 12 h light/12 h dark cycle (light: 8.00 a.m. to 8.00 p.m.) with free access to water and food (LabDiet #5k54). After weaning at post-natal Day (P) 21–22, mice were moved from the breeding room to the experimental area and housed individually. Temperature and humidity in the test room were also controlled and the light intensity was 240 lx. All experiments were performed during the daytime, from 10.00 a.m. to 5.00 p.m. All animal experiments were performed with the approval of the Academia Sinica Institutional Animal Care and Utilization Committee (Protocol No. 11-12-294) and in strict accordance with its guidelines and those of the Council of Agriculture Guidebook for the Care and Use of Laboratory Animals.

Animal behavioural assays

Four behavioural tests were conducted as described previously, i.e. open field test (OF), light/dark box (LDB), three-chamber test (3C) and reciprocal social interaction (RSI),^{18,23,24} starting at P28 and ending at P42 (Fig. 1A), to evaluate if *Cttnbp2* mutant mice exhibit sex-biased behavioural deficits. An elevated plus maze (EPM) test was also included in experiments related to zinc supplementation. Detailed descriptions of behavioural experiments are presented in the [Supplementary material](#).

C-FOS staining

C-FOS immunostaining of brain sections was conducted as described previously.²⁵ In brief, one of four consecutive 50-µm thick brain sections was selected for C-FOS staining using DAB staining (Vectastain Elite ABC kit, Vector). The region of interest (ROI) was identified according to the Allen Brain Atlas (<http://help.brain-map.org/display/api/Atlas+Drawings+and+Ontologies>, Atlas ID one, mouse P56 coronal) and manually adjusted. The numbers of C-FOS-positive (C-FOS+) cells in each ROI were then determined using ImageJ according to the following sequential steps: (i) the images were converted into 8-bit; (ii) noise signals were processed using ‘subtract background’ (setting: 10 pixels, light background) and ‘threshold’ (B&W mode); (iii) greyscale ‘Binary watershed’ was then applied to segment particles; and (iv) C-FOS+ cells in the ROI were determined using ‘Analyze particles’ with settings ‘size >10 pixels (1 µm/pixel)’ and ‘circularity 0.01–1.00’. Density of C-FOS+ cells in each ROI was then determined.

Chemogenetic modulation

Surgical injection and chemogenetic manipulation were performed as described previously.^{26,27} To evaluate the role of the ILA in controlling social behaviours of male and female mice, we bilaterally injected AAV-CamKII-hM3Dq-mCherry (4.32×10^{12} vg/ml) and AAV-CamKII-hM4Di-mCherry ($>2 \times 10^{12}$ vg/ml) into the ILA of male

and female mice, respectively, at P27/28. AAV-CAG-tdTomato (8.06×10^{12} vg/ml) was used as a negative control in both male and female mice. One hour before the behavioural assay, C21 (1 mg/kg of body weight) was injected intraperitoneally to either activate or inhibit neuronal activity of the ILA.

Liquid chromatography-tandem mass spectrometry analysis

Brains corresponding to section number 39–41 of the Allen Brain Atlas were dissected to collect the ILA. The tissue samples of three mice were pooled as one replicate and four biological repeats were used for each group. Synaptosomal fractionation, liquid chromatography-tandem mass spectrometry (LC-MS-MS) and bioinformatic analyses of the ILA were then performed as described previously^{18,28} and detailed in the [Supplementary material](#).

Immunoblotting

Five to seven biological replicates (10–15 µg per lane) of ILA or total cortical lysates were subjected to three to four independent immunoblotting assays to validate the results of our proteomic analysis or to evaluate the effect of branched-chain amino acid (BCAA) treatment. The membranes were blocked with 5% non-fat milk, washed with PBS-T (0.2% Tween-20) buffer, and then incubated with primary antibodies overnight at 4°C. After washing, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. HSP90 was used as a loading control for normalization of the protein levels. The results were visualized using a WesternBright ECL Spray or Immobilon Western Chemiluminescent HRP Substrate and recorded using an ImageQuant LAS 4000 system with ImageQuant LAS 4000 Biomolecular Imager software (GE Healthcare). Signal quantification was performed using ImageJ Fiji version 2.1.0/1.53c.

Zinc diet treatment

Mice were randomly assigned to two groups provisioned with different levels of zinc in their diets (Fig. 6A): (i) 30 ppm zinc, i.e. Research Diets D19410B representing a normal zinc diet; or (ii) 150 ppm zinc, i.e. Research Diets D19410B plus 75 ppm ZnSO₄ in drinking water representing a higher zinc diet. Behavioural assays and body weight measurements were performed during P30–P42.

Quantification and statistical analysis

All quantitative data in this report are presented as means plus standard error of the mean (SEM). Individual data-points are also presented. GraphPad9 Prism (Graphpad software, La Jolla, CA) was used to perform statistical analysis and to generate the graphs. No statistical method was applied to evaluate the sample size, but our sample sizes are similar to previous publications.^{25,29} Data collection and analysis were conducted in a random and blind manner. Statistical analyses were performed using a two-tailed unpaired Student’s *t*-test, two-tailed paired *t*-test, one-way ANOVA followed by Dunnett’s *post hoc* test, or two-way ANOVA with Tukey’s *post hoc* test. For all comparisons, *P* < 0.05 was considered significant. All statistical details and results are available in [Supplementary Tables 1–5 and 7](#).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its [Supplementary material](#).

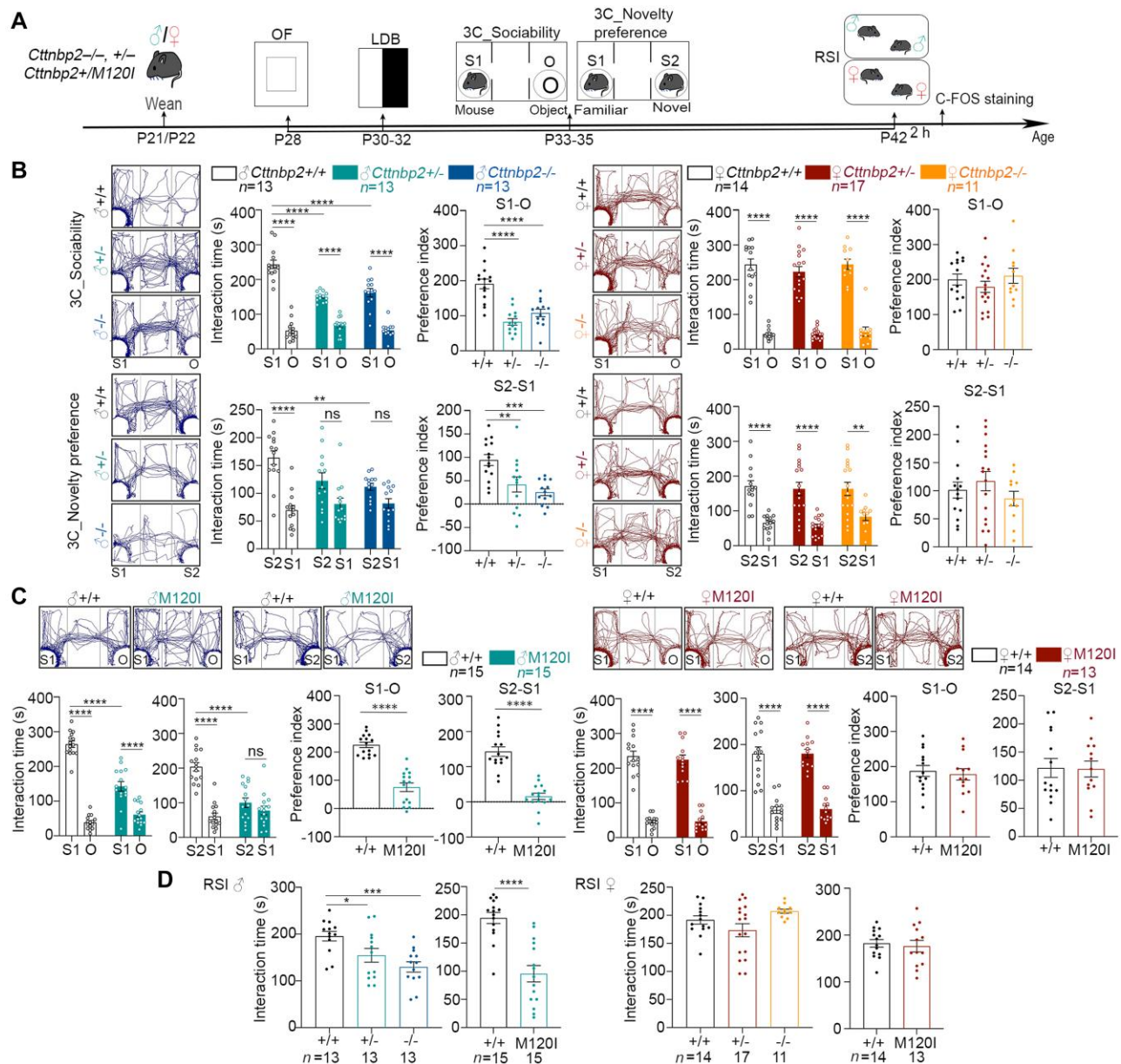


Figure 1 *Ctnnbp2* deficiency results in male-biased social deficits in mice. (A) Schematic of the experimental timeline from P28 to P42, together with the behavioural procedures conducted on both sexes of *Ctnnbp2* mutant mice. O = cage with an object; S1 = cage with unfamiliar mouse 1; S2 = cage with unfamiliar mouse 2. Two hours after RSI at P42, brains were collected for C-FOS staining. (B and C) The results of the 3C social task. Mouse trajectory in the chamber, time spent in each cage, and preference index (i.e. time difference between the indicated cages) are shown for each set of animals. Left = male; right = female. (B) Male, but not female, *Ctnnbp2*^{+/-} and *Ctnnbp2*^{-/-} mice display deficits in sociability and social novelty in the 3C test. (C) *Ctnnbp2*^{+/-}/*M120I* mice (denoted M120I mice) also display male-biased deficits. (D) The results of the reciprocal social interaction test. Left = male; right = female. Data are presented as means \pm SEM, together with data-points for individual mice. The numbers (*n*) of examined mice for each group are indicated. For all results of the 3C test, the interaction time was analysed using two-way ANOVA with Tukey's multiple comparisons *post hoc* test. The preference indices were analysed using one-way ANOVA in B and two-tailed unpaired t-test in C and D. In E, one-way ANOVA and two-tailed unpaired t-test were used for three- and two-group comparisons, respectively. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns = non-significant. All statistical analysis and results, including the actual *P*-values, are summarized in [Supplementary Table 7](#).

Results

Male-biased social deficits in *Ctnnbp2*-deficient mice

We arranged a series of behavioural paradigms from P28 to P42 (Fig. 1A) to investigate if *Ctnnbp2* deletion and autism-linked mutation result in sex-biased social deficits. In addition to *Ctnnbp2* knockout mice, *Ctnnbp2*^{+/-}/*M120I* mice serve as a mouse model of monoallelic M121I mutation of the human *CTTNBP2* gene identified in autism

patients.^{12,19,20} To confirm the observed phenotypes are not caused by the off-target effect of gene editing, we generated two independent *Ctnnbp2*^{+/-}/*M120I* mouse lines (#8 and #12, hereafter denoted M120I mice) and backcrossed the mutant mice to wild-type (WT) C57BL/6 for at least six generations before behavioural analysis. The results of line #8 have been published previously,^{19,20} revealing that the M120I mutation impairs social behaviours of adult male mice. Here, we show that adult male mice of line #12 also exhibit social

deficits (Supplementary Fig. 1A), further strengthening the evidence that *Cttnbp2* M120I mutation results in social deficits. Since both lines exhibit the same social deficits, we only subjected line #12 to the experiments presented in this report.

We subjected both male and female mice to OF, LDB, 3C and RSI tests (Fig. 1A). For all *Cttnbp2*^{+/−}, *Cttnbp2*^{−/−} and M120I mice, both male and female mutant mice behaved normally compared to their WT littermates in an OF (Supplementary Fig. 2B and C). In a LDB, male mice behaved comparably regardless of genotype, but female *Cttnbp2*^{+/−} and *Cttnbp2*^{−/−} mice spent more time in the light zone (Supplementary Fig. 2B and C). However, this anxiolytic effect was not detected for female M120I mice (Supplementary Fig. 2C). Thus, anxiolysis in females is not a shared phenotype attributable to *Cttnbp2* deficiency, so it was not pursued further in the current report.

We then used a 3C test to evaluate the sociability and novelty preference of male and female mice. Juvenile male *Cttnbp2*^{+/−}, *Cttnbp2*^{−/−} and M120I mice all exhibited a clear reduction in interactions with Stranger one (S1) for sociability and in interactions with Stranger two (S2) for novelty preference (Fig. 1B, left). In contrast to male mutant mice, none of the female mutant mice showed any deficits in sociability or novelty preference (Fig. 1B, right).

To further confirm the sex-biased social deficits, we subjected our mutant mice to an RSI assay, which revealed that all male *Cttnbp2*^{+/−}, *Cttnbp2*^{−/−} and M120I mice exhibited RSI deficits (Fig. 1C). Female *Cttnbp2* mutant mice behaved comparably to their WT littermates (Fig. 1C). Thus, *Cttnbp2* mutation elicits male-biased social defects at the juvenile stage.

In addition to the juvenile stage, we further investigated the behaviours of adult female M120I mice. Similarly, adult M120I females behaved normally in an open field (Supplementary Fig. 1B). Importantly, the reciprocal interaction of mutant adult females with female strangers was also comparable to their WT female littermates, regardless of their estrus cycle (Supplementary Fig. 1B). Thus, *Cttnbp2* autism-linked mutation results in male-biased social deficits in both juveniles and adults.

Identification of brain regions displaying sex-dependent differences

Next, we used our M120I mice as a model to investigate how autism-linked *Cttnbp2* mutation results in male-biased social deficits. First, we used structural MRI to assess neuroanatomical differences between male and female mutant mice and their same-sex WT littermates. We ran linear models to compare genotypes across all groups, and separately in males and females. Additionally, we examined the interaction between genotype and sex. However, we did not detect any significant differences in comparisons of 182 different brain regions (Supplementary Table 1), nor in our voxel-wise comparisons. Thus, there are no obvious neuroanatomical differences among these four groups.

Nevertheless, we speculated that neuronal activity may vary among the mouse groups. Accordingly, we examined C-FOS expression 2 h after RSI (Fig. 1A) across 31 brain regions (Supplementary Tables 2 and 3) involved in social interaction or associated with sexual dimorphism.^{30,31} First, we compared the mean values of C-FOS+ cell density to determine the following ratios: M120I male/WT male (to indicate a genetic effect on males); M120I female/WT female (to represent a genetic effect on females); WT female/WT male (to show an effect of sex on WT mice); and M120I female/M120I male (to reveal an effect of sex on the *Cttnbp2* M120I mutation). Based on the mean values, we observed that differences for

the M120I female/WT female and WT female/WT male groups were mainly less than 50% (Fig. 2A, region between 0.66 and 1.5 indicated by two dashed lines). Differences of >50% were mainly attributable to the groups of M120I male/WT male (Fig. 2A, blue circles) and M120I female/M120I male (Fig. 2A, orange triangles). This global comparison of neuronal activity indicates that *Cttnbp2* deficiency likely alters neuronal responses in some brain regions in a sex-biased manner.

We also performed statistical analysis for each brain region. Compared to male WT mice, male M120I mice exhibited reduced C-FOS+ cell densities in seven brain regions, including the ILA, the anteromedial area of the bed nuclei of the stria terminalis (BSTam), rostral part of the lateral septal nucleus, medial septal nucleus, dentate gyrus of the dorsal hippocampus (DGd), anterior part of basolateral amygdala (BLAa) and ventral tegmental area (VTA) (Fig. 2B–F). No such differences were detected for the comparison between female M120I and WT mice (Fig. 2B–F). When male and female M120I mice were further compared, the numbers of activated neurons in the ILA, BSTam, DGa, BLAa and VTA were also higher in female mutant mice (Fig. 2B–F, indicated with a number sign, or double number sign). Thus, the activities of brain regions were noticeably reduced by *Cttnbp2* M120I mutation in male mice.

The same analysis on female M120I mice revealed that only the lateral habenula and lateral amygdala were affected (Supplementary Fig. 3), with C-FOS+ cell density being reduced by M120I mutation in the lateral habenula but increased in the lateral amygdala (Supplementary Fig. 3 and Supplementary Tables 2 and 3). The other brain regions did not display obvious differences (Supplementary Fig. 4 and Supplementary Tables 2 and 3). Thus, the impact of M120I mutation on females is much milder.

Hence, our analysis of C-FOS expression indicates that female and male M120I mouse brains respond differently to social stimulation, with female mice proving more resilient to *Cttnbp2* deficiency in terms of neuronal activation upon social stimulation.

The critical role of the ILA-related circuits in sex-biased deficits

Among the seven male-biased regions identified above, we were particularly interested in the ILA because it links many other brain regions to control social interaction, emotional responses and other behaviours.^{32–34} Accordingly, we evaluated the role of the ILA in controlling social behaviours by means of chemogenetic manipulation (Fig. 3A and Supplementary Fig. 5). AAV-CaMKII-hM3Dq-mCherry (or AAV-CAG-tdTomato as a control) was stereotactically injected into the ILA of male M120I mice at P27–28 (Fig. 3A and Supplementary Fig. 5). We confirmed AAV injection at the ILA in all quantified mice after completing experiments (Supplementary Fig. 5).

After recovering from the surgery, the mice were subjected to behavioural assays in the order of OF–RSI–3C–RSI (Fig. 3A). C21, an agonist of designer receptor exclusively activated by designer drugs (DREADD), was intraperitoneally injected into mice one hour before the 3C and the second RSI assays (Fig. 3A). Two hours after the second RSI test at P42, we performed C-FOS staining to confirm our chemogenetic activation enhanced neuronal activation in the ILA (Fig. 3B, middle). In contrast to the ILA, neither the prelimbic area of the medial prefrontal cortex, a region adjacent to the ILA, nor the dorsal part of the anterior cingulate area (ACAd) presented differences in C-FOS+ cell numbers (Fig. 3B, middle). In the ILA, the majority of hM3Dq-expressing cells were also C-FOS+ (Fig. 3B, right), supporting that the responses of ILA neurons were regulated

by hM3Dq and C21. Together, these results support the specificity of our experimental manipulation.

Mouse performance in the OF was comparable between hM3Dq- and tdTomato-expressing male M120I mice (Supplementary Fig. 5A). Comparing data from the first and second RSI assays, we found that C21 treatment enhanced the social interaction time of hM3Dq-expressing male M120I mice, but not that of the tdTomato-expressing mutants (Fig. 3C). In the 3C test, C21 treatment increased both sociability and novelty preference of hM3Dq-expressing male M120I mice (Fig. 3C). Unlike for male M120I mice, the same chemogenetic approach did not enhance the behaviours of male WT mice expressing hM3Dq at the ILA (Fig. 3D), likely due to a ceiling effect. Together, these results support that enhanced ILA neuronal activity ameliorates the social deficits of male M120I mice.

Next, we investigated if the ILA is also critical for controlling social interaction among female mice. AAV-CaMKII-hM4Di-mCherry was infected into the ILA to inactivate neuronal activity upon C21 treatment (Fig. 3A). Similar to our observations for male mice, surgery and AAV expression did not alter the behaviours of female WT or M120I mutant mice in the OF (Supplementary Fig. 6C and D). Importantly, ILA inactivation by chemogenetic manipulation noticeably impaired the social behaviours of both WT and M120I female mice in RSI and 3C tests (Fig. 3E and F). Thus, the ILA is a critical region controlling the social behaviours of both male and female mice.

We then investigated if ILA activation is sufficient to activate other male-biased brain regions in M120I mice. Chemogenetic activation was again performed to activate the ILA in M120I male mice. After C21 treatment, we found that the numbers of C-FOS+ cells also increased in four other male-biased regions, including BSTam, DGd, BLAa and VTA (Fig. 3G). The effect was specific, because the lateral septal nucleus, medial septal nucleus, lateral amygdala and lateral habenula were not significantly affected (Fig. 3G), indicating that the ILA influences the BSTam, DGd, BLAa and VTA to control social behaviours. Each of these five regions also had more C-FOS+ cells in mutant females compared to mutant males upon social stimulation (Fig. 2), further strengthening their role in controlling sex-biased social deficits.

Altered ILA synaptic proteomes of M120I mutant mice

Since *Cttnbp2* deletion was previously shown to alter synaptic protein targeting,¹⁸ we investigated if the M120I mutation also influences the composition of synaptic proteins in the ILA and if any alteration is relevant to the male-biased social deficits of M120I mice. We collected synaptosomal fractions of the ILA from both sexes of M120I mutant mice and WT littermates and subjected them to label-free LC-MS-MS to identify differentially expressed proteins (DEPs). Principal component analysis of entire proteomes indicated that male and female mouse brains were distinguishable (Supplementary Fig. 7A). We then selected proteins with a reliable peptide signal and an adjusted *P*-value <0.05 for DEP identification (Fig. 4A–F, Supplementary Fig. 6 and Supplementary Tables 4 and 5).

The protein networks and gene ontology (GO) of these DEPs in the ILA were then analysed using STRING (<https://string-db.org>), Ingenuity Pathway Analysis (IPA, Qiagen), and Syngo (<https://www.syngoportal.org>). For male-only downregulated DEPs, the most significant GO terms were synapse-, neuron projection- and mitochondrion-related proteins (Fig. 4B, left), which may account for the ILA synaptic deficits displayed by male M120I mice. Indeed,

our IPA indicated an association of male-only DEPs with neurological and neuropsychiatric disorders, such as autism, schizophrenia, bipolar disorder, obsessive-compulsive disorders, hyperactivity, and intellectual disability (Fig. 4C and Supplementary Fig. 7C), supporting roles for these DEPs in synaptic function.

Among male-only upregulated DEPs, proteins contributing to translation were apparent (Fig. 4B, right). Since the reduced dendritic spine density attributable to the M120I mutation likely restricts synaptic activity-dependent protein synthesis, these upregulated DEPs likely represent a scenario where neurons were trying to compensate for the synaptic deficits by controlling protein synthesis.

It was unexpected to find more female-only DEPs in the ILA compared to males (Fig. 4D and E and Supplementary Table 5). The primary GO terms were related to mitochondria, neuron projection, synapse and endoplasmic reticulum (Fig. 4E), but the DEPs differed from those of males. Moreover, the IPA did not identify neuropsychiatric disorders relevant to female-only downregulated DEPs, which is consistent with the normal social behaviours displayed by female M120I mice.

The results of our Syngo analysis were similar to those of STRING. We identified several synaptic proteins at both post- and pre-synaptic sites in male up and downregulated DEPs and female downregulated DEPs. However, only two synaptic proteins were identified among female upregulated DEPs (Supplementary Fig. 7D and E). We further explored female-only upregulated DEPs to understand how females are resistant to *Cttnbp2* deficiency. STRING analysis identified several proteins related to stress responses and the proteasome, but there were no obvious GO terms associated with synaptic function (Fig. 4E, right). Accordingly, we searched the available literature manually to annotate female upregulated DEPs, which uncovered eight female upregulated DEPs reportedly linked to autism, schizophrenia, microcephaly or developmental delay (Fig. 4F and Supplementary Table 6). Among them, we noticed that two of the DEPs influence mTORC1 activity, i.e. RRAGC (Ras-related GTP-binding protein C) and TIPRL (TOR signalling pathway regulator). RRAGC regulates the relocalization of mTORC1 to lysosomes in response to the availability of amino acids, especially BCAAs, including leucine, isoleucine and valine, and consequently controls protein synthesis.^{35–37} TIPRL associates with PP2A, playing a positive role in regulating mTOR activity.³⁸ Thus, enhancing RRAGC and TIPRL expression in female M120I mice likely increases mTOR pathway activity to promote protein synthesis.

We adopted an immunoblotting approach to validate our proteomic results. Among male-only downregulated DEPs, we detected reduced synaptic protein levels of SHANK2, PSD95 and SYNPO in the ILA of male M120I mice relative to WT, but that was not the case for female counterparts (Fig. 4G). In the female-only upregulated DEPs group, protein levels of both RRAGC and TIPRL were indeed increased in female M120I mutant mice relative to WT, but again male mice did not present the same outcome (Fig. 4H). Moreover, consistent with the increased levels of RRAGC and TIPRL, we determined that mTOR phosphorylation levels were higher in female M120I mice compared to WT females (Fig. 4I). Together, these immunoblotting results strengthen the reliability of our proteomic analysis and also highlight the involvement of the mTOR pathway in responses to *Cttnbp2* deficiency.

Amelioration of the social deficits of male M120I mice via BCAA supplementation

Based on the results of our synaptic proteome analysis and our previous study,^{18,20} we deduced two potential ways of treating the

male-biased social deficits displayed by M120I mice. Our first approach was to enhance protein synthesis. Synaptic stimulation increases protein synthesis, which feeds back to strengthen synaptic responses and connectivity.^{39–41} Increased mTOR-regulated protein synthesis by means of leucine supplementation was also shown to ameliorate dendritic spine deficits, enhance neuronal activation and improve social behaviours of two other autism mouse models, i.e. *Nf1* and *Vcp* mutant mice.^{28,42} Based on this evidence, we speculated that increased mTOR activity through enhanced expression of RRAGC and TIPRL in female M120I mice may have provided a beneficial effect in terms of female behaviours. Accordingly, artificially manipulating mTOR activity by means of BCAA supplementation may also exert a beneficial effect on M120I males.

To test that hypothesis, we supplemented the drinking water of juvenile mice from P35 to P42 with BCAA (Leu:ile:Val=2:1:1) (Fig. 5A). Then, we monitored mTOR pathway activity, dendritic spine density, social behaviours, and neuronal activation (as represented by C-FOS expression). As anticipated, BCAA supplementation enhanced the levels of phospho-mTOR and phospho-S6K in the ILA of male M120I mice (Fig. 5B). BCAA supplementation also ameliorated the dendritic spine defects in the ILA of male M120I mice (Fig. 5C). However, it did not alter the body weight of WT or mutant mice (Supplementary Fig. 8A). In terms of our behavioural tests, BCAA supplementation did not influence mouse behaviours in an open field, but it did enhance the social interactions of male M120I mice to a level comparable to WT males (Fig. 5D and Supplementary Fig. 8B). Moreover, the numbers of C-FOS+ cells in the ILA were also comparable between WT and mutant mice upon BCAA supplementation (Fig. 5E). These results support our hypothesis that increasing protein synthesis can improve the social deficits of juvenile M120I mice.

We also investigated the effect of BCAA supplementation on adult M120I male mice by performing two RSI tests at 9 and 10 weeks, with BCAA supplemented for 7 days between both tests (Fig. 5F). As per our finding for juvenile mice, BCAA supplementation increased the social interactions of adult male M120I mice (Fig. 5F). Thus, BCAA supplementation ameliorates social deficits in both adult and juvenile male mice.

Differential zinc demand contributes to the sex-biased response

The second approach to tackling the male-biased social deficits displayed by M120I mutant mice was zinc supplementation. Our recent study showed that *Cttnbp2* knockout results in impaired synaptic targeting of several zinc-binding proteins, including SHANK1, SHANK2 and SHANK3.¹⁸ Zinc supplementation can restore the synaptic distribution of SHANK proteins in adult male *Cttnbp2*^{-/-} mice and improve their social interactions.¹⁸ Notably, SHANK2 and PSD-95, two critical synaptic zinc-binding proteins, were identified among the group of male-only downregulated DEPs. Therefore, we investigated the potential beneficial effect of zinc supplementation on juvenile male M120I mice.

For all of the experiments described in the previous sections, the mice were fed on a diet containing 84 ppm zinc. To test the effect of zinc, juvenile M120I mice were fed on diets with 150 ppm zinc (Fig. 6A).⁴³ These mice behaved normally in an open field and in the elevated plus maze (Supplementary Fig. 9). Importantly, male M120I mice behaved comparably to their male WT littermates in the 3C test (Fig. 6B). In the RSI test, although male zinc-supplemented M120I mice still spent a shorter time interacting with strangers compared with WT littermates (Fig. 6B), the

difference was smaller than for the groups fed on an 84 ppm zinc diet (Fig. 1D versus 6B). For female M120I mice, zinc supplementation did not appear to elicit any change in behaviour (Fig. 6B). Thus, increased zinc intake ameliorates the social interaction deficits of juvenile male M120I mice, albeit incompletely.

Next, we investigated if reducing zinc intake could induce social deficits in female M120I mice. Given that 30 ppm dietary zinc is sufficient for the entire life-cycle of WT mice,⁴⁴ we investigated if reducing dietary zinc from 84 to 30 ppm was sufficient to induce social defects in female M120I mice and found that, unlike their male littermates, female M120I mice fed on 30 ppm zinc diets still behaved normally in both 3C and RSI tests (Fig. 6C).

Overall then, our results indicate that the social behaviours of male M120I mice are susceptible to levels of zinc intake. In contrast, female mice are resilient to the change in zinc levels.

Discussion

In this report, we suggest that a deficiency of *Cttnbp2* (an autism-associated gene) influences the activity of ILA-related circuits in male mice but not in female mice, consequently impairing social behaviours in a male-biased manner. The differential synaptic proteomes of male and female mutant mice indicate the potential involvement of zinc and mTOR pathways in the sex-biased responses of *Cttnbp2* mutant mice. Indeed, zinc and BCAA supplementations both improved the social behaviours of male *Cttnbp2* mutant mice. In contrast, females proved resilient to *Cttnbp2* deficiency given that their demands for BCAA and zinc are lower than those of male mice.

Note that, to maintain health, males generally require more zinc and protein than females (<https://ods.od.nih.gov/HealthInformation/nutrientrecommendations.aspx>). Adult men are recommended to take 11–16 mg zinc daily, whereas women require 7–10 mg zinc daily.^{45–47} Thus, men require at least 30–50% more zinc than women. In terms of protein, men and women need 56 g and 46 g of protein every day, respectively, so protein demand is ~20% higher in men than women.^{48,49} A previous study reported that mutations of BCKDK (branched-chain keto acid dehydrogenase kinase) promote BCAA catabolism and reduce BCAA levels in sera and peripheral tissues, such as muscles, consequently reducing BCAA levels in the brain and contributing to autism and epilepsy.⁵⁰ Thus, it seems likely that higher demand for zinc and protein in the peripheral tissues of males sensitize them to the deficits caused by *Cttnbp2* deficiency. It will be intriguing to further explore the crosstalk between genetic variations and nutrients in mouse disease models and human patients.

In the human brain, sex-biased differences have been reported in brain volume and the percentages of grey matter and white matter.⁵¹ However, similar to the findings of a previous study using a mouse model,⁵² we did not detect a brain size difference between male and female mice. Instead, we found that *Cttnbp2* M120I mutation results in differential activation across male and female mouse brains. The M120I mutation has an obvious inhibitory effect on neuronal activation in ILA-related circuits (encompassing the BLA, DG, BST and VTA) of male but not female mice. These areas are likely critical to the resilience of female mice to *Cttnbp2* mutation, although the mechanism remains unclear.

The female protective effect and multiple-hit hypothesis are two well-known hypotheses postulated to explain the male bias in autism spectrum disorders.^{1,3,53,54} Our study reveals that genetic variation, sex and nutrients act in concert to influence the outcome of autism spectrum disorders, thus fitting the multiple-hit

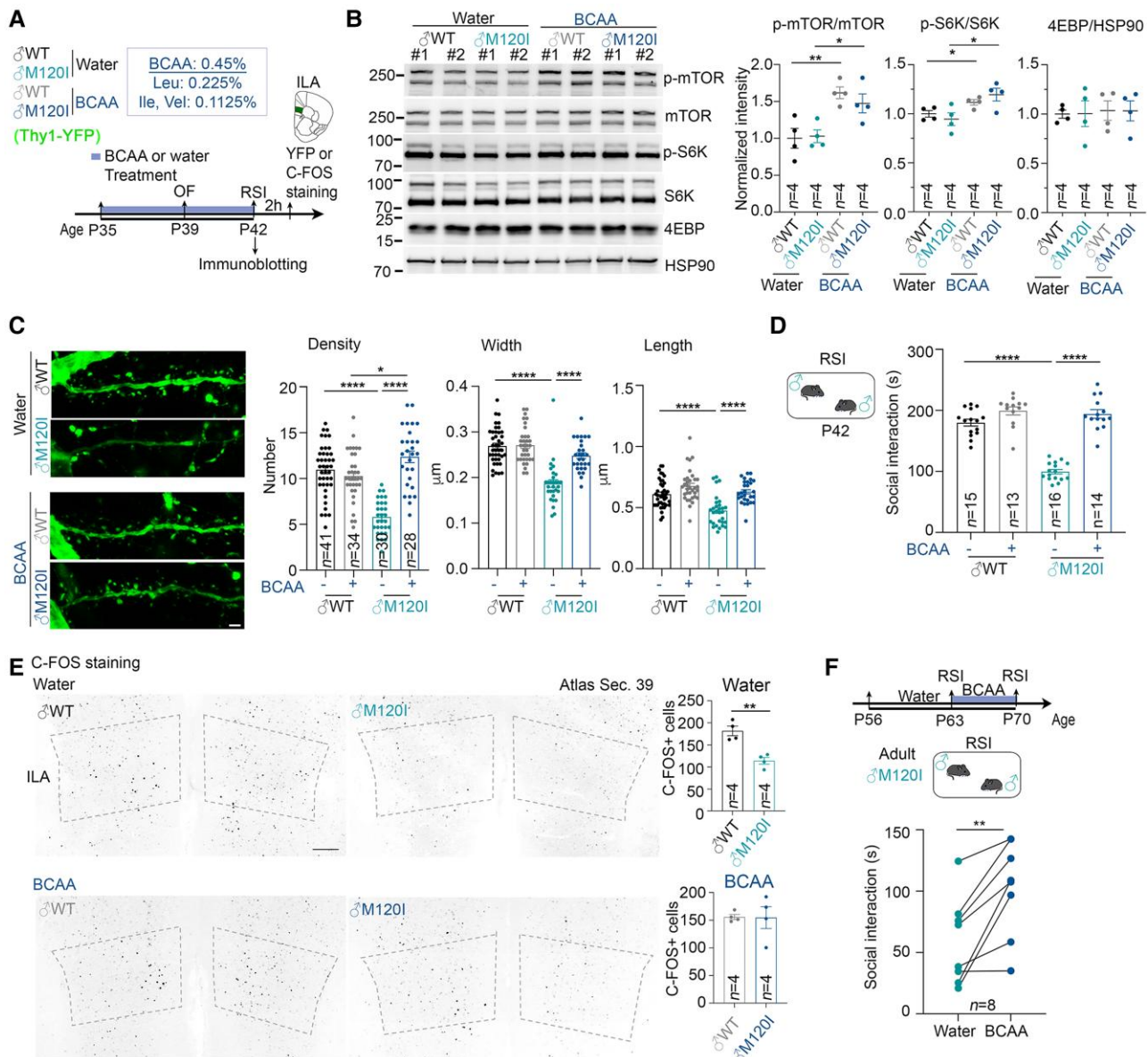


Figure 5 BCAA supplementation increases the social interactions of male *Ctnnbp2* M120I mice. (A) Schematic of the experimental timeline for BCAA treatment and behavioural tests. Male mice expressing YFP under the control of the Thy1 promoter started drinking BCAA-supplemented water at P35. Mice that drank regular drinking water were included as controls. OF and RSI tests were performed at P39 and P42, respectively. Some mouse brains were collected directly after the RSI test for immunoblotting. Some mouse brains were collected 2 h after the RSI test for C-FOS or Thy1-YFP staining. (B) BCAA treatment increases mTOR activity of the mouse cerebral cortex. Left: The results of immunoblotting using different antibodies as indicated. Right: Quantification of immunoblotting. Two lanes for each group in the blot represent synaptosomal fractions prepared from different mice, #1 and #2. Four mice per group were analysed in two separate experiments. Numbers of mice examined (*n*) are also indicated. Uncropped images are available in [Supplementary Fig. 10](#). (C) The density, width and length of dendritic spines of ILA neurons were analysed based on YFP signal. Left: Representative images of the first branches of apical dendrites of ILA neurons. Right: Quantitative results. Mouse numbers: four for both water- and BCAA-treated male WT (♂WT); three for both water- and BCAA-treated male M120I (♂M120I). Numbers (*n*) of examined neurons are indicated in the panel. (D) RSI test results at P42. Numbers of mice (*n*) are indicated. (E) C-FOS staining results. Left: Representative images of C-FOS staining at the ILA region. Right: C-FOS+ cell density in the ILA. Four mice for each genotype were analysed. (F) Adult M120I mice also respond to BCAA supplementation. Eight adult M120I mice were analysed. Data are presented as means ± SEM, and the results of individual mice are shown. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. (B) Two-tailed unpaired t-test was used to examine the effect of genotype. (C) Two-way ANOVA with Tukey's multiple comparisons post hoc test. (D) Two-way ANOVA with Tukey's multiple comparisons post hoc test. (E) Two-tailed unpaired t-test. (F) Two-tailed paired t-test. All statistical analysis and results, including the actual *P*-values, are summarized in [Supplementary Table 7](#).

hypothesis. In addition, our results are consistent with the hypothesis of a female protective effect, which suggests a higher threshold for autism spectrum disorders in females.^{53–55} The identification of more genetic variations among female patients indeed supports this hypothesis.^{53–55} However, there is only a single genetic

variation, i.e. *Ctnnbp2* M120I, in the model system we assessed here. Thus, multiple genetic variations are irrelevant to our system. Instead, our results suggest that sex-related nutrient demands elicit autism-linked phenotypes in the presence of *Ctnnbp2* mutation. Our study indicates that females hosting *Ctnnbp2* mutations are

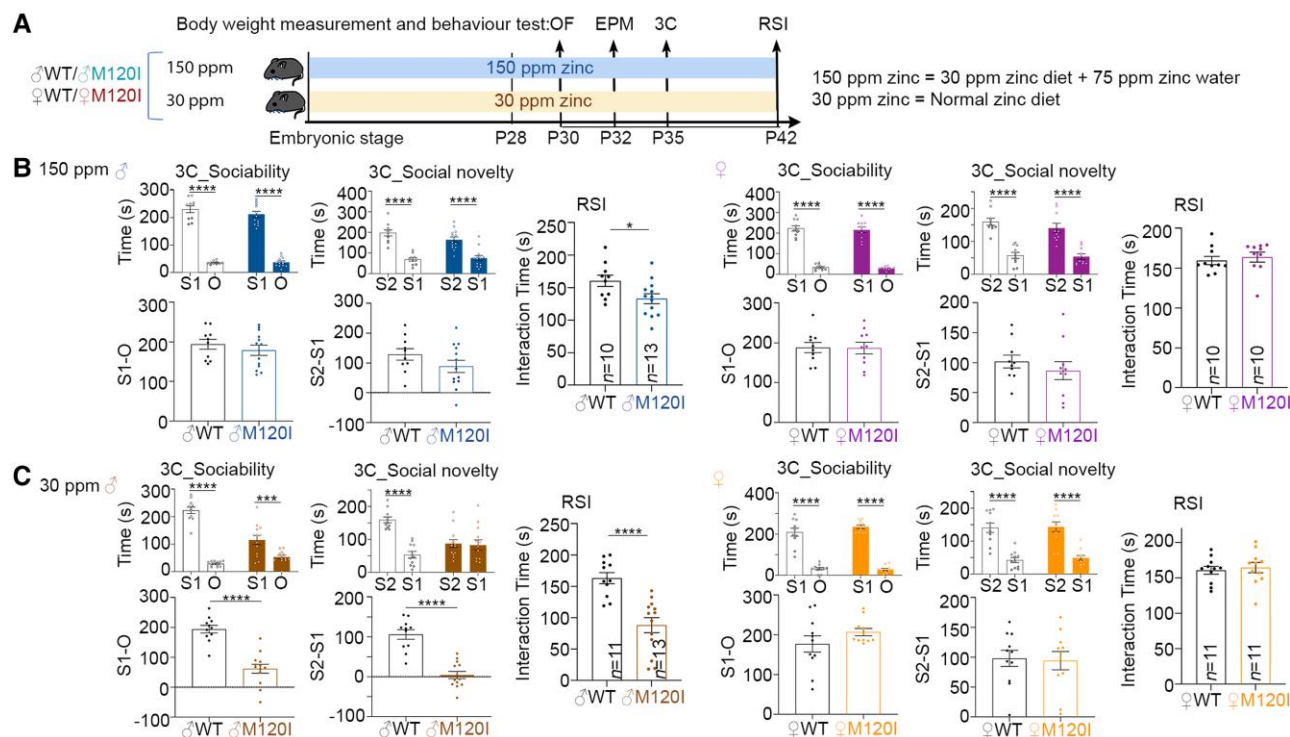


Figure 6 The social behaviours of female M120I mice are insensitive to the levels of zinc intake. (A) Schematic of the experimental timeline and behavioural tests. Mice were fed on 150 or 30 ppm zinc diets since the embryonic stage, as indicated. (B and C) The results of the 3C and RSI tests are shown as indicated. Left = male; right = female. (B) The results for mice fed on the 150 ppm zinc diet. (C) The results for mice fed on the 30 ppm zinc diet. The same sets of mice were used for the OF, EPM, 3C and RSI tests. The results for OF and EPM are summarized in [Supplementary Fig. 9](#). The sample sizes (*n*, i.e. numbers of mice) are indicated only for the results of RSI. Data are presented as means \pm SEM, and the results of individual mice are shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. For the results of the 3C test, the interaction times were analysed using two-way ANOVA with Tukey's multiple comparisons post hoc test. The preference index and RSI were analysed using a two-tailed unpaired *t*-test. All statistical analysis and results, including the actual *P*-values, are summarized in [Supplementary Table 7](#).

more protected than males because females have lower demands for BCAA and zinc. Accordingly, differing nutrient demands may be another factor modulating thresholds for the manifestation of autism spectrum disorders.

In addition to our current study, previous studies have also indicated roles for zinc and BCAA in autism spectrum disorders. Zinc directly regulates many autism-linked genes.^{56–60} For instance, zinc directly binds NMDAR, or enhances SRC kinase activity to phosphorylate NMDAR, thereby regulating NMDAR conductivity.^{59,61} Zinc also binds SHANK2 and SHANK3 to regulate their synaptic distribution and functions in the brain.^{43,62–65} Thus, zinc plays multiple roles in controlling synaptic activity. Consequently, zinc supplementation improves behavioural deficits of several autism mouse models, such as *Shank3*, *Shank2* and *Tbr1* mutant mice.^{43,64,66} Moreover, our previous study on *Cttnbp2* knockout mice demonstrated that CTTNBP2 controls synaptic targeting of 18 zinc-binding or zinc-regulated proteins.¹⁸ Zinc supplementation ameliorates the synaptic targeting deficits of CTTNBP2-regulated proteins and improves the social behaviours of *Cttnbp2* knockout mice.⁴² Here, we have further shown that M120I mutation of *Cttnbp2* also results in reduced levels of several synaptic proteins. Zinc supplementation likely improves the defects of M120I mice by bolstering direct targets of CTTNBP2 or enhancing the general activity of neurons via other molecules and/or pathways.

BCAA metabolism has also been linked to autism spectrum disorders.^{50,67,68} In addition to the BCKDK gene mentioned above, solute carrier transporter 7a5 (SLC7A5), a BCAA transporter localized at the blood–brain barrier, was also found to be relevant to autism

spectrum disorders.⁶⁷ Maintenance of BCAA levels in the brain is critical to neuronal function because deletion of SLC7A5 reduces the levels of BCAA in the brain and results in autism-linked phenotypes. Moreover, a cohort study on 516 autistic children and 164 age-matched children further indicated a reduced level of serum BCAA in the children with autism.⁶⁸ These studies strongly support a role for BCAA in autism spectrum disorders.

Our own studies have further revealed the function of BCAA in synaptic formation and responses. Previously, we have demonstrated that *Cttnbp2* M120I mutation reduces the density and size of dendritic spines in mouse brains.¹⁹ Consistent with the reduced dendritic spine density, M120I mutation also decreased the frequency of miniature excitatory postsynaptic currents.¹⁹ Here, we found that *Cttnbp2* M120I mice derive a benefit from BCAA supplementation. Dendritic spine density, neuronal activation and social behaviours of *Cttnbp2* M120I mice are all improved by BCAA supplementation in the diet. In addition to *Cttnbp2* M120I mice, our previous study showed that supplementation of leucine, the most potent BCAA for activating the mTOR pathway, induces protein synthesis, increases dendritic spine density, and improves the social behaviours and memory of *Nf1*^{+/-} and *Vcp*^{+ /R95G} mice.^{28,42,69} The molecular functions of the proteins encoded by *Nf1*, *Vcp* and *Cttnbp2* vary considerably. Neurofibromin, the protein product of *Nf1*, controls dendritic spine density via PKA activation,⁷⁰ and it interacts with VCP to control endoplasmic reticulum formation and protein synthesis.^{28,69,71} VCP functions as an AAA ATPase to control many cellular processes, with endoplasmic reticulum formation and consequent protein synthesis being the most critical downstream

targets of VCP to regulate dendritic spine formation.^{28,42,69} Since neurofibromin and VCP act in the same pathway, it is reasonable that neurofibromin and VCP deficits can be ameliorated by the same treatment, i.e. leucine supplementation. In contrast, as a cytoskeleton-associated protein, there is no evidence supporting a direct role for CTTNBP2 in controlling protein synthesis. However, fewer and/or smaller dendritic spines may limit the efficiency of protein synthesis controlled by synaptic activation. An increase in BCAA intake activates the mTOR pathway to induce protein synthesis, independently of synaptic stimulation.³⁹ Accordingly, we expect that many other autism-linked mutations that result in fewer and/or smaller dendritic spines may share the same property of their impacts being counteracted by BCAA supplementation. Thus, it will be important to apply BCAA supplementation to other autism-linked mouse models to test our hypothesis.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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