Dietary zinc supplementation rescues fear-based learning and synaptic function in the Tbr1+/− mouse model of autism spectrum disorders

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Abstract

Background: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterised by a dyad of behavioural symptoms—social and communication deficits and repetitive behaviours. Multiple aetiological genetic and environmental factors have been identified as causing or increasing the likelihood of ASD, including serum zinc deficiency. Our previous studies revealed that dietary zinc supplementation can normalise impaired social behaviours, excessive grooming, and heightened anxiety in a Shank3 mouse model of ASD, as well as the amelioration of synapse dysfunction. Here, we have examined the efficacy and breadth of dietary zinc supplementation as an effective therapeutic strategy utilising a non-Shank-related mouse model of ASD—mice with Tbr1 haploinsufficiency.

Methods: We performed behavioural assays, amygdalar slice whole-cell patch-clamp electrophysiology, and immunohistochemistry to characterise the synaptic mechanisms underlying the ASD-associated behavioural deficits observed in Tbr1+/− mice and the therapeutic potential of dietary zinc supplementation. Two-way analysis of variance (ANOVA) with Šídák’s post hoc test and one-way ANOVA with Tukey’s post hoc multiple comparisons were performed for statistical analysis.

Results: Our data show that dietary zinc supplementation prevents impairments in auditory fear memory and social interaction, but not social novelty, in the Tbr1+/− mice. Tbr1 haploinsufficiency did not induce excessive grooming nor elevate anxiety in mice. At the synaptic level, dietary zinc supplementation reversed α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartate receptor (NMDA) hypofunction and normalised presynaptic function at thalamic-lateral amygdala (LA) synapses that are crucial for auditory fear memory. In addition, the zinc supplemented diet significantly restored the synaptic puncta density of the GluN1 subunit essential for functional NMDARs as well as SHANK3 expression in both the basal and lateral amygdala (BLA) of Tbr1+/− mice.

Limitations: The therapeutic effect of dietary zinc supplementation observed in rodent models may not reproduce the same effects in human patients. The effect of dietary zinc supplementation on synaptic function in other brain regions remains to be determined.

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Background

Autism spectrum disorder (ASD) is a prevalent neurodevelopmental disorder characterised by social and communication deficits, excessive repetitive behaviours, sensory abnormalities, and cognitive dysfunction [1, 2]. Although the full understanding of the aetiology of ASD has not yet been established, a diverse range of genetic aberrations and environmental factors are thought to contribute to the heterogeneity of behavioural traits observed in children with ASD [3]. In recent years, next-generation whole-genome sequencing has identified hundreds of deleterious variants in 10–30% of ASD patients [4, 5]. Among the recurrent ASD-associated high-confidence risk genes, T-brain-1 (TBR1) has been of particular interest because it encodes a neuron-specific transcription regulator of the T-box family that modulates cortical development and brain wiring [6]. To date, genetic aberrations in one allele of TBR1 have been discovered in ASD patients who commonly display socialisation impairments, defective language skills, and intellectual disability [7–13]. Consistently, mouse models of Tbr1 haploinsufficiency (Tbr1+/−) or human TBR1 mutation knock-in (Tbr1+/K228E) also display ASD-like behavioural deficits [14–16], and thus, serve as good models to further our understanding of the pathogenic mechanisms of ASD induced via de novo TBR1 mutations.

Tbr1 is highly expressed in early-born excitatory glutamatergic neurons [17–19], and plays a critical role in neuronal differentiation, migration, laminar fate, and regional identity during brain development [20–27]. Mice carrying a homozygous mutation of the Tbr1 gene (i.e. deletion of both alleles of Tbr1, Tbr1−/−) demonstrate impaired differentiation and migration of embryonic cortical neurons as well as abnormal axonal projections in the cerebral cortex, amygdala, and olfactory bulb [14, 18, 20, 23], resulting in early postnatal death [18]. Tbr1+/− mice display significant diminishment of the anterior commissure (a bundle of white matter tract fibres connecting two cerebral hemispheres, including the two amygdala) [14], which is also observed in individuals with de novo TBR1 variants [28].

In addition to its role in brain development, Tbr1 also responds to changes in neuronal activity. Enhanced neuronal excitation upregulates Tbr1 expression in cultured mature neurons [29], and its direct interaction with calcium/calmodulin-dependent serine protein kinase (CASK), a multidomain scaffolding protein associated with neural development and synaptic function [30–33]. Together with the CASK interacting nucleosome assembly protein (CINAP) [6], TBR1, CASK, and CINAP form a tripartite complex that regulates Grin2b expression [29, 34, 35]. As the upregulation of Tbr1 expression requires activation of calcium/calmodulin-dependent protein kinase II (CaMKII) via calcium influx through NMDARs [29], Tbr1 and Grin2b form a modulatory feedback loop that links neuronal activation and synaptic function. This is evident in Tbr1 deficient mice where Grin2b expression is decreased [36], and amygdalar neurons of Tbr1+/− mice fail to show neuronal activation-induced upregulation of Grin2b expression and impaired NMDAR function at thalamic-amygdalar synapses [14, 37–39].

Our data further the understanding of the molecular mechanisms underlying the effect of dietary zinc supplementation and verify the efficacy and breadth of its application as a potential treatment strategy for ASD.

Keywords: Autism spectrum disorder, T-brain-1, Dietary zinc supplementation, Amygdala, Glutamatergic synapses, N-methyl-D-aspartate receptors
in Tbr1+/− mice. Specifically, we show that dietary zinc elevation can restore social interaction, fear memory, and functional thalamic-amygdalar synapses in the Tbr1+/− mice, thereby broadening the applicability of dietary zinc supplementation for ASD treatment.

Methods

Animals
All animal manipulations and experiments were performed under regulations approved by the University of Auckland Animal Ethics Committee and in adherence to the ARRIVE guidelines. The Tbr1+/− mice were provided by Y.-P. Hsueh (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan) [14, 15] with permission of Dr. John Rubenstein (University of California, San Francisco) and maintained at the Vernon Jansen Unit animal facility at the University of Auckland, Auckland, New Zealand. Wildtype Tbr+/+ (WT) and heterozygous Tbr+/− mice were generated from Tbr+/− male x WT female breeding pairs. All experimental animals were housed under a standard 12/12-h light–dark cycle. Offspring were ear-punched for genotyping and identification at postnatal day 10, and were kept with the dam until weaning at postnatal day 21. After weaning, animals were housed in groups of 2–4 per individually ventilated cage with mixed genotypes. Food and water were available ad libitum.

Experimental animals
At weaning, animals were randomly assigned to a control zinc diet (30 ppm [parts per million] zinc; D19410B; Research Diets, Brunswick, NJ, USA) or a zinc supplemented diet (150 ppm zinc; D06041101; Research Diets, Brunswick, NJ, USA), for 6–8 weeks. These egg white-based diets were identical in composition except for their zinc levels. As we have previously shown in Shank3ex13–16−/− mice [45, 46], which have the same C57BL/6J background, no adverse effects on animal health, weight, or development were evident on either diet. No significant differences were observed in body weight for all Tbr1 genotypes: Male mice at 9 weeks: WT30ppm 22.00 ± 0.53 g; Tbr+/− 30 ppm 21.00 ± 0.65 g; WT150ppm 22.00 ± 0.66 g; Tbr+/− 150 ppm 21.82 ± 0.99 g. Female mice at 9 weeks: WT30ppm 20.00 ± 0.51 g; Tbr+/− 30 ppm 19.75 ± 0.41 g; WT150ppm 19.70 ± 0.40 g; Tbr+/− 150 ppm 20.09 ± 0.46 g. Behavioural, electrophysiological, and imaging experiments were performed at 9–11 weeks of age on male and female mice on either diet. In total 82 WT mice and 88 Heterozygous mice were used in these experiments. Both genders were included in all experimental data sets, and WT mice were used as controls (WT: immunohistochemistry—5 males and 5 females; electrophysiology—6 males and 7 females; behavioural experiments—27 males and 22 females; Tbr+/−: immunohistochemistry—5 males and 5 females; electrophysiology—5 males and 9 females; behavioural experiments—25 males and 23 females). The animals were randomly grouped and were used independently for behavioural, electrophysiological, or imaging experiments. All experiments and analyses were conducted with the experimenter blinded to genotype and zinc diet by independent animal coding with a unique identification number at weaning.

Behavioural tests and analysis
All behavioural tests were performed in an isolated room under the light cycle, and the animals were habituated in the room for at least one hour before testing. Except for the auditory fear conditioning test, a ceiling camera DFK21AF04 (SDR Scientific) was used for recording and monitoring animal behaviour. EzTrack (Open source) [47] was used for generating heat maps and tracking animal movement. The grooming test arena, dark–light chamber, and three chamber apparatus were washed with 70% ethanol and then 3% acetic acid between each behavioural trial. The same cohort of animals underwent the grooming, light and dark, social interaction, and social novelty tests, respectively, over a period of 1–2 weeks. No two behavioural tests were performed on the same day. A separate cohort was used for the auditory fear conditioning test so that social behaviour was not influenced by fear conditioning.

Auditory fear conditioning test
Auditory fear conditioning was performed in operant chambers (17 × 17 × 25 cm³, Ugo Basile) within sound-attenuating boxes (Ugo Basile) illuminated by a 7.5 W white light and constant white noise of 60 dB playing in the background through built-in speakers. Animal motion was recorded with the built-in camera, and the freezing behaviour was analysed by EthoVision XT Version 12.0 (Noldus). The chambers were cleaned with 3% acetic acid (training days and day 0) or 70% ethanol (day 1). Each mouse was placed in the operant chamber and habituated for 8 min on training day. On day 0, each mouse was placed into the operant chamber for 4 min, and then 3 auditory conditioned trials were performed, comprising of a tone (2 kHz; 80 dB; 18 s) followed by an electric foot shock (0.6 mA; 2 s) in 1 min intervals. Freezing responses of the mice immediately after the third electric foot shock over 60 s were measured as “after shock”. Mice were returned to their home cages after the training. On day 1, mice were placed in a novel operant chamber, and forty tones (80 dB; 2 kHz; 20 s) were applied at 5 s intervals. Time spent freezing was averaged over the first ten auditory stimulations [14].
**Grooming behaviour**

Under red light conditions [15 lx], each mouse was placed in a cylindrical arena [17 cm radius] and habituated for 10 min. The behaviour of the mouse was then recorded, and the grooming behaviour was analysed over 30 min. Grooming behaviour included licking, wiping, and rubbing any body parts [45, 46].

**Dark–light emergence test**

Each mouse was placed in a dark chamber (001 lx) for 5 min. The door between the dark and light chambers was opened enabling the mouse to freely explore the chambers for 10 min. The latency to enter the light chamber (275 lx), transition number to enter the light chamber, and the time spent in the light chamber were measured.

**Three-chamber social interaction test**

The three chamber apparatus was used under low light conditions (15 lx) and conducted as previously described [14, 37, 45, 46]. Each mouse was placed in the middle chamber, and a mesh container was placed in both the left and right chambers. The doors between the chambers were then opened to enable the mouse to explore all three chambers. After 10 min, the mouse was gently guided to the centre chamber, and the doors were closed. In phase 2, a sex and age-matched stranger mouse (Stranger 1: S1) was placed in one of the containers, while the other chamber’s container remained empty (Empty cup: E). The test mouse was then allowed to explore freely for 10 min. The mouse was again gently guided to the centre chamber, and the doors were closed. In the last phase of the test, another sex and age-matched stranger mouse (Stranger 2: S2) was introduced in the previously empty container. The mouse was then allowed to explore for 10 min in all three chambers. The social interaction reference index was calculated by the time spent in close interaction with S1 subtracted by time spent in close interaction with the empty cup in the chamber. The social novelty reference index was calculated by the time spent in close interaction with S2 subtracted by time spent in close interaction with S1.

**Electrophysiology**

**Acute brain slice preparation**

Acute coronal brain slices were prepared from 9- to 11-week-old mice, as described previously [37]. Mice were euthanised with CO2 and decapitated, and the brain was rapidly sectioned at 300 µm thickness using a vibratome (VT1200S, Leica Biosystems; 1 mm x-plane blade vibration, 0.05 mm/min y-plane advancement speed, 0 mm z-axis vibration calibration), obtaining on average 3–4 coronal slices including the lateral amygdala (LA). Slices were transferred to protective cutting aCSF at 34 °C for 12 min. Before recording, slices were maintained at room temperature (RT, 24 °C) for a minimum of 1 h, submerged in a holding chamber containing carbogenated recovery aCSF: (in mM) 97 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 25 glucose, 20 HEPES, 2 CaCl2, 2 MgSO4·7H2O, 2 thiourea, 5 L-ascorbic acid, 3 Napyruvate, 0.5 CaCl2, 10 MgSO4·7H2O, pH 7.4, 295–305 mOsm. The brain was rapidly sectioned at 300 µm thickness using a vibratome (VT1200S, Leica Biosystems; 1 mm x-plane blade vibration, 0.05 mm/min y-plane advancement speed, 0 mm z-axis vibration calibration), obtaining on average 3–4 coronal slices including the lateral amygdala (LA). Slices were transferred to protective cutting aCSF at 34 °C for 12 min. Before recording, slices were maintained at room temperature (RT, 24 °C) for a minimum of 1 h, submerged in a holding chamber containing carbogenated recovery aCSF: (in mM) 97 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 25 glucose, 20 HEPES, 2 CaCl2, 2 MgSO4·7H2O, 2 thiourea, 5 L-ascorbic acid, 3 Napyruvate, pH 7.4, 295–305 mOsm. Slices were utilised for a maximum of 6 h from preparation, and any damaged, unhealthy slices were omitted from electrophysiological recordings.

**Whole-cell patch-clamp recordings**

Only brain slices with clearly defined thalamic afferents crossing the dorsolateral region of the LA, a converging location of auditory and somatosensory inputs, were utilised for whole-cell patch-clamp experiments. Slices were transferred to the recording chamber, superfused with carbogenated recording aCSF: (in mM) 119 NaCl, 2.5 KCl, 1 Na2HPO4, 1.3 MgSO4, 26.2 NaHCO3, 11 D-(-)-glucose, 2.5 CaCl2, pH 7.4, 305–310 mOsm at RT at 2–3 mL/minute. The principal neurons of the LA were visualised using infrared differential interference contrast (IR-DIC) optics with a 40× water immersion objective lens mounted on an Olympus BX-51 microscope (Olympus Corporation, Japan). A platinum-iridium concentric bipolar stimulating electrode was placed onto the thalamic afferent pathway to induce presynaptic stimulation of the principal neurons of the LA. Stimulation was performed with a constant isolated current stimulator (Model DS3; Digitimer, USA) with a duration of 500 µs at 0.05 Hz frequency. Whole-cell patch-clamp recordings of LA principal neurons were acquired with glass recording electrodes (borosilicate tubing filamented glass electrodes, BF150–86–7.5; Sutter Instrument Company, USA) of 5–7 MΩ resistance pulled by a vertical electrode puller (PC-10; Narishige, Japan), and filled with an internal solution: (in mM) 120 Cs gluconate, 40 HEPES, 5 MgCl2, 2 NaATP, 0.3 NaGTP and 5 QX314, pH 7.2, 298 mOsm. The distance between the stimulating electrode and recording electrode was approximately 200 µm. Electrophysiological signals were amplified (Multiclamp 700B; Axon Instruments, USA) and digitised (Digidata 1550B; Axon Instruments, USA). Events were sampled at 20 kHz and low-pass filtered at 2 kHz. All data were obtained and analysed using pClamp 10 acquisition software and Clampfit 10, respectively.
(Axon Instruments, USA). Series resistance (Rs) was < 25 MΩ. Rs was monitored before and after each recording paradigm, and any recordings with Rs variation greater than 20% were discarded from the analysis. Overall, we observed no differences across experimental groups in membrane capacitance (Cm; WT30ppm, 181.4 ± 7.607 pF; Tbr+/−/30 ppm, 196.4 ± 15.03 pF; WT150ppm, 171.8 ± 8.396 pF; Tbr+/−/150 ppm, 195.4 ± 8.585 pF; p > 0.05), membrane resistance (Rm; WT30ppm, 376.8 ± 33.38 MΩ; Tbr+/−/30 ppm, 323.2 ± 52.92 MΩ; WT150ppm, 424.5 ± 53.06 MΩ; Tbr+/−/150 ppm, 378.9 ± 26.02 MΩ; p > 0.05), or access resistance (Ra; WT30ppm, 20.85 ± 0.645 MΩ; Tbr+/−/30 ppm, 22.62 ± 0.575 MΩ; WT150ppm, 20.88 ± 0.765 MΩ; Tbr+/−/150 ppm, 21.33 ± 0.794 MΩ; p > 0.05).

To measure α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated excitatory postsynaptic currents (EPSCs), LA principal neurons were voltage-clamped at −70 mV and stimulated with an intensity that was adjusted to provide a half-maximal amplitude. AMPAR-mediated EPSCs were measured for 30 sweeps at 0.05 Hz. Only the peak amplitudes of monosynaptic EPSCs relative to the pre-stimulation baseline were used for data analysis. For paired-pulse ratio (PPR) and NMDAR/AMPAR ratio experiments, the stimulation intensity was modified to the level that stably evoked 200–350 pA AMPAR-mediated EPSCs. For paired-pulse stimulation, two consecutive stimuli separated by 50 ms were delivered to LA principal neurons (voltage-clamped at −70 mV, 30 sweeps, 0.05 Hz). Only recordings that displayed stable evoked first EPSCs were included for data analysis. PPR was determined by dividing the peak amplitude of the second AMPAR-mediated EPSC response by that of the first peak current amplitude. For NMDAR/AMPAR ratio experiments, LA principal neurons were first voltage-clamped at −70 mV, and 30 consecutive AMPAR-mediated EPSCs at a stimulus intensity that stably evoked 200–350 pA current responses were recorded (0.05 Hz) to ensure consistent presynaptic stimulation and AMPAR-mediated responses when measuring NMDAR EPSCs. Then, 10 μM CNQX was bath applied to block AMPAR-mediated current responses, and the holding potential was switched to +40 mV to record NMDAR-mediated EPSCs (30 sweeps, 0.05 Hz). The NMDAR/AMPAR ratio was calculated as the average peak amplitude of NMDAR-mediated EPSCs divided by the average peak amplitude of AMPAR-mediated EPSCs. Picrotoxin (100 μM) was included in the recording aCSF to block GABA_A receptor-mediated inhibitory currents during stimulation. Decay kinetics of the NMDAR-mediated responses were measured by fitting a double exponential function to the decay phase of normalised NMDAR-mediated EPSCs (Clampfit 10) [46, 48]. To compare decay times between different experimental groups directly, we calculated a weighted mean decay time constant: \( r_m = r_f \left[ \frac{I_f}{I_f + I_s} \right] + r_s \left[ \frac{I_s}{I_f + I_s} \right] \), where \( I_f \) and \( I_s \) represent the amplitudes of the fast and slow decay components, respectively, and \( r_f \) and \( r_s \) represent fast and slow decay time constants, respectively.

**Luxol fast blue staining**

After dietary supplementation with normal (30 ppm) and increased (150 ppm) zinc for 6–8 weeks, WT and Tbr+/−/ mice were euthanised with CO2 and transcardially perfused with 20 mL of ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The brains were collected and postfixed in 4% PFA for 24 h at 4 °C and then incubated in 30% sucrose in 0.1 M phosphate buffer for 72 h at 4 °C. Coronal brain sections were prepared at 50 μm thickness. Brain sections from 7 animals were used for each group (i.e. WT30ppm vs. Tbr+/−/30 ppm vs. WT150ppm vs. Tbr+/−/150 ppm). Brain sections were placed in alcohol/chloroform solution and then rinsed with 70% ethanol, 5 min × 3 times. The sections were then placed in 0.1% Luxol fast blue solution overnight at 56 °C and were destained with 0.05% lithium carbonate solution. Each whole brain section was imaged with a Leica MZ6 stereomicroscope using a Leica Flexacam C1 camera at 1.6X magnification.

**Antibodies and immunohistochemistry**

Coronal sections (50 μm) were first permeabilised overnight with 0.25% Triton X-100 in 1 x phosphate-buffered saline (PBST, pH 7.4) at 4 °C. After 3 washes with 1 × PBST, non-specific binding was blocked by incubating the sections with 10% normal goat serum (NGS) in 1 × PBST for 1 h at RT. The sections were immunolabelled for GluN1 (1:500; AGC-001, Alomone Labs), Shank2 (1:500; 162 302, Synaptic Systems) and synapsin1/2 (1:500; 106 004, Synaptic Systems) or Shank3 (1:500; 162 004, Synaptic Systems) with primary antibody solution prepared in 1 × PBST containing 1% NGS for 72 h at 4 °C. The sections were washed in 1 × PBST, incubated for 4 h at RT with secondary antibodies (goat anti-guinea pig IgG-Alexa Fluor 594, 1:500; A11076, Molecular Probes; goat anti-rabbit IgG-Alexa Fluor 594, 1:500; A11012, Molecular Probes; goat anti-rabbit IgG-Alexa Fluor 488, 1:500; Molecular Probes, A21245). No primary antibody controls showed no immunostaining signal for all antibodies used. The sections were further washed in 1 × PBST and incubated with Hoechst (Sigma) for 30 min at RT and mounted on microscope slides (Menzel Glaser) in Citifluor mounting medium (Agar Scientific, AF1).
Confocal imaging and image analysis
GluN1, Shank2 or Shank3 and synapsin1/2 immunostaining in the LA and basal amygdala (BA) was imaged via confocal microscopy (FV1000; Olympus Corporation, Japan) at 63X magnification (UPLSAPO, 1.35 NA) with 3X digital zoom using FluoView 4.0 image acquisition software, yielding images with a size of 512×512 pixels (0.138 μm/pixel, both x and y). Images were taken sequentially, and two counts of the line Kalman integration method were applied. For each section, z-stacks were obtained (30–35 images taken at 0.3 μm apart) from a minimum of two regions in the LA and BA per brain slice, and a minimum of three brain slices were imaged per brain. Imaging parameters including laser power, amplifier gain, and offset, were optimised for each antibody and kept consistent for all subsequent imaging to enable direct comparisons between images of different genotype and zinc diet groups. ImageJ software (NIH, USA) was used for puncta-by-puncta and colocalisation analysis. The postsynaptic GluN1, Shank2 or Shank3, and presynaptic synapsin1/2 immunolabelling was corrected for background intensity variation by subtracting a Gaussian (σ=3 pixels) blurred version of the substack. The 3D Objects Counter tool in ImageJ was then utilised to analyse the number and intensity of GluN1, Shank2 or Shank3, and synapsin1/2 puncta and identify their colocalisation in a 3-dimensional space captured by the z-stack. Only GluN1, Shank2 or Shank3, and synapsin1/2 puncta colocalised in each z-plane were counted as colocalised. GluN1, Shank2 or Shank3 puncta that colocalised with synapsin1/2 were defined as synaptic to ensure data are directly relevant to changes occurring at synapses. For each data set, the image analysis criteria including intensity threshold and detection voxel size range were kept consistent for each experimental set, regardless of the genotype and diet manipulation. The intensity and puncta density (number of puncta per 10,000 μm³) measurements were compared between treatment groups by normalising to the average intensity and puncta density values, respectively, calculated from images from the WT 30 ppm dietary zinc group.

Statistical analysis
All data are presented as mean±standard error of the mean (SEM). Statistical analyses were conducted using Graphpad Prism 9.0, with a p value <0.05 considered significant. The D’Agostino and Pearson normality test was performed to assess normality and homogeneity of variances in the data. Statistical significance was determined by two-way analysis of variance (ANOVA) with Šídák’s post hoc test, one-way ANOVA with Tukey’s post hoc multiple comparisons, or nonparametric Kruskal–Wallis test, depending on the normality of data distribution and data comparison. Significant results are marked with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Details of each statistical test for each data set, experimental ‘n’ number of neurons, images, and animals are provided in the figure legends.

Results
To examine the effects of dietary zinc supplementation on ASD-related behaviours and synaptic deficits beyond Shank3-ASD mutations, we fed wildtype Tbr1+/− (WT) and heterozygous mutant Tbr1−/− mice control (30 ppm) or high (150 ppm) dietary zinc in their normal chow for 6–8 weeks after weaning [45]. This was followed by behavioural testing as well as glutamatergic excitatory synaptic transmission and immunocytotoxic analysis in the amygdala at 9–11 weeks of age.

Auditory fear memory deficits in Tbr1−/− mice are rescued by dietary zinc supplementation
Previously it has been shown that one of the profound behavioural deficits observed in mice with haploinsufficiency in Tbr1 (i.e. Tbr1−/−) is the impairment in fear conditioning [14]. Therefore, we performed an auditory fear conditioning test to examine the efficacy of our zinc supplementation strategy on this ASD phenotype (Fig. 1A). As expected [14], auditory fear conditioning behaviour was significantly altered in Tbr1−/− mice. Specifically, Tbr1−/− mice fed the control 30 ppm zinc diet showed a significant deficit in the percentage of time freezing in response to the auditory tone compared with WT mice, with a value of 45.95% compared to 64.27% in WT mice, reflecting a deficit in fear memory. Dietary supplementation with 150 ppm zinc in WT mice did not induce any significant change in fear memory (WT30ppm, 64.27±4.70%; Tbr1−/− 30 ppm, 46.67±3.64%; WT30ppm vs. Tbr1−/− 30 ppm, p value=0.0060; Fig. 1B), reflecting a deficit in fear memory. Dietary supplementation with 150 ppm zinc in WT mice did not induce any significant change in fear memory (WT150ppm, 69.23±7.48%; Fig. 1C). However, in Tbr1−/− mice fed 150 ppm dietary zinc, fear memory was significantly improved, resulting in no significant difference between WT and Tbr1−/− mice in fear memory (Tbr1−/− 150 ppm, 56.43±6.44%; WT150ppm vs. Tbr1−/− 150 ppm, p value=0.2867; Fig. 1C). Increasing dietary zinc can therefore remove the deficit in fear memory in mice expressing the ASD-associated Tbr1 mutation.

ASD-associated social interaction deficits in Tbr1−/− mice can be prevented by dietary zinc supplementation
We also examined the effect of dietary zinc supplementation on ASD-associated social interaction deficits in WT and Tbr1−/− mice. We observed no significant phenotypic difference in the social interaction test measured as time spent in close interaction, with both WT and Tbr1−/− mice fed the 30 ppm zinc diet both spending significantly more time interacting with the stranger 1 mouse
compared to the empty cup in a chamber (WT\textsubscript{30ppm}, empty = 23.82 ± 4.27 s, stranger 1 = 134.09 ± 14.12 s, \(p\) value < 0.0001; Tbr\textsubscript{+/-}30 ppm, empty = 35.00 ± 4.78 s, stranger 1 = 72.09 ± 7.45 s, \(p\) value = 0.0091; Fig. 2A and B). Increasing dietary zinc levels to 150 ppm did not alter the social interaction phenotype in either WT or Tbr\textsubscript{+/-} mice (WT\textsubscript{150ppm}, empty = 40.89 ± 6.65 s, stranger 1 = 131.00 ± 10.45 s, \(p\) value < 0.0001; Tbr\textsubscript{+/-}150 ppm, empty = 26.00 ± 5.07 s, stranger 1 = 115.27 ± 14.11 s, \(p\) value < 0.0001; Fig. 2A and B). However, Tbr\textsubscript{+/-} mice fed the control 30 ppm diet did show a deficit in preference index, calculated as the difference in time spent with the stranger mouse and empty cup. Specifically, Tbr\textsubscript{+/-} mice fed the 30 ppm control zinc diet spent significantly less time with the stranger mouse relative to the exploration time spent closely interacting with the empty cup, compared with WT mice (WT\textsubscript{30ppm}, 110.27 ± 13.03 s; Tbr\textsubscript{+/-}30 ppm, 37.09 ± 7.28 s; WT\textsubscript{30ppm} vs. Tbr\textsubscript{+/-}30 ppm, \(p\) value = 0.003; Fig. 2C). Increasing dietary zinc did not alter this time in the WT mice, however high dietary zinc did remove the deficit in Tbr\textsubscript{+/-} mice such that the preference index was no longer significantly different between WT and Tbr\textsubscript{+/-} mice (WT\textsubscript{150ppm}, 90.11 ± 13.09 s; Tbr\textsubscript{+/-}150 ppm, 89.27 ± 12.39 s; WT\textsubscript{150ppm} vs. Tbr\textsubscript{+/-}150 ppm, \(p\) value > 0.9999; Fig. 2C). Moreover, Tbr\textsubscript{+/-} mice fed with the 150 ppm zinc diet spent significantly more time with the stranger mouse versus the empty cup than Tbr\textsubscript{+/-} mice fed with the control 30 ppm zinc diet, demonstrating a zinc diet-induced improvement in preference index (Tbr\textsubscript{+/-}30 ppm vs. Tbr\textsubscript{+/-}150 ppm, \(p\) value = 0.0123; Fig. 2C).

Social novelty was then tested in the second phase of the social behaviour test: while the WT mice on the control 30 ppm zinc diet showed a significant difference in interaction time between stranger 1 (i.e. familiar mouse) and stranger 2 (i.e. novel mouse), Tbr\textsubscript{+/-} mice did not (WT\textsubscript{30ppm}, stranger 1 = 55.09 ± 6.60 s, stranger 2 = 92.64 ± 16.43 s, \(p\) value = 0.0254; Tbr\textsubscript{+/-}30 ppm,

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**Fig. 1** High dietary zinc prevents auditory fear conditioning deficits in the heterozygous Tbr\textsubscript{+/-} mouse model of autism spectrum disorder (ASD). A Schematic of the auditory fear conditioning test. B Freezing responses of the wildtype Tbr\textsubscript{+/+} mice (WT) and heterozygous Tbr\textsubscript{+/-} mice fed with the normal zinc diet (30 parts per million) before electric shock (Basal), immediately after the third electric shock (After Shock), and 1 day after training (Auditory Fear Memory) are shown. Tbr\textsubscript{+/-} mice (\(n\) = 14) display impaired auditory fear memory represented by significantly reduced freezing responses compared to WT (\(n\) = 12). C Tbr\textsubscript{+/-} mice (\(n\) = 12) fed with high dietary zinc (150 parts per million) no longer display significantly different freezing responses when compared to WT mice (\(n\) = 11). All data represent mean ± standard error of the mean, analysed using two-way analysis of variance (ANOVA) with Šídák’s post hoc test and one-way ANOVA with Tukey’s post hoc test. ns = not significant, **\(p\) < 0.01
stranger 1 = 46.64 ± 5.32 s, stranger 2 = 57.09 ± 6.37 s, p value = 0.8920; Fig. 2D and E). Furthermore, dietary zinc supplementation did not rescue the deficit in time in close interaction with stranger 1 mouse versus stranger 2 mouse in Tbr1+/− mice (WT150 ppm, stranger 1 = 57.56 ± 7.42 s, stranger 2 = 97.33 ± 8.00 s, p value = 0.0345; Tbr1+/−150 ppm, stranger 1 = 59.45 ± 9.05 s, stranger 2 = 70.63 ± 11.15 s, p value = 0.8665; Fig. 2D and E). However, the preference index, measured as the difference in time spent with the stranger 1 mouse and stranger 2 mouse, showed no significant difference across the 4 experimental groups (WT30 ppm, 37.55 ± 19.61 s; Tbr1+/−30 ppm, 10.45 ± 8.06 s; WT150 ppm, 39.78 ± 11.76 s; Tbr1+/−150 ppm, 11.18 ± 10.17; Fig. 2F).
Tbr1<sup>+/−</sup> mice do not display ASD-related anxiety behaviours nor excessive grooming

Next, we examined other ASD-associated behaviours such as heightened anxiety traits and excessive grooming in Tbr1<sup>+/−</sup> mice (Fig. 3). The dark–light emergence anxiety test revealed no significant differences between the WT controls and Tbr1<sup>+/−</sup> mice in either the latency to enter the bright arena nor time spent in the bright arena (Latency: WT<sub>30ppm</sub> 7.79 ± 1.66 s; Tbr<sup>+/−</sup> 30 ppm 7.79 ± 1.29 s; WT<sub>150ppm</sub> vs. Tbr<sup>+/−</sup> 30 ppm, p value = 0.9999; Time spent: WT<sub>30ppm</sub> 306.47 ± 22.28 s; Tbr<sup>+/−</sup> 30 ppm, 287.07 ± 19.49 s; WT<sub>30ppm</sub> vs. Tbr<sup>+/−</sup> 30 ppm, p value = 0.9078; Fig. 3A–C), and increased dietary zinc did not alter this lack of difference (Latency: WT<sub>150ppm</sub> 8.20 ± 2.15 s; Tbr<sup>+/−</sup> 150 ppm, 7.36 ± 1.67 s; WT<sub>150ppm</sub> vs. Tbr<sup>+/−</sup> 150 ppm, p value = 0.9876; Time spent: WT<sub>150ppm</sub> 297.10 ± 21.65 s; Tbr<sup>+/−</sup> 150 ppm, 322.64 ± 24.02 s; WT<sub>150ppm</sub> vs. Tbr<sup>+/−</sup> 150 ppm, p value = 0.8760; Fig. 3A–C). Similarly, Tbr1<sup>+/−</sup> mice showed no significant increase in time spent grooming (% time spent grooming: WT<sub>30ppm</sub> 10.11 ± 1.22%; Tbr<sup>+/−</sup> 30 ppm, 7.41 ± 1.44%; WT<sub>30ppm</sub> vs. Tbr<sup>+/−</sup> 30 ppm, p value = 0.3558; Fig. 3D), and this was also not significantly altered with the high zinc diet (WT<sub>150ppm</sub> 8.32 ± 1.25%; Tbr<sup>+/−</sup> 150 ppm, 5.94 ± 0.56%; WT<sub>150ppm</sub> vs. Tbr<sup>+/−</sup> 150 ppm, p value = 0.5467; Fig. 3D). These results were not surprising as previous work has also described unaltered grooming behaviours and normal anxiety phenotypes measured by open field or elevated plus maze test, respectively, in Tbr1<sup>+/−</sup> mice, when compared to the WT mice [14].

![Fig. 3](image-url)
Dysfunctional glutamatergic thalamic-LA synapses in Tbr1\(^{+-}\) mice are normalised by high dietary zinc

Auditory inputs from the thalamus and cortex are concentrated in the lateral nuclei of the amygdala (LA) [49], and proper synaptic function onto the pyramidal neurons in the LA is crucial for fear memory [50, 51]. Due to the significant deficit in fear conditioning observed in Tbr1\(^{+-}\) mice and its rescue by dietary zinc, we also performed an electrophysiological analysis of excitatory synaptic function in the LA. Specifically, whole-cell patch-clamp recordings of thalamic-LA synapses revealed that excitatory glutamatergic synaptic transmission mediated by AMPARs was significantly decreased in Tbr1\(^{+-}\) mice: half-maximal AMPAR EPSC amplitude averaged \(-771.0 \pm 67.94\) pA in WT\(_{30\text{ppm}}\) mice and \(-469.1 \pm 52.92\) pA in Tbr1\(^{+-}\)\(_{30\text{ppm}}\) mice, with a p value of 0.0028 (Fig. 4A–C). Increasing dietary zinc did not significantly alter AMPAR EPSC amplitude in WT mice (WT\(_{150\text{ppm}}\) -648.0 \pm 60.30 pA; WT\(_{30\text{ppm}}\) vs. WT\(_{150\text{ppm}}\), p value = 0.5157; Fig. 4A–C). However, dietary zinc supplementation significantly increased half-maximal AMPAR EPSC amplitude in Tbr1\(^{+-}\) mice (Tbr1\(^{+-}\)\(_{150\text{ppm}}\), -909.2 \pm 60.37 pA; Tbr1\(^{+-}\)\(_{30\text{ppm}}\) vs. Tbr1\(^{+-}\)\(_{150\text{ppm}}\), p value < 0.0001; Fig. 4A–C) so there was no longer a

![Fig. 4](image-url)
significant difference from WT mice fed with 30 ppm dietary zinc (WT<sub>30ppm</sub> vs. Tbr<sup>+/− 150ppm</sup> p value = 0.4318).

Similarly, we observed that the amplitude of evoked isolated NMDAR-mediated EPSCs, as well as the NMDAR/AMPAR EPSC ratio, were also significantly decreased in heterozygous Tbr<sup>+/−</sup> mice (NMDAR-EPSC: WT<sub>30ppm</sub>, 138.3 ± 12.11 pA; Tbr<sup>+/− 30 ppm</sup>, 98.35 ± 4.76 pA; WT<sub>30ppm</sub> vs. Tbr<sup>+/− 30 ppm</sup>, p value = 0.0402; NMDAR/AMPAR ratio: WT<sub>30ppm</sub>, 0.5056 ± 0.0349; Het<sub>30ppm</sub>, 0.3774 ± 0.0308; WT<sub>30ppm</sub> vs. Het<sub>30ppm</sub>, p value = 0.0498; Fig. 4D–F), revealing a deficit in NMDAR-mediated synaptic transmission in thalamic-LA synapses of Tbr<sup>+/−</sup> mice [37]. Increasing dietary zinc significantly increased both the amplitude of evoked NMDAR-mediated EPSCs and the NMDAR/AMPAR EPSC ratio in Tbr<sup>+/−</sup> mice, rescuing the deficit in NMDAR function (NMDAR-EPSC: WT<sub>150ppm</sub>, 139.8 ± 15.70 pA; Tbr<sup>+/− 150 ppm</sup>, 159.3 ± 9.996 pA; Tbr<sup>+/− 30 ppm</sup> vs. Tbr<sup>+/− 150 ppm</sup>, p value = 0.0003; NMDAR/AMPAR ratio: WT<sub>150ppm</sub>, 0.4922 ± 0.0467; Het<sub>150ppm</sub>, 0.5720 ± 0.0353; Het<sub>30ppm</sub> vs. Het<sub>150ppm</sub>, p value = 0.0024; Fig. 4D–F). In contrast to the amplitude measurements, no significant differences in the weighted NMDAR decay time were observed between WT and Tbr<sup>+/−</sup> mice fed with either normal or high dietary zinc (WT<sub>30ppm</sub>, 203.2 ± 31.26 ms; Tbr<sup>+/− 30 ppm</sup>, 162.1 ± 27.97 ms; WT<sub>150ppm</sub>, 247.9 ± 41.53 ms; Tbr<sup>+/− 150 ppm</sup>, 267.9 ± 51.33 ms), suggesting that no change in NMDAR subunit expression occurs in Tbr<sup>+/−</sup> mice.

**High zinc diet supplementation restores presynaptic function at glutamatergic thalamic-LA synapses in Tbr<sup>+/−</sup> mice**

We also examined AMPAR-mediated paired-pulse ratio (PPR) in principle neurons of the LA to assess presynaptic function in WT and Tbr<sup>+/−</sup> mice. The ratio of the first to second AMPAR EPSC amplitudes (separated by 50 ms) was measured in all four experimental groups. In the control dietary zinc group, we observed a significant increase in PPR in Tbr<sup>+/−</sup> mice compared with WT mice (WT<sub>30ppm</sub>, 0.7725 ± 0.0257; Tbr<sup>+/− 30 ppm</sup>, 0.9348 ± 0.0388; WT<sub>30ppm</sub> vs. Tbr<sup>+/− 30 ppm</sup>, p value = 0.0039; Fig. 5A and B), supporting a lower release probability occurring at thalamic-LA synapses in these ASD mutant mice. Interestingly in mice fed the high zinc diet, there was no significant difference in PPR between Tbr<sup>+/−</sup> mice compared with WT mice (WT<sub>150ppm</sub>, 0.8486 ± 0.0340; Tbr<sup>+/− 150 ppm</sup>, 0.8370 ± 0.0278; WT<sub>150ppm</sub> vs. Tbr<sup>+/− 150 ppm</sup>, p value = 0.9953; Fig. 5A and B), showing that increased dietary zinc levels return the PPR to control level, suggesting a return to normal presynaptic function.
High dietary zinc restores synaptic GluN1 and Shank3, but not Shank2, puncta density in the amygdala of Tbr1+/− mice

To examine whether dietary zinc supplementation normalises thalamic-LA synapse function in Tbr1+/− mice via restoration of inter-amygdalar axonal projections, we performed Luxol fast blue staining of brain sections in WT and Tbr1+/− mice (n = 7/group; example in Fig. 6). Luxol fast blue staining clearly showed the significant reduction in inter-amygdalar axonal projections in Tbr1+/− mice, however we observed no improvement in these impaired axonal projections in Tbr1+/− mice with the increase in dietary zinc (Fig. 6). Next, we performed immunocytochemical analysis of the GluN1 subunit of the NMDAR in the LA to validate whether changes in the synaptic expression of NMDARs contribute to our behavioural and electrophysiological differences observed in WT and Tbr1+/− mice (Fig. 7A–E). Supporting our electrophysiological data (Fig. 4), we observed that synaptic NMDARs, as measured by colocalised GluN1 and synapsin1/2 puncta, were significantly decreased in the LA of Tbr1+/− mice compared with WT mice fed with normal 30 ppm zinc diet (WT30ppm 1.0000 ± 0.1304; Tbr+/− 30 ppm, 0.5776 ± 0.0413; WT30ppm vs. Tbr+/− 30 ppm, p value = 0.0144; Fig. 7B and C). Moreover, high dietary zinc restored the deficits in synaptic GluN1 density in the LA of Tbr1+/− mice, such that the synaptic GluN1 densities measured from Tbr1+/− mice fed 150 ppm zinc were comparable with WT mice fed with the normal zinc diet (WT150ppm 0.6610 ± 0.1014; Tbr+/− 150 ppm, 0.9583 ± 0.0858; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value = 0.0041; WT30ppm vs. Tbr+/− 150 ppm, p value > 0.9999; Fig. 7B and C). We also conducted immunocytochemical analysis of the GluN1 subunit of the NMDAR in the basal nuclei of the amygdala (BA), the major target for LA pyramidal neurons that are involved in the expression and extinction of conditioned fear memory [52]. As observed in the LA, the synaptic density of GluN1 in mice fed normal dietary zinc levels was significantly reduced in the BA of Tbr1+/− mice compared with the WTmice (WT30ppm 1.0000 ± 0.0661; Tbr+/− 30 ppm, 0.5206 ± 0.0432; WT30ppm vs. Tbr+/− 30 ppm, p value = 0.0015; Fig. 7D and E). In the BA, dietary zinc supplementation also reversed the reduced synaptic GluN1 density in Tbr1+/− mice (WT150ppm 0.8009 ± 0.0920; Tbr+/− 150 ppm, 1.072 ± 0.0998; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value = 0.0015; WT30ppm vs. Tbr+/− 150 ppm, p value = 0.8619; Fig. 7D and E).

As Shank2 and Shank3 proteins are highly regulated by zinc, and play a critical role in driving postsynaptic glutamate receptor recruitment to the synapse, we also examined the synaptic localisation of Shank2 and Shank3 in WT and Tbr1+/− mice fed normal and high dietary zinc. We observed no significant differences in Shank2 expression in either genotype with dietary zinc in either in LA or BA (Shank2 LA: WT30ppm 1.0000 ± 0.0785; Tbr+/− 30 ppm, 0.9117 ± 0.1000; WT30ppm, 0.4924 ± 0.0744; Tbr+/− 150 ppm, 0.7267 ± 0.0851; WT30ppm vs. Tbr+/− 30 ppm, p value > 0.9999; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value = 0.9509; WT30ppm vs. Tbr+/− 150 ppm, p value = 0.1723; Fig. 8A and B. Shank2 BA: WT30ppm 1.0000 ± 0.0867; Tbr+/− 30 ppm, 0.7369 ± 0.1042; WT30ppm, 0.4391 ± 0.0653; Tbr+/− 150 ppm, 0.7453 ± 0.0684; WT30ppm vs. Tbr+/− 30 ppm, p value = 0.4383; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value > 0.9999; WT30ppm vs. Tbr+/− 150 ppm, p value = 0.6654; Fig. 8C and D). However, interestingly we did observe a significant increase in Shank3 synaptic expression in both the LA and BA in Tbr1+/− mice fed high dietary zinc, such that Shank3 levels were returned to WT levels (Shank3 LA:
WT30ppm, 1.0000 ± 0.1209; Tbr+/− 30 ppm, 0.5783 ± 0.1266;
WT150ppm, 1.164 ± 0.1870; Tbr+/− 150 ppm, 1.396 ± 0.1357;
WT30ppm vs. Tbr+/− 30 ppm, p value = 0.0241; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value < 0.0001;
WT150ppm vs. Tbr+/− 30 ppm, 0.7823 ± 0.1476; Tbr+/− 150 ppm, 1.301 ± 0.0790;
WT30ppm vs. Tbr+/− 30 ppm, p value < 0.0001; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value < 0.0001; WT30ppm vs. Tbr+/− 150 ppm, p value = 0.6833; Fig. 8E and F; Shank3 BA: WT30ppm, 1.0000 ± 0.08972; Tbr+/− 30 ppm, 0.4526 ± 0.0610;
WT150ppm, 0.7823 ± 0.1476; Tbr+/− 150 ppm, 1.301 ± 0.0790;
WT30ppm vs. Tbr+/− 30 ppm, p value < 0.0001; Tbr+/− 30 ppm vs. Tbr+/− 150 ppm, p value < 0.0001; WT30ppm vs. Tbr+/− 150 ppm, p value = 0.0621; Fig. 8G and H). Together
these data show that a deficit in synaptic NMDAR expression occurs in both the basal and lateral nuclei of the amygdala (BLA) in Tbr1+/− mice which likely underpins the decrease in NMDAR-mediated currents and the NMDAR/AMPA ratio, and that this deficit can be rescued by dietary zinc supplementation in Tbr1+/− mice, in part, through Shank3-related mechanism.

**Discussion**

Here, we show that dietary zinc supplementation prevents impairments in auditory fear memory and social interaction, but not social novelty, in the Tbr1+/− mouse model of ASD. At the synaptic level, dietary zinc supplementation also reversed AMPAR and NMDAR hypofunction and normalised presynaptic function at glutamatergic thalamic-LA synapses that are crucial for auditory fear memory [49, 50], and restored synaptic density of the GluN1 subunit essential for functional NMDARs. Together, our data further the understanding of the molecular mechanisms underlying the effect of dietary zinc supplementation and verify the efficacy and breadth of its application as a potential treatment strategy for ASD.

Zinc supplementation is a viable therapeutic approach beyond Shank mutations for ASD phenotypes stemming from the amygdala.

The concept of zinc supplementation as a potential ASD treatment has been based on observations that (1) reduced serum zinc levels have been identified in individuals with ASD [53–57], and (2) prenatal zinc-deficient animals display ASD-like behaviours including increased anxiety, social interaction deficits and altered ultrasonic vocalisation [58, 59]. The efficacy and mechanistic underpinnings of zinc supplementation as a treatment strategy have so far been primarily examined in rodent models carrying ASD-associated SHANK2 or SHANK3 mutations, but also in the maternal immune activation model of ASD [37, 41, 44, 45, 59–62]. A focus on SHANK models is likely due to (1) the high prevalence of SHANK2 and SHANK3 mutations in people affected by ASD [63]; (2) SHANK2 and SHANK3, which are excitatory glutamatergic postsynaptic proteins, contain a SAM domain that directly binds zinc [41, 43, 64]; (3) synaptic localisation and expression of SHANK2 and SHANK3 are highly dependent on zinc [42–44, 64–66]; and (4) zinc deficiency dysregulates the postsynaptic SHANK scaffold, which is thought to contribute to the synaptic mechanisms underlying behavioural deficits in ASD [41, 44, 58]. The data described in the current study is one of the few that have identified zinc supplementation as a viable therapeutic approach beyond ASD-associated Shank mutations and provided potential mechanisms underlying its beneficial effect [37, 67], specifically in the amygdala. Together with previous studies [37, 41, 44, 45, 59–62], this shows impairment of zinc sensitive pathways may be a shared biological substrate for ASD behaviours across several ASD models.

The amygdala forms intricate neural circuits with other brain regions such as the prefrontal cortex, thalamus, hypothalamus, hippocampus, and striatum, and plays a principal role in emotional memory and social behaviours [68, 69]. Defective inter- and intra-amygdalar axonal projections and impaired neuronal activation in the amygdala are major phenotypes observed in Tbr1+/− mice, and consistent with this, amygdala-dependent behaviours such as social interaction, social novelty recognition, and auditory fear memory are impaired in Tbr1+/− mice [14, 37]. In particular, thalamic-LA synapse function is significantly impaired, likely contributing to auditory fear memory deficits observed in Tbr1+/− mice. We observed that the amplitudes of both AMPAR- and NMDAR-mediated EPSCs were decreased, and that presynaptic function (as measured by PPR) was altered at thalamic-LA synapses in Tbr1+/− mice. Also, the synaptic puncta density of GluN1 and Shank3, was reduced in Tbr1+/− amygdala, indicating that diminished synaptic expression of
Shank3 proteins and NMDARs contributes to NMDAR hypofunction at $Tbr1^{+/-}$ thalamic-LA synapses. A critical result is that dietary zinc supplementation can restore excitatory glutamergic pre- and postsynaptic function at $Tbr1^{+/-}$ thalamic-LA synapses, as well as the synaptic GluN1 and Shank3 density, comparable to that of WT.
mice. Together our data suggest that normalisation of thalamic-LA synapse function in \textit{Tbr1}^{+/−} mice is a central mechanism behind the dietary zinc-induced prevention of auditory fear memory impairment.

**Potential zinc-related pathways involved**

The majority of zinc ions are extensively bound within proteins as structural or catalytic cofactors in the brain, while a pool of free (or chelatable) zinc is highly localised within synaptic vesicles, accumulated by the vesicular zinc transporter ZnT3, at glutamatergic nerve terminals [40, 70, 71]. In the amygdala, only the excitatory cortical-amygdalar synapses implicated in auditory fear conditioning contain vesicular zinc. In contrast, thalamic projections to the LA involved in auditory fear conditioning lack free zinc, as demonstrated by minimal expression of the \textit{ZnT3} gene [72]. Therefore, the high dietary zinc-induced restoration of glutamatergic thalamic-LA synapse function in the \textit{Tbr1}^{+/−} mice is unlikely to be caused by the enrichment of free zinc, but rather by changes in the availability of protein-bound zinc at these synapses. Further analysis measuring total zinc levels by inductively coupled plasma mass spectrometry, and free or intracellular zinc with zinc indicators (e.g. TFL-Zn or ZnAF-2DA, respectively) [37] would be required in the amygdala to assess this possibility.

At excitatory glutamatergic synapses, bound zinc maintains the organisation of a complex assembly of postsynaptic density proteins where it associates with SAP102, SHANK2, and SHANK3 [43, 64, 73]. The exact role of the zinc-binding motif of SAP102 remains elusive [73], although it may link to its trafficking of AMPARs and NMDARs during synaptogenesis to regulate synaptic organisation and plasticity [74–76]. Conversely, much more is known about zinc’s role in driving synaptic localisation and expression of SHANK2 and SHANK3, and subsequently AMPARs and NMDARs [41, 42, 44, 45, 59, 77, 78]. In \textit{Ctnnbp2}^{−/−} ASD mice (independent of \textit{Shank} ASD mutations), zinc supplementation induced a significant increase in synaptic expression of SHANK2 and SHANK3, as well as NMDAR (GluN1 and GluN2B) [67]. Our observed zinc-induced rescue of glutamatergic receptor function and GluN1 puncta density in \textit{Tbr1}^{+/−} mice appears to be SHANK3, but not SHANK2, related, suggesting that dietary zinc-induced increases in SHANK3 can drive NMDAR expression at synapses in \textit{Tbr1}^{+/−} mice. Moreover, SHANK3 can regulate glutamatergic presynaptic function via trans-synaptic signalling through the neuromodulation of neuromodulator cell adhesion molecules to recruit presynaptic structural proteins and increase transmitter release, which could further contribute to our observed dietary zinc-related changes in presynaptic and postsynaptic function in \textit{Tbr1}^{+/−} mice [79]. In addition, zinc-binding proteins are located in the presynapse, including Piccolo, Bassoon, Munc13, and RIM, that control the priming, tethering, and exocytosis of synaptic vesicles, and thereby determine presynaptic release probability [80–82]. Dietary zinc supplementation may drive zinc-binding to presynaptic proteins together with zinc-dependent recruitment of SHANK protein complexes pre- and postsynaptically to modify vesicular release probability and prevent abnormal PPR observed in the \textit{Tbr1}^{+/−} mice. Altogether, the supplemented zinc may exert its therapeutic effect through association with zinc-responsive proteins at both pre- and postsynapses that synergistically work together to repair glutamatergic thalamic-LA synapse structure and function.

**Timing and duration are critical in dietary zinc supplementation.**

In \textit{Tbr1}^{+/−} mice, dietary zinc supplementation prevents impairments in auditory fear memory and social interaction, but not social novelty recognition impairments. This selective therapeutic effect of dietary zinc supplementation may be attributed to (1) differences in brain circuitry and regions involved in distinct social behaviours [83, 84], (2) discrete zinc responsiveness in different neural pathways [72, 85], and (3) timing of zinc supplementation [86]. The detrimental behavioural phenotypes induced by \textit{Tbr1} haploinsufficiency likely manifest from early stages in brain development [17, 20, 24–26]. Indeed we have recently shown that both social interaction and social novelty deficits are present in \textit{Shank3}^{−/−} mice as early as 3 weeks of age, indicating that the neural circuitry responsible for social behaviours is established before weaning [46]. In the current study, dietary zinc supplementation was performed post-weaning and our data suggest this is after the “window” to modulate this circuitry has closed. Indeed, our Luxol fast blue staining suggests that some structural deficits, such as in the anterior commissure, are not rescued by dietary zinc supplementation. Similarly adult restoration of \textit{Shank3} expression in 2- to 4.5-month-old \textit{Shank3}^{−/−} mice only partially prevented ASD-associated behavioural phenotypes [87], and cloquinol-induced increases in free zinc in 2- to 4-month-old \textit{Shank2}^{−/−} mice recovered social interaction deficits but did not restore defective social novelty recognition behaviour nor normalise heightened anxiety [37]. Together with our data, these studies suggest that specific ASD behaviours are more difficult to alter as development and maturation progresses, and therefore the timing of treatment is an essential factor. The underpinning neural pathways do appear to have a window of modulation during early development however, as supported by the intriguing observation that dietary maternal zinc supplementation during pregnancy and lactation...
can prevent ASD-associated social behaviour deficits developing in 3-week-old Shank3−/− offspring, and this persists into adulthood [46]. Timing therefore appears to be critical for zinc-based therapies for ASD-associated mutations in genes such as Tbr1 and Shanks that encode proteins involved in early brain development.

Our dietary zinc treatment strategy, in which animals receive dietary zinc in their food for a minimum of 6 weeks [45, 46], induces not only functional but also structural changes at the synaptic level, and effects on ASD behaviours can be observed 2–3 months after zinc supplementation [46]. In contrast, zinc supplementation for a shorter period through the drinking water for 7 days in Cttnbp2-deficient mouse does not restore decreased spine density in the hippocampus, and the initial rescue of social interaction behaviours deteriorated 1 week after discontinuation of zinc [67]. Therefore, not only the timing but also the duration of the zinc treatment could be pivotal in the persistence of synaptic remodelling and subsequent behavioural outcomes. Another notable feature of the dietary zinc supplementation approach in this study is that when used at a physiological level (30 ppm or 150 ppm) [88, 89], the increased zinc levels did not influence behaviours in the WT mice. This is consistent with previous findings that WT mice fed with elevated dietary zinc displayed normal behaviours regardless of the timing, duration, or method of delivery [45, 46, 67], even in the presence of small changes at the molecular or synaptic levels.

Rationale and translational potential of dietary zinc supplementation.

The supplemented dietary zinc level of 150 ppm was used based on our previous studies examining the effects of supplemented zinc diets to ensure a direct comparison was possible across ASD model genotype effects [45, 46]. We have previously identified the large variance in zinc levels in rodent chow ranging from 25 ppm to 120 ppm [45], resulting in a large “normal” range. How this translates to human dosage is difficult to directly compare. ASD children show lower serum zinc levels, and a recent study showed an increase in cognitive-motor performance following daily zinc supplementation for 12 weeks in ASD children with daily zinc dose to each child equal to their body weight in kg plus 15–20 mg [90]. Nevertheless, a conservative approach is required to discover the effective dosage for ASD treatment. The therapeutic window of zinc concentration is narrow, and zinc can influence the metabolism of other metal ions, such as copper [91–95]. Moreover, zinc does not cross the blood–brain barrier (BBB) freely [61], yet the recent development of nano-particle carriers to aid BBB crossover may prove to be encouraging [96, 97]. Together, dietary zinc supplementation has a great therapeutic potential as an ASD treatment strategy, but additional research is required to bring forward safe and effective clinical use.

Limitations

The therapeutic effects of zinc supplementation have only been evaluated in the mouse models of ASD [37, 45, 46, 64], including Tbr1+−/− mice in the current study. Whether zinc-based treatment strategies can induce the same beneficial outcomes in human-derived models and ASD patients, that is reversal of ASD-related behaviours through the restoration of synapse function, requires further examination. An additional challenge will be determining whether ASD diagnosis can precede the therapeutic window in humans. Also, the effects of dietary zinc supplementation on neural circuits other than the thalamic-LA projection pathway such as the olfactory system and anterior commissure-related interhemispheric connectivity that have previously been shown to be dysfunctional in Tbr1−/− mice [15, 38, 39], or those that may underlie ASD-associated behaviours observed in Tbr1+−/− mice (i.e. prefrontal cortex and ventral hippocampus for social memory and behaviours) require examination in future studies. We also recognise the current study focussed on the synaptic axis for the therapeutic role of dietary zinc, and it is possible that given the many roles of zinc, that its therapeutic actions are also indirect, for example via gut-brain signalling.

Conclusion

In summary, here we demonstrate the effectiveness of dietary zinc supplementation in the reversal of ASD-associated synaptic and behavioural deficits in the Tbr1+/− ASD rodent model, highlighting the breadth of this treatment strategy beyond Shank ASD mutations. Zinc supplementation can restore impaired glutamatergic synapse function and density, particularly in the thalamic-LA projection pathway, and prevent ASD-associated behaviours in Tbr1+/− mice. These data improve our understanding of the mechanisms and the potential of dietary zinc supplementation as a treatment strategy for ASD.

Abbreviations

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ASD: Autism spectrum disorder; BA: Basal amygdala; BLA: Basolateral amygdala; CaMKII: Calcium/calmodulin-dependent protein kinase II; CASK: Calcium/calmodulin-dependent serine protein kinase; CINAP: CASK interacting nucleosome assembly protein; CNQX: 6-Cyano-7-nitroquinoxaline-2,3-dione; EPSC: Excitatory postsynaptic current; HET: Heterozygous; LA: Lateral amygdala; NMDAR: N-Methyl-D-aspartate receptor; PPM: Parts per million; PPR: Paired-pulse ratio; TBR1: T-box brain 1; WT: Wildtype; ZnT3: Zinc transporter 3.
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Authors’ contributions

KL conducted electrophysiology and histological experiments, analysed data, and carried out the manuscript writing. YJ performed all animal behavioural tests and carried out the manuscript writing. YV carried out electrophysiology experiments and contributed to the manuscript writing. IS performed the auditory fear conditioning experiments, and provided the Tbr1+/- mice breeders to establish the colony at the University of Auckland. JMM planned and conceived the project, designed the experiments, secured the funding, and performed manuscript writing. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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