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The role of the *CNTNAP2* gene in the development of autism spectrum disorder

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder in which genetic and environmental factors interact in its development. Research suggests that the contactin associated protein 2 (*CNTNAP2*) gene may play a role in ASD pathophysiology, yet more studies involving human participants and animal models of autism are needed. One such model may be the use of prenatal valproic acid (VPA) model to induce autism-like behaviors in offspring rats. The aim of this study was twofold: (1) to examine the association of the *CNTNAP2* gene rs2710102 variant with ASD in children; and (2) to examine the effect of prenatal exposure to VPA on *Cntnap2* gene expression in the rat brain. The study included 167 children of European ancestry—81 diagnosed with ASD (20 girls, 61 boys; age 4.9 ± 1.4 years) and 86 controls (44 girls, 42 boys; 5.1 ± 1.2 years). *In vivo* experiments were conducted in 80 rats (40 with the VPA model of autism), with *Cntnap2* gene expression analysis in the amygdala, hippocampus, prefrontal cortex, and cerebellum. Results demonstrated that the frequency of the *CNTNAP2* gene rs2710102 GG genotype was significantly higher in children with ASD when compared with controls (33.3 vs 19.8%; OR=2.03, 95%CI [1.004, 4.102], $p = 0.035$), although, potentially due to bias in cohort selection, in the ASD children this polymorphism did not meet Hardy-Weinberg expectations ($\chi^2 = 5.40$, $p = 0.02$). In addition, *Cntnap2* gene expression was significantly lower ($p < 0.01$) in the amygdala and hippocampus of VPA rats when compared with controls, regardless of sex. These results support previous research and provide evidence for the *CNTNAP2* gene as a risk factor for ASD.

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1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder, characterized by impaired social, verbal, and nonverbal communication, repetitive behavior, and restricted interests (Semina et al., 2019; Hirota & King, 2023). Currently, 1 in 100 children worldwide are diagnosed with ASD (Zeidan et al., 2022). Childhood autism is the most severe form of ASD, usually diagnosed between the ages of 6 and 10, with a higher prevalence in males (Fombonne, MacFarlane, & Salem, 2021)—the average male to female ratio is 4:1 (Werling et al., 2013).

ASD is genetically heterogeneous. According to the Simons Foundation Autism Research Initiative (The SFARI; https://gene.sfari.org/), most of the genes encoding synaptic cell adhesion molecules cause the development of this pathology (Baig et al., 2017). One strong candidate is the contactin associated protein 2 (*CNTNAP2*) gene, which spans 1.5% of chromosome 7 (7q35-q36.1) and is the largest in the human genome (2.3 Mb, 25 exons) (NCBI Gene; https://www.ncbi.nlm.nih.gov/gene/). The gene is expressed at high levels in the central nervous system (in the cerebral cortex and the basal ganglia). *CNTNAP2* interacts with genes associated with ASD that encode voltage-gated potassium channel type 2 and calcium/calmodulin-dependent serine protein kinase (*KCNA2* and *CASK*). The CNTNAP2 protein is a cell adhesion molecule that mediates the interaction between neurons and glia during nervous system development. It is also involved in the localization of potassium channels in differentiating axons (NCBI Gene; https://www.ncbi.nlm.nih.gov/gene/).

Neurobiological studies using animals, and in vivo experiments, show that Cntnap2 contributes to nervous system abnormalities and diseases, including epilepsy, deficits in social interaction, reduced auditory processing and responsiveness to stimuli, and stereotyped behavior (Scott et al., 2020; Scott et al., 2018; Peñagarikano et al., 2011). Cultured induced pluripotent stem cells from a neurotypical patient with a shortened *CNTNAP2* gene showed reduced neurite branching and simplified complex neuronal networks (St George-Hyslop et al., 2023).

Genetic studies suggest a link between intragenic deletions and copy number variants (CNVs) in *CNTNAP2* and developing ASD (Strauss et al., 2006; Bakkaloglu et al., 2008; Nord et al., 2011; O'Roak et al., 2011; Poot et al., 2010). Gene mutations have led to the development of characteristic ASD endophenotypes and associated disorders, such as delayed language development and language processing disorder, stuttering, hyperactivity, intellectual disability, epilepsy, obsessive-compulsive disorder, schizophrenia, bipolar disorder, and anxiety (Strauss et al., 2006; Sehested et al., 2010; Friedman et al., 2008; Gu et al., 2018; Poot, 2017; Smogavec et al., 2016; Enikeeva et al., 2020). Several studies show phenotypic diversity in gene function, reflecting an association with cognitive processes (rs34438057 variant / polymorphism), development of Alzheimer's disease, and associated biochemical changes: beta-amyloid levels and LDL concentrations (rs9271192, rs12154459, and rs117834366 variants) (Gouveia et al., 2022; Jansen et al., 2019; Lee et al., 2018; Moreno-Grau et al., 2019). *CNTNAP2* rs2710119 and rs144958708 variants have also been associated with altered gut microbiota, while rs11773362, rs6944674, and rs1026412 have been associated with the obesity characteristic of ASD patients (Qin et al., 2022; Scepanovic et al., 2019; Huang et al., 2022; Kichaev et al., 2019; Sakaue et al., 2021).

One of the most intriguing SNPs for investigating its association with ASD is the rs2710102 located in the intron 13 of the *CNTNAP2* gene. Although the *CNTNAP2* rs2710102 variant has not exhibited genome-wide association with ASD, it has consistently demonstrated replication, with evidence across various populations, suggesting that the risk allele (G) of the rs2710102 has functional effects. Numerous studies have linked *CNTNAP2* rs2710102 to language impairments, encompassing language development, specific language impairment, oral and written language delay, dyslexia, and age at first words in both healthy populations and individuals with ASD (Alarcón et al., 2008; Newbury et al., 2011; Peter et al., 2011; Whitehouse et al., 2011; Poot, 2014; Uddin et al., 2021). The G allele of *CNTNAP2* rs2710102 has previously been shown to be a risk factor for developmental delay and language impairment in children with ASD, and also leads to specific functional brain changes in ASD patients (Scott-Van Zeeland et al., 2010; Alarcón et al., 2008; Fang et al., 2021; Uddin et al., 2021). It is important to note, however, that several studies have found no association between *CNTNAP2* gene variants and the development of ASD (Toma et al., 2018; Murdoch et al., 2015; Werling et al., 2016). Therefore, additional replication studies involving different ethnicities are needed.

It is well known that the *CNTNAP2* gene is highly conserved between humans and rodents (Abrahams et al., 2007; Poot, 2017). Previous studies using *Cntnap2* knockout rat and mouse models have shown that loss of *Cntnap2* causes autism-related phenotypes (Peñagarikano et al., 2011; Scott et al., 2020). We therefore hypothesized that rats exposed to valproic acid *in utero* (VPA model of autism: Nicolini & Fahnestock, 2018) would have decreased expression of the *Cntnap2* gene in the brain.

The aim of this study was thus twofold: (1) to examine the association of the *CNTNAP2* gene rs2710102 variant with ASD in children; and (2) to examine the effect of prenatal exposure to valproic acid (VPA model of autism) on *Cntnap2* gene expression in the rat brain.

2. Methods

2.1. Ethics Statement

Both (human and animal) studies in the present research were approved by the Ethics committee of the Kazan State Medical University (protocol number 199, 4 January 2016; and protocol number 3, 29 March 2019). The studies were conducted according to the guidelines of the Declaration of Helsinki, and we adhered to the Strengthening The Reporting of Genetic Association Studies (STREGA) guidelines: An extension of the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement recommendations. Written informed consent was obtained from the parents or legal guardians of the children.

2.2. Study samples

2.2.1. Human study participants

The study involved 167 children of European (Russian) ancestry—81 children with diagnosed ASD (20 girls, 61 boys; age 4.9 ± 1.4 years) and 86 controls (44 girls, 42 boys; 5.1 ± 1.2 years) (see Fig. 1). Children diagnosed with ASD underwent hospital examination in the Republican Clinical Psychiatric Hospital (named after Academician V.M. Bekhterev of the Ministry of Health of the Republic of Tatarstan, Kazan) between March 2019 and December 2021. With the child's parent or guardian in attendance, ASD severity was assessed by two board-certified developmental pediatricians, using the Autism Diagnostic Observation Schedule-Second Edition (ADOS-2: Lord et al., 2012). A total of three modules (1, 2, and 3) of the ADOS-2 were used in this study and the appropriate module was selected according to the age and language level of the individual.

Inclusion criteria for participants were (a) a diagnosis of ASD according to the International Classification of Diseases (ICD-10), and (b) an age between 2 and 7 years. Exclusion criteria were any other comorbid condition.

2.2.2. DNA extraction and genotyping

DNA was extracted from saliva samples using LumiPure (Moscow, Russia). *CNTNAP2* rs2710102 was genotyped by multiplex PCR followed by hybridization on low-density biochips (Biochip-IMB, LLC, Moscow).

PCR was performed in 25 μl of the following composition: PCR buffer with HotTaqMulti polymerase, 4 units (Asfogen, Russia), 5 mM $MgSO_4$, 0.2 mM of each of the dNTPs (Sibenzym, Russia), primer mixture, 200 pmol of Cy5-TCATTGGATCTCATTA universal primer, 1 ul of DNA. Amplification was carried out in 0.2 ml PCR-tubes on a SpeedCycler (AnalytikJena, Germany) with the following conditions: 95 °C for 2 min and 50 cycles in the first stage (95 °C for 20 s, 65 °C for 30 s, 66 °C for 30 s, 69 °C for 40 s), then 40 cycles in the second stage (95 °C - 20 s, 56 °C - 30 s, 72 °C - 30 s).

All allele-specific oligonucleotides were produced by Lumiprobe (Russia). The fabrication of hydrogel biochips was carried out on Qarray2 (Genetix, UK) in dust-free cleanrooms, according to the original technology of the IMB RAS, as previously described (Fesenko et al., 2014).

Hybridization. The 30-μl chamber of the biochip was filled with a mixture of the following composition: 7.5 μl formamide, 7.5 μl 20 ×SSPE, 15 μl PCR product. After incubation (10 h, 37 °C) and washing (10 min in 1x SSPE at room temperature), the biochips were washed with distilled water, dried with compressed air, placed in a portable analyzer "Picodetect" (BIOCHIP-IMB, LLC, Moscow) and fluorescence was registered with an exposure of 0.5–2 s. Image analysis was performed using ImaGelStudio software (IMB RAS).

2.2.3. Animal study

Wistar rats were purchased from the Stolbovaya branch office of the Scientific Center for Biomedical Technologies (Federal Medical and Biological Agency, Russia). The animals were individually-housed and maintained at room temperature, with a 12-hour light/12-hour dark cycle, with a complete balanced diet.

In our previous study, the detailed design of the valproic acid (VPA) rat model was described (Semina et al., 2023). Experiments were performed on 80 rats (age 100 ± 3 days): VPA groups with prenatal administration of 500 mg/kg valproic acid on day 13 of pregnancy of their mothers (n = 20 rats of each sex) and control groups with saline at the same time in the same volume (n = 20 rats of each sex) (Fig. 1). The administration of valproic acid on day 13 of gestation is crucial for embryonic brain development. This is because it coincides with the peak of neurogenesis, gene expression in neurotransmitter systems, and the completion of the formation

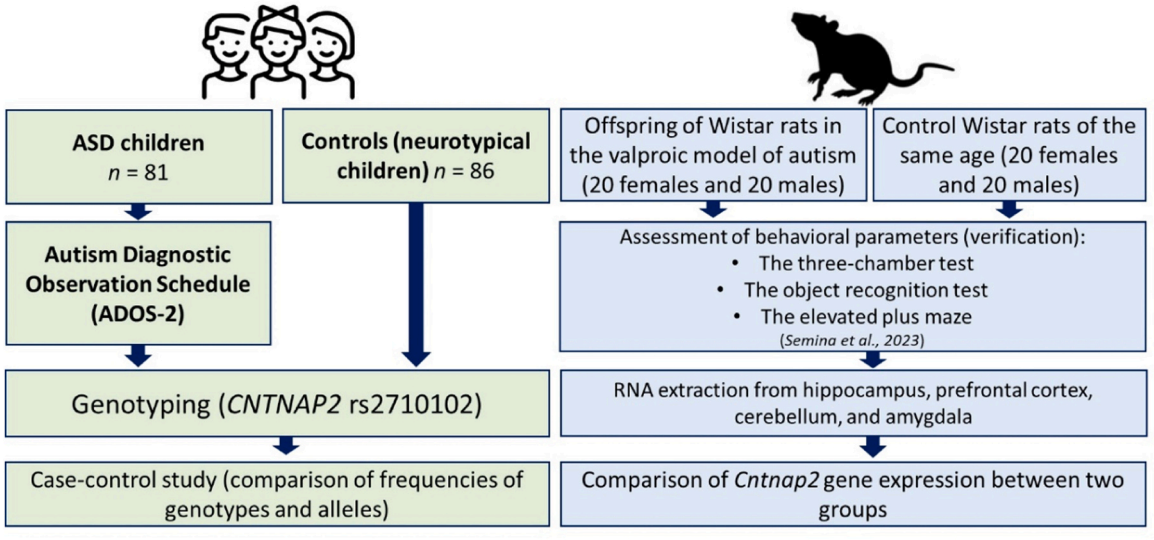

Fig. 1. Design for Human and Animal Studies.

of the cytoarchitectonics of the fetal cortex and neural tube (Ingram et al., 2000; Schneider & Przewłocki, 2005; pp. 518, 1300; Markram et al., 2008; pp. 453, 1301; Servadio et al., 2016). The offspring of these rats exhibit symptoms similar to those of humans with ASD, such as increased anxiety, antisocial behaviour, and stereotypy (Schneider & Przewłocki, 2005; pp. 518, 1300; Ingram et al., 2000; Nicolini & Fahnestock, 2018). The model has been verified using the material described in a recent study (Semina et al., 2023).

2.2.4. Total RNA extraction, reverse transcription reaction and quantitative PCR

All rats of VPA groups and control groups were decapitated using a guillotine (NPO Open Science, Russia). The brain was removed for subsequent isolation of the prefrontal cortex (PFC), hippocampus, cerebellum, and amygdala. Brain structures were isolated in the cold with separate brain samples from each rat were placed into chilled tubes and frozen at –80 °C.

Total RNA was isolated from each rat structure using the ExtractRNA (BC032, Evrogen, Russia) according to the manufacturer's instructions. The concentration RNA was assessed using a NanoDrop Lite spectrophotometer (ThermoFisher, USA). The reverse transcription reaction was assessed using the moloney murine leukemia virus (MMLV) reverse transcriptase kit (SK021, Evrogen, Russia) and 500 ng of total RNA. The resulting cDNA samples were then subjected to real-time qPCR on a CFX96 (BioRad, USA) using commercial TaqMan kits for the *Cntnap2* (Cat. 4351372, Applied Biosystems, USA) and the *Gapdh* as reference gene (Cat. 4448490, Applied Biosystems, USA) and with TaqMan Fast Advanced MasterMix (Cat. 4444965, Applied Biosystems, USA). Each analysis was performed in duplicate. Relative levels of *Cntnap2* expression were analyzed using the $2^{-\Delta\Delta Ct}$ method [Livak & Schmittgen (2001)].

2.2.5. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.0.1 (GraphPad Software, Inc., San Diego, CA, USA) software. We used chi-squared (χ^2) to test for Hardy-Weinberg equilibrium in the human data, developed by Michael H. Court (2005–2008) http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls. The frequencies of genotypes and alleles were compared between children with autism and controls using Fisher's exact test. The relationship between *CNTNAP2* genotypes and ASD severity was performed using multiple regression analysis (adjusted for age and sex). A nominal association was considered significant at $p < 0.05$. The normality of human data was assessed using the Shapiro–Wilk test. In the rat data, we compared gene expression between VPA rats and controls using Student's t-test, with a Bonferroni correction for multiple tests across the four brain structures of interest, meaning that results were considered statistically significant if their p-values fell below an alpha of 0.00625 (i.e., 0.05/[4 brain structures * 2 sexes]). Data are presented as mean ± SEM.

3. Results

3.1. Human study

The rs2710102 polymorphism of the *CNTNAP2* gene met Hardy-Weinberg expectations in the control group (χ^2 =1.21, p = 0.27), but not in children with ASD (χ^2 =5.40, p = 0.02). The frequency of the *CNTNAP2* gene rs2710102 GG (risk) genotype was significantly higher in the children with ASD when compared with controls (33.3% vs 19.8%; OR=2.03, 95% CI [1.004, 4.102], $p = 0.035$) (see Table 1). There was no association between the *CNTNAP2* gene rs2710102 polymorphism and ASD severity (adjusted for age and sex).

3.2. Animal study

Relative *Cntnap2* mRNA expressions in VPA rats and controls are shown separately in female (Fig. 2a) and male (Fig. 2b) rats. The hippocampal *Cntnap2* gene expression was significantly lower in VPA rats compared to controls in both females (0.14 ± 0.03 vs. 1.00 ± 0.08; $p = 5.1 \times 10^{-5}$) and males (0.34 ± 0.05 vs. 1.00 ± 0.19; $p = 6.0 \times 10^{-4}$). Similar differences in relative *Cntnap2* gene expression were observed in the amygdala in both females (0.26 ± 0.07 vs. 1.00 ± 0.19; $p = 0.0068$) and males (0.14 ± 0.02 vs. 1.00 ± 0.26; $p = 3.0 \times 10^{-4}$). After adjustment for multiple testing, these differences remained significant in male rats only ($p < 0.00625$). In the cerebellum, only male VPA rats had significantly lower *Cntnap2* gene expression compared to controls (0.19 ± 0.05 vs. 1.00 ± 0.17, $p = 0.002$). In female VPA rats, there was a trend towards increased gene expression in the cerebellum (1.37 ± 0.34 vs. 1.00 ± 0.11, $p = 0.0241$). No significant differences were observed in the prefrontal cortex of rats.

Table 1
Genotype Distribution and Allele Frequencies of CNTNAP2 rs2710102 in Children with ASD and Controls.

Groups	Genotypes			G (risk) allele frequency, %
	GG	GA	AA	
ASD children ($n = 81$)	27 (33.3%)*	30 (37.0%)	24 (29.6%)	51.9
Controls ($n = 86$)	17 (19.8%)	48 (55.8%)	21 (24.4%)	47.7

Note. * $p = 0.035$, statistically significant differences between ASD children and controls.

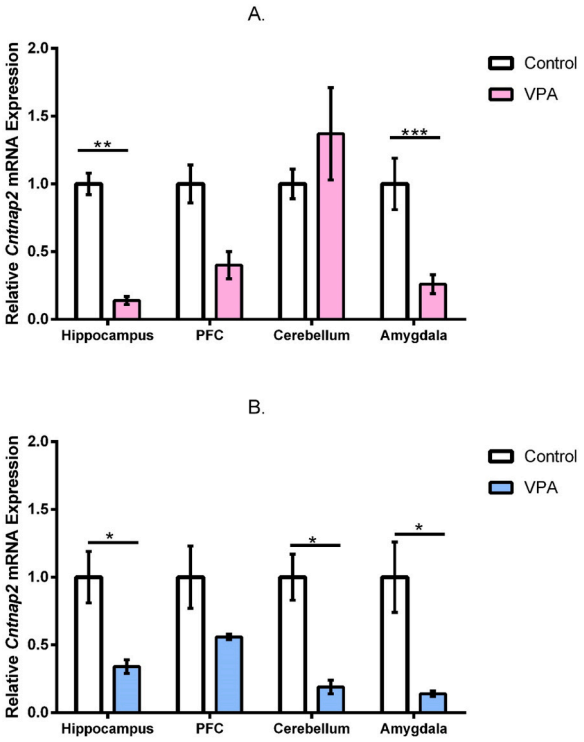

Fig. 2. Change of Relative Cntnap2 mRNA Expression in Different Brain Structures in VPA and Control Female (a) and Male (b) Rats. *Note.* Data are presented as mean ± SEM. * $p \leq 0.002$, * * $p = 5.1 \times 10^{-5}$, * ** $p = 0.0068$. PFC = prefrontal cortex.

4. Discussion

In line with previous work, in the present study we observed that the *CNTNAP2* rs2710102 G allele is a risk factor for ASD. Additionally, to the best of our knowledge, this is the first study to demonstrate that prenatal exposure to valproic acid may reduce the expression of the *Cntnap2* gene in the amygdala and hippocampus of rats, regardless of sex. These findings, when considered alongside previous *Cntnap2* gene knockout studies in rodents, indicate that the *CNTNAP2* gene may be implicated in ASD pathophysiology.

CNTNAP2 is highly expressed in brain structures and is also involved in neuronal development, synaptogenesis, and neuronal migration (Peñagarikano et al., 2011). During development, the CNTNAP2 protein acts as a neuronal adhesion molecule and receptor (Strauss et al., 2006). Previously, the *CNTNAP2* rs2710102 G allele was reported to be associated with language disorders, dyslexia, and ASD (Alarcón et al., 2008; Newbury et al., 2011; Peter et al., 2011; Whitehouse et al., 2011; Poot, 2014; Uddin et al., 2021). In our sample, we observed the expected association between the *CNTNAP2* rs2710102 GG genotype and ASD. The role of the *CNTNAP2* rs2710102 gene in contributing to the development of language delay in children with ASD has been shown in previous studies (Alarcón et al., 2008; Fang et al., 2021; Uddin et al., 2021). Another independent polymorphism (i.e., rs7794745 in the *CNTNAP2* gene) has also been shown to have an association with ASD and severity of language impairment in other populations (Nascimento et al., 2016; Uddin et al., 2021; Fang et al., 2021). The above said, there is some debate about the role of these two SNPs. In one meta-analysis (Zhang et al., 2019), these two SNPs (rs2710102 and rs7794745) were not shown to be associated with ASD development; in another meta-analysis (Uddin et al., 2021), these same two SNPs were indeed found to be associated with ASD. Using integrative multiomic analysis, Jang and colleagues (Jang et al., 2023) sought to explain that the development of *Cntnap2*-related ASD is largely due to mitochondrial dysfunction, axonal impairment, and synaptic activity. Panyard and colleagues (Panyard et al., 2021) demonstrated that the G allele of the *CNTNAP2* rs2710102 polymorphism was associated with high levels of the metabolite 3-hydroxy-3-methylglutarate, leading to abnormalities in the cholesterol synthesis pathway and the development of inflammation characteristic of children with ASD (Kwon et al., 2022).

Several studies have found an association between the *CNTNAP2* gene and activity in the brain, using functional magnetic resonance imaging (fMRI). One study demonstrated that variants of the *CNTNAP2* rs7794745 gene were associated with changes in activity of certain regions of the brain that are responsible for language function in healthy volunteers (Tan et al., 2010). Another study found an association between the *CNTNAP2* rs2710102 variant and brain activity in areas related to social and communication skills in children with autism (Scott-Van Zeeland et al., 2010). In particular, children carrying the risk allele (rs2710102 G) were found to have decreased long-range functional connections (i.e., fronto-occipital) but increased short-range functional connections (Scott-Van Zeeland et al., 2010). Importantly, in an animal model, homozygous loss of the *Cntnap2*$^{-/-}$ gene resulted in a reduction in local and distant prefrontal functional connectivity. This suggests that neurodevelopmental disorders and autism can be explained by selective

dysregulation of connections in integrative prefrontal areas (Liska et al., 2018).

The role and functions of *Cntnap2* can be studied by knocking out the gene in animals. Knocking out (KO) the *Cntnap2* gene in mice leads to various phenotypic changes, including impaired social behaviour, communication, learning, and memory. A complete KO in mice led to epileptic seizures and a reduction in the number of interneurons. Neuronal migration was impaired, and the activity of the neural network was abnormal (Peñagarikano et al., 2011). In another study, *Cntnap2* KO in rats and mice affected behavior and electroencephalogram scores in the same way, but with differences in phenotypic expression. *Cntnap2* KO rats exhibited severe motor seizures, hyperactivity, and increased consolidation of wakefulness and REM sleep. Although *Cntnap2* KO mice did not show any seizure-like events, they showed hypoactivity and fragmentation of wakefulness (Thomas et al., 2017). In a study by Poulin and Fox (2021), gene knockout rat models showed a deficit in social interaction and increased repetitive and anxious behavior. *Cntnap2* KO mice showed deficiencies in silent gap detection but a surprising superiority in pitch discrimination over control animals. Stereological analysis revealed a reduction in the number and density of neurons. There was also a shift in the size distribution of neurons towards smaller neurons in the medial geniculate nucleus of mutant mice (Truong et al., 2015). Knocking out the *Cntnap2* gene also affects learning and memory. *Cntnap2* KO mice have problems learning new tasks and remembering and recalling information (Rendall et al., 2016). These findings suggest that this gene is important for normal brain development and function, particularly in social behavior and communication. They also highlight the link between changes in the *CNTNAP2* gene and different aspects of ASD.

Our previous work with these rats demonstrated that prenatal administration of valproic acid induced behavioural changes characteristic of autistic patients (Semina et al., 2023). More specifically, VPA rats showed impaired social and anxiety behaviour. Male and female rats prenatally administered valproic acid at a dose of 500 mg/kg showed increased contact with a familiar non-social object in the Three-Chamber Social Test. In addition, the VPA rats showed a preference for closed arms in the Elevated Plus Maze Test. Mitsuhashi et al. (2023) discovered changes in social interaction in mice that received valproic acid at a lower concentration from embryonic day 1 to birth. Male mice showed a preference for a new object (an unfamiliar mouse), and there were no differences in arm preference in the Elevated Plus Maze between control and test mice (Mitsuhashi et al., 2023). The observed differences may be attributed to interspecific convergence and differences between rats and mice (Viggars et al., 2023; Till et al., 2022), as well as variations in the route, duration, and dosage of valproic acid administration.

The present study suggests that the reduction in hippocampal and amygdala *Cntnap2* gene expression in rats in the VPA model of autism could result from several factors. First, the hippocampus and the amygdala are key structures associated with social behavior and emotional responses (Machado et al., 2008; Jonason & Enloe, 1971). The hippocampus plays an important role in memory formation and learning, as well as in the regulation of emotions and stress (Kim & Diamond, 2002). The amygdala is involved in processing emotional signals and forming social bonds (Machado et al., 2008; Jonason & Enloe, 1971). Changes in expression of the *Cntnap2* gene in these structures may therefore disrupt these functions. Second, valproic acid may have a direct effect on expression of the *Cntnap2* gene. Valproic acid affects DNA methylation, which can lead to changes in gene activity, including *Cntnap2* (Wang et al., 2010; Hamza et al., 2017). Valproic acid can directly reduce *Cntnap2* gene expression in the hippocampus and amygdala (Lauber et al., 2016; Zang et al., 2022). Finally, genes associated with ASD often show vulnerability to the environment and stress (Hamza et al., 2017). The VPA rat model of autism can create stressful conditions for brain development, which can lead to altered *Cntnap2* gene expression in the hippocampus and amygdala (Zang et al., 2022). Overall, the reduction in *Cntnap2* gene expression in the rat hippocampus and amygdala in the VPA model of autism may be the result of the interaction of several factors, including the role of these structures in social behavior and emotional responses, the direct effect of valproic acid on *Cntnap2* gene expression, and the effects of stress and the environment on susceptibility.

It was noted a decade ago that the cerebellum plays an important role in cognitive processes and social behaviour (Becker & Stoodley, 2013; Basson & Wingate, 2013). Cerebellum aberrant development has been observed in individuals with ASD (Scott et al., 2009; Sathyanesan et al., 2019). Furthermore, prenatal administration of VPA in mice models has resulted in cerebellar developmental defects and an impaired gene expression profile (Becker & Stoodley, 2013; Basson & Wingate, 2013; Guerra et al., 2023). In particular, Wang and colleagues (2018) observed premature migration of granular cell precursors and a decrease in the number of Purkinje cells (PC) in the cerebellar cortex, which correlates with their impaired synaptic functionality. The *Cntnap2* gene is highly expressed in PC and regulates their morphology (Gogolla et al., 2009; Wang et al., 2018). Other studies have demonstrated that *Cntnap2* KO mice show changes in cerebellar volume and defects in cerebellar sensory learning (Ellegood & Crawley, 2015; Kloth et al., 2015). Further, several studies have noted that *Cntnap2* expression in various brain tissues of the KO model is sex-dependent (Dawson et al., 2023; Schaafsma et al., 2017; Townsend & Smith, 2017). Townsend and Smith (2017) observed that mutations of *Cntnap2* had a stronger effect on the functional responses of cortical circuits and its expression in male mice than in female mice. In contrast to female mice, reduced expression of *Cntnap2* only in KO or heterozygous male mice resulted in reduced visually evoked activity in upper visual areas associated with the dorsal stream. The sex specificity of *Cntnap2* expression was also demonstrated in a mice model by Schaafsma and colleagues (2017). These authors demonstrated that corticotropin receptor 1-releasing hormone (*Crhr1*) expression was increased in males whose mothers experienced early stress-induced maternal immune activation (Schaafsma et al., 2017). A neurobiological study found that male *Cntnap2* KO mice microglia showed increased activated morphology and phagocytosis of synaptic structures compared to wild type (WT) mice, although no differences were observed in female KO and WT mice (Dawson et al., 2023). The above examples notwithstanding, there is a lack of data on sex-dependent changes in *Cntnap2* expression in the cerebellum. In the present study, we used rats that were exposed prenatally to valproic acid to complement previous findings. In a study by Ojiro and colleagues (2022) the cerebral cortex of male rats showed a significant 0.65-fold downregulation in *Cntnap2* expression when offspring of post-pubertal male rats were orally administered a high dose (900 mg/kg) of valproic acid for 28 days (Ojiro et al., 2022). Our results indicate that only males showed a significant decrease in *Cntnap2* expression in the cerebellum, while females tended to show an increase. This observation may be related to the influence of sex hormones. Hoffman et al. (2016) demonstrated the effect of 17β-estradiol on

experiments with danio-mutant *cntnap2* fish. The mutants had a deficit of inhibitory neurons and impaired regulation of signal transduction in excitatory and inhibitory neurons, leading to the nocturnal hyperactivity observed in individuals with ASD. Estrogen receptor agonists suppressed this phenotype (Hoffman et al., 2016). Furthermore, it is possible that sex hormones may influence *Cntnap2* gene expression in cerebellar tissue through perinatal effects of testosterone in males, which contribute to the development of differences in male and female microglia throughout the cerebellum (McCarthy et al., 2017). According to Donna M. Werling's hypothesis, however, such an observed difference may be modulated by natural sexually dimorphic processes in sexually dimorphic microglia, specifically the prenatal effects of VPA on *Cntnap2* expression (A.M. Werling et al., 2016; D.M. Werling et al., 2016). Taken together, these findings emphasize the significance of gender as a factor when evaluating the functional activity of a gene in animal models. Further neurobiological studies are required to observe individual brain tissue cell types and explore molecular pathways.

A key strength of the present research is the combined study of the *CNTNAP2* gene in humans and animals. The findings from our human study support previous research and provide evidence for the *CNTNAP2* gene rs2710102 polymorphism as a risk factor for ASD. We also found, in the first study of its kind, that *Cntnap2* gene expression was significantly lower in the amygdala and hippocampus of VPA rats compared to controls, regardless of sex. Against the backdrop of this study's strengths, we should note some potential limitations—the relatively limited sample size of ASD children and controls, and the lack expression in rats at the protein level. Regardless, however, our findings further highlight the importance of the *CNTNAP2* gene in ASD and its potential link to the changes in brain activity seen with this disorder.

CRediT authorship contribution statement

Liliya R. Safiullina: Data curation. **Ilnur S. Sabirov:** Data curation. **Elena V. Valeeva:** Writing – original draft, Visualization, Investigation, Formal analysis. **Ildus I. Ahmetov:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Denis O. Fesenko:** Investigation. **Tim Rees:** Writing – review & editing. **Irina I. Semina:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Dmitriy O. Nikitin:** Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data Availability

Data will be made available on request.

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