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mS-11, a mimetic of the mSin3-binding helix in NRSF, ameliorates social interaction deficits in a prenatal valproic acid-induced autism mouse model

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ABSTRACT

Growing evidence suggests pivotal roles for epigenetic mechanisms in both animal models of and individuals with autism spectrum disorders (ASD). Neuron-restrictive silencer factor (NRSF) binds to neuron-restrictive silencing elements in neuronal genes and recruits co-repressors, such as mSin3, to epigenetically inhibit neuronal gene expression. Because dysregulation of NRSF is related to ASD, here we examined the effects of mS-11, a chemically optimized mimetic of the mSin3-binding helix in NRSF, on the behavioral and morphological abnormalities found in a mouse model of valproic acid (VPA)-induced ASD. Chronic treatment with mS-11 improved prenatal VPA-induced deficits in social interaction. Additionally, we found that NRSF mRNA expression was greater in the somatosensory cortex of VPA-exposed mice than of controls. Agreeing with these behavioral findings, mice that were prenatally exposed to VPA showed lower dendritic spine density in the somatosensory cortex, which was reversed by chronic treatment with mS-11. These findings suggest that mS-11 has the potential for improving ASD-related symptoms through inhibition of mSin3-NRSF binding.

1. Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by core symptoms that include impairments in social behavior and communication (Geschwind and Levitt, 2007; Persico and Bourgeron, 2006). Children with ASD also exhibit high rates of irritability and aggressive behavior (Lecavalier, 2006). Although the atypical antipsychotic drugs risperidone and aripiprazole effectively treat irritability in ASD patients, no pharmacological agents have been approved for treating the core symptoms of ASD (Accordino et al., 2016; Goel et al., 2018). Thus, developing novel treatments is imperative and understanding the pathogenetic factors underlying ASD is of utmost importance.

The multigenic condition of ASD has been speculated to depend on epigenetic effects (Loke et al., 2015), although the exact factors remain unclear. Epigenetic regulation is crucial for nervous system development and several common mental retardation syndromes and related neurodevelopmental disorders are caused by abnormalities in chromatin remodeling (Dall'Aglio et al., 2018; Eshraghi et al., 2018; Tsankova et al., 2007). The transcription factor neuron-restrictive silencer factor (NRSF; also known as repressor element 1 silencing transcription factor [REST]) plays a fundamental role in neurodevelopment and represses neuronal differentiation by binding to conserved NRS elements (NRSE) in the gene promoters of non-neuronal cells,

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where it associates with one of several large repressor complexes, including the transcriptional co-repressor mSin3, CoREST, and histone deacetylases (HDACs) (Hwang and Zukin, 2018; Zhao et al., 2017). NRSF also modulates the expression of NRSE-containing genes in mature neurons; inhibition of NRSF leads to neuronal activation and the promotion of neurogenesis (Kuwabara et al., 2004). Patients with ASD exhibit significant downregulation of NRSF target genes, suggesting that NRSF activity is associated with ASD in humans (Katayama et al., 2016). Additionally, CHD8, a member of the chromodomain helicase DNA-binding (CHD) family of proteins, is known to interact physically with NRSF, and CHD8 haploinsufficiency causes aberrant NRSF activation, resulting in an autistic-like phenotype in mice (Katayama et al., 2016).

A mimetic of the mSin3-binding helix in NRSF has been recently developed, named mS-11 (Molecular Weight, 429.57) (Ueda et al., 2017). mS-11 strongly binds to mSin3 with a binding mode similar to that of NRSF, thus inhibiting endogenous mSin3-NRSF binding. In the separate experiment, the plasma and brain levels of mS-11 at 5 min after intraperitoneal (i.p.) administration of mS-11 (30 mg/kg) were about 4 μg/mL and 1 μg/g tissue for adult male ICR mice, respectively, indicating that mS-11 can cross the blood-brain barrier (unpublished data). mS-11 had a short half-life of less than an hour and it was not detected in mouse brain tissue 24 h after i.p. administration. In a mouse model of neuropathic pain, mS-11 was found to ameliorate abnormal pain-related behavior and to reverse the loss of peripheral morphine analgesia that is caused by epigenetic μ-opioid receptor-gene silencing *via* NRSF and HDAC (Matsumoto et al., 2006). To assess the potential that inhibiting mSin3-NRSF binding can treat ASD-related symptoms, the present study examined the effects of mS-11 on the social interaction deficits found in a mouse model of ASD in which mice are prenatally exposed to valproic acid (VPA) (Hara et al., 2012; Kataoka et al., 2013). Maternal use of VPA during pregnancy has been implicated in the etiology of ASD in children (Christensen et al., 2013; Ornoy, 2009). Rodents prenatally exposed to VPA show behavioral alterations similar to those observed in humans with ASD (Roullet et al., 2010; Schneider and Przewłocki, 2005; Wagner et al., 2006) and thus may be useful as an animal model of ASD. We also analyzed the mRNA expression of NRSF and mSin3 as well as the dendritic morphology in the brains of VPA-exposed mice.

2. Materials and methods

2.1. Animals

We purchased 8-week-old male and female ICR (CD1) mice from Japan SLC Inc. (Hamamatsu, Japan). The mice were housed individually in plastic cages (28 × 17 × 12 cm) under a standard light/dark cycle (12-h light cycle starting at 8:00) and at a constant temperature of 22 ± 1 °C. The animals had *ad libitum* access to food and water, and were handled in accordance with the guidelines established by the Animal Care and Use Committee of the Graduate Schools of Pharmaceutical Sciences and Dentistry, Osaka University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Vaginal smear checks were conducted as previously reported (Hara et al., 2015, 2016). Vaginal smears were collected from all female mice every day and stained using Giemsa solution. Female mice were mated with male mice overnight when a vaginal smear indicated proestrus or early estrus. The next day was defined as gestation day 0.

2.2. Drug administration

mS-11 was synthesized at PRISM BioLab Co., Ltd. (Yokohama, Japan). VPA, Kolliphor® HS 15, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). mS-11 was dissolved in distilled water with 2% Kolliphor® HS 15 and 8% DMSO. VPA was dissolved in saline (0.9% NaCl solution; Otsuka pharmaceutical Co., Ltd., Tokushima, Japan). Administration of VPA was conducted as previously reported (Hara et al., 2012, 2015, 2016; Kataoka et al., 2013; Takuma et al., 2014). Either VPA (500 mg/kg) or saline was i.p. administered to pregnant mice on E12.5 at 10 mL/kg body weight. All animals were returned to their home cages immediately after VPA administration and left undisturbed until offspring were weaned. After weaning, offspring were caged in groups of 5–6 mice of the same sex at postnatal day 21. We used only male offspring in this study to avoid the sex differences that we reported in previous publications (Hara et al., 2012, 2015; Kataoka et al., 2013). mS-11 (10, 30 mg/kg) or vehicle was i.p. administered at 10 mL/kg body weight once daily for 2 weeks from the age of 8 weeks, and behavioral and morphological analysis were carried out 24 h after the last administration. mS-11 at dose of 10 mg/kg partially but significantly ameliorated abnormal pain behavior in mouse models of neuropathic pain and fibromyalgia (Ueda et al., 2017). Then, the dose could affect a potential mechanism in the central nervous system as well as the peripheral nervous system. Because 30 mg/kg of mS-11 might achieve sufficient concentrations in mouse brain, we have chosen 10 and 30 mg/kg of mS-11 in this study. The duration of the treatment was based on our previous studies that chronic (2 weeks), but not acute, treatment with the attention deficit/hyperactivity disorder drugs methylphenidate and atomoxetine improved VPA-induced impairments in social interaction and the decreased spine density in the prefrontal cortex (Hara et al., 2016). Because the effects of mS-11 may be achieved at least in part *via* epigenetic mechanisms, we firstly sought to identify the effects of chronic treatment with mS-11.

2.3. Social interaction test

The social interaction test was carried out according to our previous reports (Hara et al., 2016; Kataoka et al., 2013). Briefly, an intruder mouse was placed in the test cage after the test mouse had been habituated to the cage for 60 min. Each pair was socially naïve to each other before the test session. Over the total experimental period (20 min), the duration of sniffing behaviors of the test mouse to face, back and anogenital area of the intruder mouse and allogrooming behaviors was measured as a social interaction time by a well-trained observer blind to experimental groups. This test was carried out between 10:00 and 14:00.

2.4. Real-time RT-PCR

Total RNA was isolated using QIAzol Lysis Reagent (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. Reverse transcription of total RNA (500 ng) and real-time RT-PCR were performed as described previously (Inoue et al., 2018). Real-time RT-PCR was conducted with GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The following primers were used: 5′-CACCTGCGAGCTGGCGAGAAC-3′ (forward) and 5′-CACATTTTAAATGGCTTCTCTCACCTG-3′ (reverse) for NRSF; 5′-CGCTGTGAGGATGAGAGATTT3′ (forward) and 5′-CTCCAGC ACTCGGATGGT-3′ (reverse) for mSin3; and 5′-GTGTTCCTACCCCCAA TGTG-3′ (forward) and 5′-TACCAGGAAATGAGCTTGAC-3′ (reverse) for GAPDH. All data were normalized to GAPDH mRNA levels and expressed as relative change in mRNA.

2.5. Dendritic spine analysis

Dendritic spine morphological analysis was carried out as previously described (Hara et al., 2016; Takuma et al., 2014). Golgi–Cox impregnation was performed using the FD Rapid GolgiStain TM Kit (FD Neurotechnologies, Ellicott, MD, USA) according to the manufacturer's instructions. In brief, mice were deeply anesthetized and then decapitated. The brains were removed and immersed in an impregnation

solution comprising potassium dichromate, mercuric chloride, and potassium chromate at room temperature for 2 weeks. Then, the brains were transferred to a cryoprotectant solution at 4 °C for 2.5 days in the dark. Coronal sections (thickness:100 μm) were cut on a cryostat (CM1950; Leica Microsystems GmbH, Wetzlar, Germany) at −22 °C, mounted on gelatin-coated microscope slides (FD Neurotechnologies), and allowed to air dry at room temperature in the dark for up to 1 week. After drying, the sections were placed in the cryoprotectant solution, dehydrated with a graded ethanol series, defatted in xylene, and finally coverslipped using Mount Quick (Daido Sangyo, Saitama, Japan). Digitized images were obtained using an upright light microscope with a cooled CCD digital camera system (Axio Imager.M2/AxioCam MRc5; Carl Zeiss, Jena, Germany) with a 100× oil immersion lens. The 30–40 serial Z-stack images were collected at 0.2-μm intervals to cover the entire depth of the dendritic arbors (25–50 μm), then projected into a single TIFF image using the Z-Stack and Extended Focus modules (AxioVision 4.8.2; Carl Zeiss). All dendritic protrusions were counted as spines using NIH ImageJ 1.46 for Windows (http://rsb.info.nih.gov/ij) by a well-trained observer blind to experimental groups. The dendritic spine density was averaged from at least three sections per mouse.

2.6. Statistical analysis

All results are presented as the mean ± standard error of the mean (SEM). Data for mRNA expression were analyzed using the Student's *t*-test. Other data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey–Kramer *post hoc* correction for multiple comparisons. Statistical analyses were performed using Statview 5.0 J software for Apple Macintosh (SAS Institute Inc., Cary, NC, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

We examined the effects of chronic treatment with mS-11 (10, 30 mg/kg, once daily for 2 weeks) on social interaction deficits in mice prenatally exposed to VPA (Fig. 1). Two-way ANOVA revealed a significant main effect of prenatal VPA exposure ($F_{1,75} = 9.7$, $P < 0.01$), but not of mS-11 treatment ($F_{2,75} = 1.6$, $P > 0.05$), and the significant

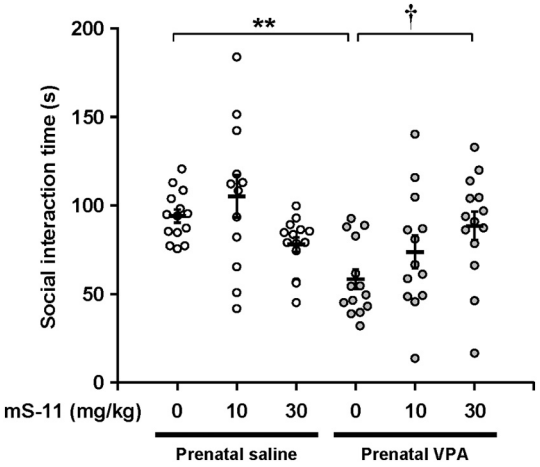

Fig. 1. Effects of chronic treatment with mS-11 on social interaction deficits in mice prenatally exposed to VPA. Pregnant female mice were intraperitoneally (i.p.) injected with VPA (500 mg/kg) or saline at E12.5. Male offspring were i.p. injected with mS-11 (10 or 30 mg/kg) or vehicle once daily for 2 weeks from the age of 8 weeks. A social interaction test was performed at 8 weeks of age. The duration of sniffing and allogrooming was measured during a 20-min test period. Data are expressed as the means ± SEM (n = 12–14/group). $^{**}P < 0.01$, compared with control mice prenatally exposed to saline and postnatally treated with vehicle. $^{\dagger}P < 0.05$, compared with mice prenatally exposed to VPA and postnatally treated with vehicle. *VPA*, valproic acid.

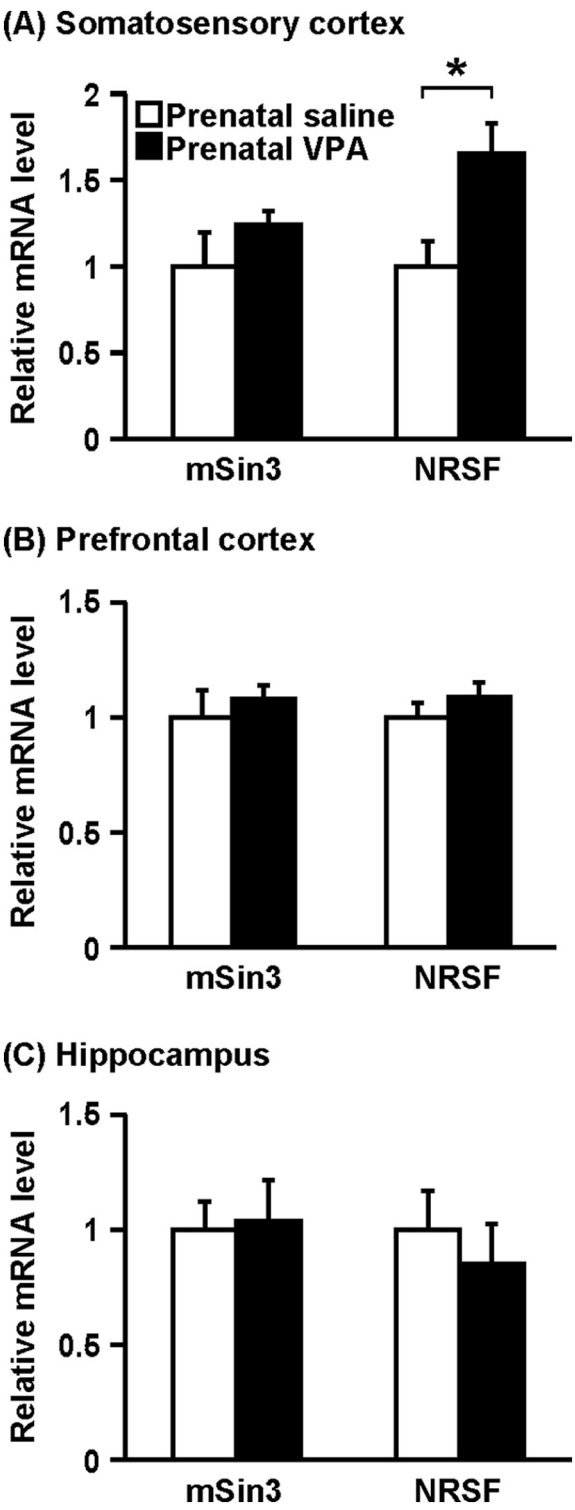

Fig. 2. Effects of prenatal VPA exposure on NRSF and mSin3 mRNA expression in the brain of mice. Pregnant female mice were intraperitoneally (i.p.) injected with VPA (500 mg/kg, i.p.) or saline at E12.5. Expression of NRSF and mSin3 in the somatosensory cortex (A), prefrontal cortex (B), and hippocampus (C) of adult male offspring was quantified by real-time PCR. The values obtained from the prenatal saline-exposed mice were arbitrarily set to 1. Data are expressed as the means ± SEM (n = 8/group). $^{*}P < 0.05$, compared with control mice prenatally exposed to saline. *VPA*, valproic acid; *NRSF*, Neuron-restrictive silencer factor.

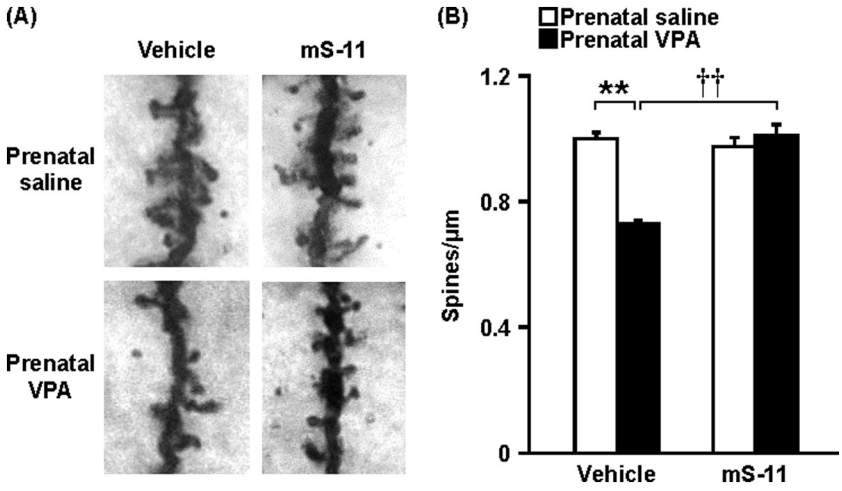

Fig. 3. Effects of chronic treatment with mS-11 on dendritic spine density in the somatosensory cortex of mice prenatally exposed to VPA. Male offspring were intraperitoneally (i.p.) injected with mS-11 (30 mg/kg) or vehicle once daily for 2 weeks from the age of 8 weeks. Golgi–Cox staining was performed at 10 weeks of age. Typical microscopic images of Golgi–Cox-impregnated neurons in the somatosensory cortex (A) and quantification of dendritic spine density (B) are shown. Data are expressed as the means ± SEM (n = 4/group). $^{**}P < 0.01$, compared with control mice prenatally exposed to saline and postnatally treated with vehicle. $^{††}P < 0.01$, compared with mice prenatally exposed to VPA and postnatally treated with vehicle. *VPA*, valproic acid.

interaction between the two ($F_{2,75} = 6.0$, $P < 0.01$). *Post-hoc* analysis revealed that mS-11 at 30 mg/kg alleviated the social interaction deficits in mice that were prenatally exposed to VPA.

We have previously observed that the prefrontal cortex, somatosensory cortex, and hippocampus are involved in the expression of behavioral abnormalities in VPA-exposed mice (Hara et al., 2012, 2016; Takuma et al., 2014). Thus, we investigated the changes in NRSF and mSin3 mRNA expression in the adult brain of VPA-exposed mice (Fig. 2). We found significantly greater NRSF mRNA expression in the somatosensory cortex of the VPA-exposed mice than in that of the control mice ($P < 0.05$, analyzed by Student's *t*-test). Similar results were not found for mSin3 ($P > 0.05$). Neither NRSF nor mSin3 mRNA expression was altered in the prefrontal cortex or in the hippocampus.

Finally, we examined the effects of chronic treatment with mS-11 (30 mg/kg, once daily for 2 weeks) on the loss of dendritic spines in the somatosensory cortex of mice prenatally exposed to VPA (Fig. 3). Two-way ANOVA revealed significant main effects of mS-11 treatment ($F_{1,12} = 25.2$, $P < 0.001$), prenatal VPA exposure ($F_{1,12} = 20.5$, $P < 0.001$), and the interaction between the two ($F_{1,12} = 35.9$, $P < 0.0001$). *Post-hoc* analysis revealed that mS-11 at 30 mg/kg reversed the spine loss in the somatosensory cortex.

4. Discussion

In the present study, we first demonstrated that chronic administration of mS-11 improved social interaction deficits in VPA-exposed mice. Although the etiology of ASD remains elusive, clinical research has indicated that receiving VPA during pregnancy increases the incidence of autism in children (Christensen et al., 2013; Ornoy, 2009). VPA is an antiepileptic drug widely used for treating epilepsy, migraine, and bipolar disorder (Calabresi et al., 2007; Henry, 2003). VPA acts as an HDAC inhibitor as well as a sodium- and calcium-channel blocker and a GABA-transaminase inhibitor (Göttlicher et al., 2001). VPA inhibits class I (HDAC1, 2, 3, 8) and class IIa (HDAC4, 5, 7, 9) HDACs (Gurvich et al., 2004). We previously reported that prenatal VPA exposure at E12.5 induces ASD-like behavioral abnormalities in male mice, including social interaction deficits (Kataoka et al., 2013). Because mice prenatally exposed to valpromide (which exerts an equivalent pharmacological effect to VPA without affecting HDAC activity; Eikel et al., 2006; Nishikawa and Scatton, 1985) at E12.5 did not experience any changes in behavior or embryonic brain development, we can conclude that transient HDAC inhibition plays a fundamental role in the pathogenesis of ASD in offspring exposed to VPA. Of note, the neuropeptide oxytocin, which clinically improves impairments in social communication and reciprocity in people with ASD (Watanabe et al., 2014, 2015), also reversed prenatal VPA-induced social interaction deficits (Hara et al., 2017a). Thus, VPA-exposed offspring meet at least face and predictive validities as an ASD-like model. Interestingly, chronic treatment with sodium butyrate, another HDAC inhibitor, as well as VPA in adult VPA-exposed offspring reversed prenatal VPA-induced behavioral abnormalities, suggesting the postnatal HDAC inhibition could restore the ASD-related symptoms (Takuma et al., 2014). NRSF binds to NRSE and recruits co-repressor mSin3 and HDAC, and then suppress the gene expression. Thus, HDAC plays a role in NRSF-mediated effects. mS-11 is a mimetic of the mSin3-binding helix of NRSF that binds strongly to mSin3 with a binding mode similar to that of NRSF (Ueda et al., 2017), thus it inhibits endogenous mSin3-NRSF binding. Similar to the action of HDAC inhibitors, mS-11 might then also inhibit HDAC-mediated effects, leading to reduced social interaction deficits.

We also observed greater NRSF mRNA expression in the somatosensory cortex, but not in the prefrontal cortex or hippocampus, of VPA-exposed mice when they reached adulthood. We have previously found that the prefrontal cortex, somatosensory cortex, and hippocampus are involved in the expression of behavioral abnormalities in VPA-exposed mice (Hara et al., 2012, 2016; Takuma et al., 2014). The reason for the region-specific effects of prenatal VPA on adult NRSF expression is unclear, but NRSF activation in the brain has been implicated in patients with ASD (Katayama et al., 2016). In animal models, mice heterozygous for *Chd8* mutations manifest ASD-like behavioral characteristics including altered social behavior, which is associated with aberrant NRSF activation (Katayama et al., 2016). Although many genes contain the NRSE motif, we have not yet identified the molecules associated with VPA-induced social interaction deficits that are targeted by NRSF. Further experiments are required in near future.

Dendritic spines are the site of neuronal plasticity at excitatory synapses, and spine morphology has been demonstrated to contribute or be related to synapse formation and synaptic transmission (Matsuzaki et al., 2001). Altered density of dendritic spines has been observed in ASD and related neurodevelopmental diseases, both in humans and in animal models of autism, both genetic and non-genetic (Martínez-Cerdeño, 2017). VPA-exposed mice (Hara et al., 2016, 2017b) and rats (Bringas et al., 2013; Raza et al., 2015) also present with reduced numbers of spines in the prefrontal cortex. In this study, we observed that mice prenatally exposed to VPA showed reduced dendritic spine density in the somatosensory cortex. Furthermore, and consistent with the behavioral changes, mS-11 restored the spines that had been lost in the somatosensory cortex of VPA-exposed mice. Thus, the effects of mS-11 on social interaction deficits might be associated with regulation of dendritic spines, although the exact mechanisms are still unknown.

5. Conclusions

The present study demonstrated that chronic treatment with mS-11

improved prenatal VPA-induced deficits in social interaction. We also found that NRSF mRNA expression was greater in the somatosensory cortex of VPA-exposed mice and that mS-11 reversed prenatal VPA-induced spine loss in the somatosensory cortex. These findings suggest that mS-11 might have the potential for improving ASD-related symptoms.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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