Maternal stress in Shank3<sup>ex4-9</sup> mice increases pup-directed care and alters brain white matter in male offspring

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Abstract

Gene-environment interactions contribute to the risk for Autism Spectrum Disorder (ASD). Among environmental factors, prenatal exposure to stress may increase the risk for ASD. To examine if there is an interaction between exposure to maternal stress and reduced dosage or loss of Shank3, wild-type (WT), heterozygous (HET) and homozygous (HOM) female mice carrying a deletion of exons four through nine of Shank3 (<i>Shank3<sup>ex4-9</sup></i>) were exposed to chronic unpredictable mild stress (CUMS) from prior to conception throughout gestation. This study examined maternal care of these dams and the white matter microstructure in the brains of their adult male offspring. Overall, our findings suggest that maternal exposure to CUMS increased pup-directed care for dams of all three genotypes. Compared to WT and HET dams, HOM dams also exhibited increased maternal care behaviors with increased time spent in the nest and reduced cage exploration, regardless of exposure to CUMS. Diffusion tensor imaging showed higher mean fractional anisotropy in the hippocampal stratum radiatum of WT and HOM male offspring from dams exposed to CUMS and HOM offspring from unexposed dams, compared to WT male offspring from unexposed dams. These data support that CUMS in Shank3-mutant dams results in subtle maternal.
Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by social and communication impairments along with repetitive and stereotypic behaviors [1]. With a prevalence of 1 in 68 children and 1 in 42 in males [2], ASD has become an increasingly important public health concern. The etiology of ASD is multifactorial with highly heterogeneous genetic contribution [3]. Many putative ASD risk genes have been identified [4–6], with major contributing genes those that encode proteins with a role in synaptic function, such as NRXN1, NLGN3, NLGN4, and SHANK3 [5, 7, 8]. SHANK3 encodes a predominantly neuronal scaffolding protein that localizes to the postsynaptic density of glutamatergic synapses [9, 10]. The Shank3ex4-9 deletion mice lack all major isoforms of the SHANK3 protein and display abnormal social and repetitive behaviors [11]. Adult Shank3ex4-9 deletion mice have reduced dendritic spine density and deficits in glutamatergic transmission [12]. It has also been described that there are white matter structural abnormalities thought to be associated with social impairment in the brains of individuals with ASD [13].

Although genetic predisposition is a well-established risk factor for the etiology of ASD, recent evidence suggests that environmental factors such as prenatal stress are also associated with an increased risk for ASD [14, 15]. Psychological stress during pregnancy affects a significant number of women, with up to 78% experiencing low-to-moderate levels of antenatal stress [16, 17]. Prenatal stress is also a risk factor for shortened gestation, preterm birth and obstetric complications [18] and has been linked to poor coping with adverse experiences in offspring [19]. Retrospective survey and population-based studies have found a higher incidence of ASD in children born to mothers who experienced more stress during pregnancy [15, 20–25]. In contrast, others found no increased risk for ASD with prenatal adverse life events [26], and even reported that mild to moderate levels of psychological stress can enhance fetal organ and neuromaturation in healthy populations [27, 28]. While the evidence linking ASD risk to increased maternal stress is limited and inconclusive, there is increasing support for the notion that individual variations in neuropsychiatric risk may result from genetic differences that can cause either resilience or vulnerability to environmental stressors [29–31]. Prenatal stress can also affect maternal behaviors, adding another layer of complexity to the impact of the perinatal environment [32].

In this study, we tested in a mouse model the hypothesis that maternal exposure to chronic unpredictable mild stress (CUMS), in the context of a Shank3 mutation that models an ASD-associated human mutation, influences maternal care behaviors of exposed dams. We also tested if neuronal connectivity in relevant brain regions in exposed offspring was altered.

Materials and methods

Mice

All methods and animal care procedures were approved by Baylor College of Medicine (BCM) Institutional Animal Care and Use Committee (IACUC). The approved protocol number is AN-5888. All experiments were conducted according to institutional and governmental regulations concerning the ethical use of animals in research. The animal facilities are approved by the Association for Assessment and Accreditation for Laboratory Animal Care International (NIGMS). The project described was also supported by Grant Number C06RR029965 from the National Center for Research Resources (NCRR). This work was also supported by the neurobehavioral core of the Baylor College of Medicine Intellectual and Developmental Disabilities Research Center (IDDRC) supported by award 1U54HD083092-04 from the Eunice Kennedy Shriver National Institute for Child Health and Human Development (NICHD). The content is solely the responsibility of the authors and does not represent the official views of the BWF and the NIGMS, NCRR and NICHD of the National Institutes of Health. We note that while BKYW is a current employee of Noldus Information Technology Inc., she was not affiliated with Noldus Information Technology Inc. in any shape or form during the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Animals were housed in AAALAC approved Transgenic Mouse Facility (TMF) at BCM. All maternal behavioral testing were conducted in the Neurobehavioral core facility of the BCM Intellectual and Developmental Disabilities Research Center (IDDRC) situated in the TMF. All experimental animals were generated by mating heterozygous (HET) Shank3<sup>ex4-9</sup> (Shank3<sup>tm1Yhj</sup>, JAX # 017442) male and female mice, generously donated by Dr. Arthur Beaudet (Baylor College of Medicine, Houston, TX). The mouse line was maintained on a C57BL/6J background. Standard rodent chow (Pico LabRodent Diet, #5053, Purina USA) and water were available ad libitum. The colony room was maintained on a 12:12 light/dark cycle with lights on at 07:00 hour. Genotyping of Shank3<sup>ex4-9</sup> mice was performed per Wang et al. 2011 [33] with minor modifications. A group of mice comprising of 6–13 dams per genotype and CUMS exposure were examined for maternal behavior. All behavioral testing were performed during the light period (09:00–17:00 hour) and mice were acclimated to the testing rooms for ≥ 30 minutes prior to each assay.

Detailed materials and methods including mouse breeding strategy, CUMS protocol, genotyping, and exact number of mice used for each test are provided in S1 File (Supplemental materials and methods).

**Maternal care behavioral testing**

Maternal care behavioral testing was conducted during the first eight days postpartum (PND 1–8) on wildtype (WT), heterozygous (HET), and homozygous (HOM) Shank3<sup>ex4-9</sup> adult females who were at least six weeks old. These dams had either been exposed to chronic unpredictable mild stress (CUMS) from one week before mating with HET sires until just prior to parturition or were unexposed to CUMS (Control). The order of testing was as follows and was the same for all dams tested: nesting quality scores (PND 1 and 3), maternal in-nest care assessments (PND 1, 3, and 5), pup retrieval test (PND 3), and maternal intruder test (PND 8) [34–37]. For the maternal in-nest care assessments, pup retrieval, and maternal intruder tests, videos were recorded under room lighting in the home cage with a Plexiglas lid in a biological fume hood with the fan running to produce background noise. During data acquisition, the test administrator was blinded to genotype and exposure, and videos were de-identified. Videos were scored using a Psion 5.0 hand-held computer, and data was extracted using the Noldus Observer (Leesburg, VA). Details of individual tests are provided in S1 File (Supplemental materials and methods).

**Diffusion tensor imaging**

Diffusion tensor imaging (DTI) was performed on eight-week-old WT and HOM adult male offspring from control and CUMS-exposed Shank3<sup>ex4-9</sup> HET dams (Control: 5 WT, 4 HOM; CUMS: 2 WT, 3 HOM). The mice were anesthetized with isoflurane (Cat# NDC 11695-6776-2, Henry Schein Animal Health, Dublin, OH) and transcardially perfused with heparinized phosphate buffered saline (PBS) followed by fixation with 4% paraformaldehyde (PFA). Perfusion was carried out using a diffusion pressurization set up from Warner Instruments (Model VPP-6, Hamden, CT). The skulls were exposed, fixed in PFA and stored in 5mM gadopentate dimeglumine (Cat# NDC 50419-188-15; Bayer, Leverkusen, Germany) at 4°C until imaging was performed to assess for white matter tractography in the brains [38]. For structural characterization of offspring brains, whole brain voxel-voxel analysis was applied, and the average fractional anisotropy (FA) values in regions of interest (ROI) were reported. Further details of image acquisition and data processing are in S1 File (Supplemental materials and methods).
Statistical analysis

All data were analyzed by a two-way ANOVA for main effects of genotype (G) and exposure (E) and for gene × exposure interaction (G×E) using Prism GraphPad Version 6 (La Jolla, CA). Significant main effects and interactions were followed up with Tukey’s post hoc analysis for multiple comparisons. Data are presented as mean ± standard error of the means (SEM) and the statistical significance was set at \( P < 0.05 \).

Results

CUMS exposure does not significantly affect pregnancy outcomes of \( \text{Shank}3^{ex4-9} \) mutant dams

To evaluate the impact of preconceptional and gestational exposure to CUMS on pregnancy outcomes, we assessed gestational length and litter size of WT, HET and HOM \( \text{Shank}3^{ex4-9} \) mutant dams. Gestational length (Fig 1A) was not affected by genotype (F\(_{2,45}\) = 2.902, ns) and CUMS exposure (F\(_{1,45}\) = 1.032, ns), and there was no G×E interaction (F\(_{2,45}\) = 0.484, ns). Similarly, there was no main effect of genotype (F\(_{2,47}\) = 1.080, ns) and exposure (F\(_{1,47}\) = 3.213, ns), and no G×E interaction (F\(_{2,47}\) = 0.939, ns) on litter size at birth (Fig 1B). These findings suggest that the maternal \( \text{Shank}3 \) genotype and maternal exposure to CUMS did not affect pregnancy outcomes.

Exposure to CUMS increases pup-directed care and \( \text{Shank}3^{ex4-9} \) mutant dams exhibit more maternal in-nest care behaviors

Maternal care behaviors, including nest building, nursing and grooming are necessary for pup survival [11]. It has been reported that mild chronic stress increases nursing and grooming but does not modulate nesting or nurturing behaviors, while ultra-mild chronic stress does not alter maternal care [39, 40]. There was a significant G×E interaction on nest quality scores (Fig 2A; F\(_{2,50}\) = 4.079, \( P < 0.05 \)). Post-hoc analysis identified a statistical trend (\( P = 0.055 \)) of lower quality nest built by HET \( \text{Shank}3 \) dams exposed to CUMS compared to nests built by unstressed WT dams. There were no differences in nest qualities scores between all other groups.

![Fig 1. CUMS exposure in Shank3^{ex4-9} mutant dams did not affect pregnancy outcomes.](https://doi.org/10.1371/journal.pone.0224876.g001)
The time spent nursing was unaffected by genotype (Fig 2B; G: $F_{2,48} = 2.522$, $P = 0.09$). CUMS exposure increased pup-directed care behaviors of dams across all genotypes (Fig 2C; E: $F_{1,48} = 6.126$, $P < 0.05$), while the Shank3 genotype did not change pup-directed care behaviors (Fig 2C; G: $F_{2,48} = 0.530$, ns).

Self-care behaviors of dams was not affected by CUMS exposure (Fig 2D; E: $F_{1,48} = 1.807$, ns; G×E: $F_{2,48} = 0.236$, ns), or by genotype ($F_{2,48} = 2.571$, $P = 0.087$). The amount of time that dams spent exploring their cages was also not altered by CUMS exposure (Fig 2E; E: $F_{1,48} = 0.6016$, ns; G×E: $F_{2,48} = 0.536$, ns), but there was a genotype effect ($F_{2,48} = 3.332$, $P < 0.05$) with HOM dams spending less time exploring their cages compared to HET dams (Post-hoc $P < 0.05$).

Shank3ex4-9 HOM dams also spent more time in the nest compared to WT and Shank3ex4-9 HET dams (Fig 2F; G: $F_{2,48} = 12.03$, $P < 0.0001$; post-hoc $P < 0.003$), and this was not affected by exposure to CUMS (E: $F_{1,48} = 1.428$, ns; G×E: $F_{2,48} = 1.842$, ns).

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CUMS exposure of Shank3\textsuperscript{ex4-9} mutant dams does not alter their pup retrieval and maternal aggression responses to intruders

Studies have shown that maternal exposure to mild stress can robustly increase the pup retrieval response, but that exposure to ultra-mild stress increases the latency to first pup retrieval [39, 40]. In our study, no observable differences were identified in total pup handling time (Fig 3A; G: F\textsubscript{2,46} = 2.541, ns; E: F\textsubscript{1,46} = 1.411, ns; G×E: F\textsubscript{2,46} = 1.842, ns), or in the time it took dams to crouch or hover over the nest while all pups were in the nest (Fig 3B; G: F\textsubscript{2,46} = 0.1779, ns; E: F\textsubscript{1,46} = 0.0951, ns; G×E: F\textsubscript{2,46} = 0.6203, ns). When pups were removed from the nest and placed in a corner of the cage, all dams took a similar amount of time to collect the pups and place them back in the nest, irrespective of genotype or exposure to CUMS (Fig 3C–3E; ns).

Gestational stress can also result in altered maternal responses towards an intruder manifesting as either heightened aggression or impaired defense behaviors depending on the severity of the stress paradigm [39, 40]. There were no differences between groups in the number of and time spent on active or passive contacts (Fig 4A–4D; ns), or in total time engaged in social interactions (Fig 4E; G: F\textsubscript{2,46} = 2.988, ns; E: F\textsubscript{1,46} = 0.04353, ns; G×E: F\textsubscript{2,46} = 1.672, ns).

Fractional anisotropy of white matter is significantly altered in brains of offspring from heterozygous Shank3\textsuperscript{ex4-9} mutant dams exposed to CUMS

Maternal stress can cause changes in white matter in brains of offspring that can persist into adulthood [41]. Additionally, there is DTI evidence of white matter structural abnormalities in children with ASD that is associated with social impairment [13, 42]. Adult WT and HOM...
male offspring were examined by DTI and the fractional anisotropy (FA) values compared. A voxel by voxel analysis was used to evaluate 29 brain regions of interest based on the Hopkins mouse brain template (S1 Table). Compared to the unexposed control group, WT male offspring from dams exposed to CUMS had increased mean FA values in the stratum radiatum, a distinct region of the CA1 area in the hippocampus (Fig 5A; G: $F_{1,10} = 72.61, P < 0.0001$; E: $F_{1,10} = 52.10, P < 0.0001$; G×E: $F_{1,10} = 38.97, P < 0.0001$; post-hoc $P < 0.0001$). This CUMS-associated increase in stratum radiatum FA value was not observed in HOM male offspring (Fig 5A; post-hoc $P > 0.5$). The FA changes in the stratum radiatum (Fig 5B, i-iv) can be seen in the direction-encoded color maps (DEC) (Fig 5B, v-viii), the zoomed-in images for the DEC panel (Fig 5B, ix-xii) and the outline of the stratum radiatum segmentation (Fig 5B, xiii-xvi) from representative coronal images comparing adult WT and HOM male offspring with and without CUMS exposure. These data show potential changes in directionality and diffusion in this contiguous region (Fig 5B, ix-xii; yellow arrow).

Amongst the other examined brain regions, the neocortex and nucleus accumbens of offspring exposed to maternal CUMS had reduced mean FA values irrespective of the Shank3 genotype indicating an effect of stress on these regions (S1 Fig: Neocortex: G: $F_{1,10} = 4.974, P < 0.05$; G×E: $F_{1,10} = 1.103, ns$; Nucleus accumbens: G: $F_{1,10} = 2.663, ns$; E: $F_{1,10} = 7.517, P < 0.05$; G×E: $F_{1,10} = 0.00851, ns$).

HOM males showed higher mean FA values in the amygdala and septum irrespective of exposure to maternal CUMS (S1 Fig: Amygdala: G: $F_{1,10} = 4.974, P < 0.05$; E: $F_{1,10} = 3.015, ns$; G×E: $F_{1,10} = 1.790, ns$; Septum: G: $F_{1,10} = 6.891, P < 0.05$; E: $F_{1,10} = 0.5451, ns$; G×E: $F_{1,10} = 1.064, ns$). In the piriform cortex, stress exposure and genotype both showed a reduction in...
Discussion

Through the study of numerous mouse models carrying different Shank3 alleles, from complete disruption of the locus to specific inactivation of the major full-length isoforms, it is clear that disruption of Shank3 in mice causes varying degrees of ASD-like behaviors and synaptic dysfunction [33, 43–46]. However, the incomplete penetrance of SHANK3 mutations in humans suggests that there may be non-genetic contributing factors as well as modifier loci. Given the conflicting reports on the effects of maternal stress as a risk factor for ASD in humans [15, 20–26] and the limited ability of human studies to control or account for parental genotypes, presence of underlying maternal psychiatric illness, and presence of adverse

mean FA values (S1 Fig: G: $F_{1,10} = 6.08, P < 0.05$; E: $F_{1,10} = 9.648, P < 0.05$; G×E: $F_{1,10} = 0.408$, ns).
postnatal environments [15], we sought to evaluate the effect of CUMS exposure during pregnancy in an isogenic mouse model.

The Shank3<sup>ex4-9</sup> mutant mouse is a well-characterized model of human ASD, with HOM mice exhibiting abnormal social and repetitive behaviors [33, 43–45, 47]. This study is the first to evaluate the direct effects of CUMS exposure on maternal behaviors of dams in relationship to their Shank3<sup>ex4-9</sup> genotype.

With respect to stress, we found that CUMS had mild effect on maternal behaviors of dams of all Shank3<sup>ex4-9</sup> genotypes consisting only of increased time spent on pup-directed care. There was also a decrease in nest quality score for CUMS-exposed HET dams, but all other measures of maternal care (i.e. time spent nursing, pup retrieval, and maternal aggressive behaviors) and pregnancy outcome (i.e. gestational length and litter size) were not altered by CUMS exposure. Most of our findings are consistent with previous reports that mild to ultra-mild prenatal stress did not alter nest building or nurturing behaviors [39, 40], but we could not replicate the observation by Meek <i>et al.</i> [39] that exposure to mild stress in Swiss-Webster mice, which are not known to be more sensitive to stress [48, 49], robustly increased the pup retrieval response. In contrast, our observation of increased pup-directed behavior in the CUMS group was consistent with findings from Maestripieri <i>et al.</i> [50], who described a positive correlation between prenatal chronic stress-induced anxiety and increased maternal care behaviors. The overall findings likely differed between these studies because they used a slightly different stress protocol, indicating that even minor changes in the timing and types of applied environmental stressors modifies how maternal care behavior is affected.

Since the Shank3<sup>ex4-9</sup> mutant mouse is a model for ASD, previous studies of this model have not focused on maternal care behavior. Here we demonstrate that Shank3<sup>ex4-9</sup> mutation does not negatively impact maternal care of dams. In addition to more CUMS-induced pup-directed behavior, Shank3<sup>ex4-9</sup> HOM dams spent more time in the nest and less time in cage exploration also suggesting more nurturing behavior. The lower cage exploration is not because Shank3<sup>ex4-9</sup> HOM females are less active, as Wang <i>et al.</i> showed that they have typical locomotion in the open field test [33]. Shank3<sup>ex4-9</sup> HOM mice also investigate novel objects more often from within than from outside the nest, which is not due to increased anxiety levels as these mice perform the same as WT mice on anxiety tests [33]. This finding combined with other behavioral anomalies they observed led Wang <i>et al.</i> to suggest that Shank3<sup>ex4-9</sup> HOM mice have less behavioral plasticity, which may model impairments in novelty processing associated with human ASD [33, 51]. Therefore, although their increased time spent in the nest and lower cage exploration seem to suggest that Shank3<sup>ex4-9</sup> HOM dams are better caregivers to their pups, these behavioral changes may actually not be driven by desire to care for their pups.

We and others have postulated that gene-environment interactions modulate the severity of the ASD phenotype [7]. Data in rodent models indicate that chronic prenatal stress alters brain development with the severity of changes being influenced by genetic background [29]. Prenatal stress alters development of the hippocampus, prefrontal cortex and amygdala, causing decreased neurogenesis and neuronal connectivity [29]. In addition to synaptic dysfunction, abnormal development of neural connectivity is also observed in children with ASD and has been implicated in pathologies of social behavior [41, 52–54]. The majority of DTI studies on ASD brains in humans and in animal models have found FA value changes in various brain regions that rarely overlap between individuals, but changes in either direction have been correlated with the severity of ASD symptoms [52]. We found that prenatal CUMS exposure significantly increased FA of the stratum radiatum, located in the CA1 region of the hippocampus, but only in WT males with FA values similar to those observed in HOM males with and without prenatal CUMS exposure. In previous characterizations of different Shank3
knockout mouse models, there were fewer hippocampal CA1 pyramidal neurons with altered neurite structure and lower current amplitude. In addition, impaired neuroplasticity, as measured by long-term potentiation (LTP), is also predominantly observed in the stratum radiatum [43, 55]. Similarly, mice with a mutation in NLGN3, another synaptic protein linked to ASD, also have altered synaptic structures in the stratum radiatum [56], thus these mouse models share structural changes in the stratum radiatum and social interaction deficits [43, 56].

In addition to the CUMS-associated FA changes in the WT stratum radiatum, we also found lower FA values in the neocortex and nucleus accumbens in the CUMS group, irrespective of the Shank3<sup>ex4-9</sup> genotype. The changes in FA in the neocortex are consistent with previous reports that prenatal stress alters the medial prefrontal cortex, a region within the neocortex [57]. In a large human case-control study, ASD was associated with lower volume of nucleus accumbens [58]. The nucleus accumbens also plays a role in the mesolimbic reward pathway with connections to the amygdala [59], where we observed a significant genotype effect with an increased FA value in HOM males compared with WT mice, regardless of CUMS exposure. Although it is currently unclear through which mechanism these structural alterations correlate with social behavior in the context of prenatal stress and the Shank3 genotype, the affected regions have previously been identified as abnormal in individuals with ASD or have been associated with social deficits in humans and mouse models. Finally, we detected significant effects of CUMS exposure and Shank3<sup>ex4-9</sup> genotype, but no G×E interaction on FA values, in the piriform cortex, a region susceptible to structural changes upon stress [60].

In summary, our data indicate that perinatal maternal exposure to chronic mild stress influences maternal behavior. We found that CUMS-exposed dams have more pup-directed care behaviors, the HOM Shank3 mutation caused dams to spend more time in the nest and less time exploring the cage, suggestive of increased maternal care behaviors. Furthermore, in male offspring we correlated FA changes in the stratum radiatum of the hippocampal CA1 region, with exposure to maternal CUMS in WT mice leading to white matter connectivity that mimics that of Shank3<sup>ex4-9</sup> HOM mice. Characterization of this Shank3 mutant mouse, a genetic mouse model of ASD, in the context of a chronic mild stress paradigm allowed us to determine that incremental burden of an adverse environmental exposure contributes to the severity of some maternal behaviors and alterations in brain white matter tractography.

Supporting information

S1 Data. Supplemental datatables. These are the accompanying supplemental datatables that for the data used to generate all figures. Excel sheet labels correspond to each figure panel. (XLSX)

S1 Fig. DTI analysis in the white matter of male offspring from CUMS-exposed dams.
Fractional anisotropy (FA) values for the indicated brain regions in WT and HOM male offspring of CUMS-exposed dams are presented. CUMS exposure reduced FA values in both the neocortex (A) and nucleus accumbens (B), regardless of genotypes. There was an increase in FA values in the amygdala (C) and septum (D) of male offspring of all three genotypes, irrespective of CUMS exposure. There were also significant main effects of genotype and exposure in the piriform cortex, with higher FA values in HOM compared to WT; CUMS exposure reduced the FA values in both groups. WT: Wildtype; HOM: Homozygous. N = Control (5 WT, 4 HOM) and CUMS (2 WT, 3 HOM). Individual data points are represented by diamonds. G: Main effect of genotype, E: Main effect of exposure; *P < 0.05. (TIF)
S1 File. Supplemental materials and methods. This file contains all supplemental materials and methods.

S1 Table. Most offspring brain regions analyzed by DTI do not show significant differences between groups. Results of diffusion tensor imaging (DTI) conducted in 24 additional brain regions of offspring from HET dams with and without CUMS exposure (Control/CUMS). The number of male offspring in each group was Control (5 WT, 4 HOM) and CUMS (2 WT, 3 HOM).

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References
1. American Psychiatric Association., American Psychiatric Association. DSM-5 Task Force. Diagnostic and statistical manual of mental disorders: DSM-5. 5th ed. Washington, D.C.: American Psychiatric Association; 2013. xlv, 947 p. p.
2. Christensen DL, Baio J, Braun KV, Bilder D, Constantino JN, et al. Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years—Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012. MMWR Surveill Summ. 2016; 65(3):1–23. https://doi.org/10.15585/mmwr.ss6503a1 PMID: 27031587.
3. Levy SE, Mandell DS, Schultz RT. Autism. Lancet. 2009; 374(9701):1627–38. https://doi.org/10.1016/S0140-6736(09)61376-3 PMID: 19819542.
4. Geishecker MR, Heymann G, Wang T, Coe BP, Turner TN, Stessman HAF, et al. Hotspots of missense mutation identify neurodevelopmental disorder genes and functional domains. Nat Neurosci. 2017; 20(8):1043–51. Epub 2017/06/20. https://doi.org/10.1038/nn.4589 PMID: 28628100.
5. Miles JH. Autism spectrum disorders—a genetics review. Genet Med. 2011; 13(4):278–94. https://doi.org/10.1097/GIM.0b013e3181f67ba PMID: 21358411.
6. Stessman HA, Xiong B, Coe BP, Wang T, Hoekzema K, Fenckova M, et al. Targeted sequencing identifies 91 neurodevelopmental-disorder risk genes with autism and developmental-disability biases. Nat Genet. 2017; 49(4):515–26. Epub 2017/02/14. https://doi.org/10.1038/ng.3792 PMID: 28191889.
7. Chaste P, Leboyer M. Autism risk factors: genes, environment, and gene-environment interactions. Dialogues Clin Neurosci. 2012; 14(3):281–92. PMID: 23226853.
8. Kumar RA, Christian SL. Genetics of autism spectrum disorders. Current neurology and neuroscience reports. 2009; 9(3):188–97. PMID: 19348707.

9. Betancur C, Buxbaum JD. SHANK3 haploinsufficiency: a "common" but underdiagnosed highly penetrant monogenic cause of autism spectrum disorders. Molecular autism. 2013; 4(1):17. https://doi.org/10.1186/2040-2394-4-17 PMID: 23758743

10. Uchino S, Waga C. SHANK3 as an autism spectrum disorder-associated gene. Brain Dev. 2013; 35(2):106–10. https://doi.org/10.1016/j.braindev.2012.05.013 PMID: 22749736.

11. Wang Z, Storm DR. Maternal behavior is impaired in female mice lacking type 3 adenylyl cyclase. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology. 2011; 36(4):772–81. https://doi.org/10.1038/npp.2010.211 PMID: 21150908

12. Varghese M, Keshav N, Jacot-Descombes S, Warda T, Wicinski B, Dickstein DL, et al. Autism spectrum disorder: neuropathology and animal models. Acta neuropathologica. 2017; 134(4):537–66. https://doi.org/10.1007/s00401-017-1736-4 PMID: 28584888

13. Noriuchi M, Kikuchi Y, Yoshiura T, Kira R, Shigeto H, Hara T, et al. Altered white matter fractional anisotropy and social impairment in children with autism spectrum disorder. Brain research. 2010; 1362:141–9. https://doi.org/10.1016/j.brainres.2010.09.051 PMID: 20858472.

14. Beversdorf DQ, Stevens HE, Jones KL. Prenatal Stress, Maternal Immune Dysregulation, and Their Association With Autism Spectrum Disorders. Current psychiatry reports. 2018; 20(9):76. https://doi.org/10.1007/s11920-018-0945-4 PMID: 30094645.

15. Kinney DK, Munir KM, Crowley DJ, Miller AM. Prenatal stress and risk for autism. Neuroscience and biobehavioral reviews. 2008; 32(8):1519–32. https://doi.org/10.1016/j.neubiorev.2008.06.004 PMID: 18598714

16. Whitehead NS, Brogan DJ, Blackmore-Prince C, Hill HA. Correlates of experiencing life events just before or during pregnancy. Journal of psychosomatic obstetrics and gynaecology. 2003; 24(2):77–86. PMID: 12854392.

17. Woods SM, Melville JL, Guo Y, Fan MY, Gavin A. Psychosocial stress during pregnancy. American journal of obstetrics and gynecology. 2010; 202(1):61 e1–7. https://doi.org/10.1016/j.ajog.2009.07.041 PMID: 19766975

18. Wadhwa PD, Ernong S, Buss C, Lu MC. The contribution of maternal stress to preterm birth: issues and considerations. Clinics in perinatology. 2011; 38(3):351–84. https://doi.org/10.1016/j.clp.2011.06.007 PMID: 21989014

19. Meijer A. Child psychiatric sequelae of maternal war stress. Acta psychiatrica Scandinavica. 1985; 72(6):505–11. https://doi.org/10.1111/j.1600-0447.1985.tb02647.x PMID: 2417452.

20. Beversdorf DQ, Manning SE, Hillier A, Anderson SL, Nordgren RE, Walters SE, et al. Timing of prenatal stressors and autism. Journal of autism and developmental disorders. 2005; 35(4):471–8. https://doi.org/10.1007/s10803-005-5037-8 PMID: 16134032.

21. Class QA, Abel KM, Khashan AS, Rickert ME, Dalman C, Larsson H, et al. Offspring psychopathology following preconception, prenatal and postnatal maternal bereavement stress. Psychological medicine. 2014; 44(1):71–84. https://doi.org/10.1017/S0033291713000780 PMID: 23591021

22. Roberts AL, Lyall K, Rich-Edwards JW, Ascherio A, Weisskopf MG. Maternal exposure to intimate partner abuse before birth is associated with autism spectrum disorder in offspring. Autism: the international journal of research and practice. 2016; 20(1):26–36. https://doi.org/10.1177/1362361314566049 PMID: 25662292

23. Ronald A, Pennell CE, Whitehouse AJ. Prenatal Maternal Stress Associated with ADHD and Autistic Traits in early Childhood. Frontiers in psychology. 2010; 1:223. https://doi.org/10.3389/fpsyg.2010.00223 PMID: 21833278

24. Walder DJ, Laplante DP, Sousa-Pires A, Veru F, Brunet A, King S. Prenatal maternal stress predicts autism traits in 6(1/2) year-old children: Project Ice Storm. Psychiatry research. 2014; 219(2):353–60. https://doi.org/10.1016/j.psychres.2014.04.034 PMID: 24907222.

25. Ward AJ. A comparison and analysis of the presence of family problems during pregnancy of mothers of "autistic" children and mothers of normal children. Child psychiatry and human development. 1990; 20(4):279–88. https://doi.org/10.1007/bf00706020 PMID: 2376213.

26. Rai D, Golding J, Magnusson C, Steer C, Lewis G, Dalman C. Prenatal and early life exposure to stressful life events and risk of autism spectrum disorders: population-based studies in Sweden and England. PloS one. 2012; 7(6):e38893. https://doi.org/10.1371/journal.pone.0038893 PMID: 22719977

27. Allen MC, Donohue PK. Neuromaturation of multiples. Semin Neonatal. 2002; 7(3):211–21. PMID: 12234745.
28. DiPietro JA, Novak MF, Costigan KA, Atella LD, Reusing SP. Maternal psychological distress during pregnancy in relation to child development at age two. Child development. 2006; 77(3):573–87. https://doi.org/10.1111/j.1467-8624.2006.00891.x PMID: 16686789.

29. Boersma GJ, Tamashiro KL. Individual differences in the effects of prenatal stress exposure in rodents. Neurobiol Stress. 2015; 1:100–8. http://dx.doi.org/10.1016/j.nysr.2014.10.006 PMID: 27589662.

30. Sharma S, Powers A, Bradley B, Ressler KJ. Gene x Environment Determinants of Stress- and Anxiety-Related Disorders. Annual review of psychology. 2016; 67:239–61. https://doi.org/10.1146/annurev-psych-122414-033408 PMID: 26442668.

31. Wermter AK, Laucht M, Schimmelmann BG, Banaschewski T, Sonuga-Barke EJ, Rietschel M, et al. From nature versus nurture, via nature and nurture, to gene x environment interaction in mental disorders. European child & adolescent psychiatry. 2010; 19(3):199–210. https://doi.org/10.1007/s00787-009-0082-z PMID: 20024596.

32. Beery AK, Kauer D. Stress, social behavior, and resilience: insights from rodents. Neurobiol Stress. 2015; 1:116–27. https://doi.org/10.1016/j.nysr.2014.10.004 PMID: 25562050.

33. Wang X, McCoy PA, Rodriguiz RM, Pan Y, Je HS, Roberts AC, et al. Synaptic dysfunction and abnormal behaviors in mice lacking major isoforms of Shank3. Hum Mol Genet. 2011; 20(15):3093–108. https://doi.org/10.1093/hmg/ddr212 PMID: 21558424.

34. Chourbaji S, Hoyer C, Richter SH, Brandwein C, Pfeiffer N, Vogt MA, et al. Differences in mouse maternal care behavior—is there a genetic impact of the glucocorticoid receptor? PloS one. 2011; 6(4): e19218. https://doi.org/10.1371/journal.pone.0019218 PMID: 21525222.

35. Deacon RM. Assessing nest building in mice. Nature protocols. 2006; 1(3):1117–9. https://doi.org/10.1038/nprot.2006.170 PMID: 17406392.

36. Macbeth AH, Stepp JE, Lee HJ, Young WS 3rd, Caldwell HK. Normal maternal behavior, but increased pup mortality, in conditional oxytocin receptor knockout females. Behavioral neuroscience. 2010; 124(5):677–85. https://doi.org/10.1037/a0020799 PMID: 20939667.

37. Martin-Sanchez A, Valera-Marin G, Hernandez-Martinez A, Lanuza E, Martinez-Garcia F, Agustin-Pavon C. Wired for motherhood: induction of maternal care but not maternal aggression in virgin female CD1 mice. Frontiers in behavioral neuroscience. 2015; 9:197. https://doi.org/10.3389/fnbeh.2015.00197 PMID: 26257621.

38. Bitner BR, Perez-Torres CJ, Hu L, Inoue T, Pautler RG. Improvements in a Mouse Model of Alzheimer’s Disease Through SOD2 Overexpression are Due to Functional and Not Structural Alterations. Magnetic resonance insights. 2012; 5:1–6. https://doi.org/10.4137/MRI.S9352 PMID: 22639527.

39. Meek LR, Dittel PL, Sheehan MC, Chan JY, Kjolhaug SR. Effects of stress during pregnancy on maternal behavior in mice. Physiology & behavior. 2001; 72(4):473–9. https://doi.org/10.1016/s0031-9384(00)00431-5 PMID: 11282130.

40. Pardon M, Gerardin P, Joubert C, Perez-Diaz F, Cohen-Salmon C. Influence of prepartum chronic ultramild stress on maternal pup care behavior in mice. Biological psychiatry. 2000; 47(10):858–63. https://doi.org/10.1016/s0006-3223 (99)00253-x PMID: 10807958.

41. Sarkar S, Craig MC, Dell’Acqua F, O’Connor TG, Catani M, Deeley Q, et al. Prenatal stress and limbic-prefrontal white matter microstructure in children aged 6–9 years: a preliminary diffusion tensor imaging study. The world journal of biological psychiatry: the official journal of the World Federation of Societies of Biological Psychiatry. 2014; 15(4):346–52. https://doi.org/10.3109/15622975.2014.903336 PMID: 24815522.

42. Alexander AL, Lee JE, Lazar M, Field AS. Diffusion tensor imaging of the brain. Neurotherapeutics: the journal of the American Society for Experimental NeuroTherapeutics. 2007; 4(3):316–29. https://doi.org/10.1016/j.nurt.2007.05.011 PMID: 17599689.

43. Bozdagi O, Sakurai T, Papapetrou D, Wang X, Dickstein DL, Takahashi N, et al. Haploinsufficiency of the autism-associated Shank3 gene leads to deficits in synaptic function, social interaction, and social communication. Molecular autism. 2010; 1(1):15. https://doi.org/10.1186/2040-2392-1-15 PMID: 21167025.

44. Kouser M, Speed HE, Dewey CM, Reimers JM, Widman AJ, Gupta N, et al. Loss of predominant Shank3 isoforms results in hippocampus-dependent impairments in behavior and synaptic transmission. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2013; 33(47):18448–68. https://doi.org/10.1523/JNEUROSCI.3017-13.2013 PMID: 24259569.

45. Peca J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN, et al. Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. Nature. 2011; 472(7344):437–42. https://doi.org/10.1038/nature09965 PMID: 21423165.

46. Zhou Y, Kaiser T, Monteiro P, Zhang X, Van der Goes MS, Wang D, et al. Mice with Shank3 Mutations Associated with ASD and Schizophrenia Display Both Shared and Distinct Defects. Neuron. 2016; 89(1):147–62. https://doi.org/10.1016/j.neuron.2015.11.023 PMID: 26687841.
47. Wang X, Xu Q, Bey AL, Lee Y, Jiang YH. Transcriptional and functional complexity of Shank3 provides a molecular framework to understand the phenotypic heterogeneity of SHANK3 causing autism and Shank3 mutant mice. Molecular autism. 2014; 5:30. https://doi.org/10.1186/2040-2392-5-30 PMID: 25071925
48. van Bogaert MJ, Groenink L, Oosting RS, Westphal KG, van der Gugten J, Olivier B. Mouse strain differences in autonomic responses to stress. Genes, brain, and behavior. 2006; 5(2):139–49. Epub 2006/ 03/02. https://doi.org/10.1111/j.1601-183X.2005.00143.x PMID: 16507005.
49. Crawley JN. Behavioral phenotyping strategies for mutant mice. Neuron. 2008; 57(6):809–18. Epub 2008/03/28. https://doi.org/10.1016/j.neuron.2008.03.001 PMID: 18367082.
50. Maestripieri D, Badiani A, Puglisi-Allega S. Prepartal chronic stress increases anxiety and decreases aggression in lactating female mice. Behavioral neuroscience. 1991; 105(5):663–8. https://doi.org/10.1037/0735-7044.105.5.663 PMID: 1815617.
51. Maes JH, Eling PA, Wezenberg E, Vissers CT, Kan CC. Attentional set shifting in autism spectrum disorder: differentiating between the role of perseveration, learned irrelevance, and novelty processing. Journal of clinical and experimental neuropsychology. 2011; 33(2):210–7. https://doi.org/10.1080/ 13803395.2010.501327 PMID: 20694871.
52. Amois SH, Catani M. Altered white matter connectivity as a neural substrate for social impairment in Autism Spectrum Disorder. Cortex. 2015; 62:158–81. https://doi.org/10.1016/j.cortex.2014.10.014 PMID: 25433958.
53. Mak-Fan KM, Morris D, Vidal J, Anagnostou E, Roberts W, Taylor MJ. White matter and development in children with an autism spectrum disorder. Autism: the international journal of research and practice. 2013; 17(5):541–57. https://doi.org/10.1177/1362361312442596 PMID: 22700988.
54. Zoghbi HY, Bear MF. Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities. Cold Spring Harb Perspect Biol. 2012; 4(3). https://doi.org/10.1101/ cshperspect.a009886 PMID: 22258914
55. Uppal N, Puri R, Yuk F, Janssen WG, Bozdagi-Gunal O, Harony-Nicolas H, et al. Ultrastructural analyses in the hippocampus CA1 field in Shank3-deficient mice. Molecular autism. 2015; 6:41. https://doi. org/10.1186/s13229-015-0036-x PMID: 26137200
56. Etherton M, Foldy C, Sharma M, Tabuchi K, Liu X, Shamloo M, et al. Autism-linked neuroligin-3 R451C mutation differentially alters hippocampal and cortical synaptic function. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(33):13764–9. https://doi.org/10.1073/ pnas.111093108 PMID: 21808020
57. Negron-Oyarzo I, Neira D, Espinosa N, Fuentetalia P, Aboitiz F. Prenatal Stress Produces Persistence of Remote Memory and Disrupts Functional Connectivity in the Hippocampal-Prefrontal Cortex Axis. Cereb Cortex. 2015; 25(9):3132–43. https://doi.org/10.1093/cercor/bhu108 PMID: 24860018.
58. van Rooij D, Anagnostou E, Arango C, Auzias G, Behrmann M, Busatto GF, et al. Cortical and Subcortical Brain Morphometry Differences Between Patients With Autism Spectrum Disorder and Healthy Individuals Across the Lifespan: Results From the ENIGMA ASD Working Group. The American journal of psychiatry. 2018; 175(4):359–69. https://doi.org/10.1176/appi.ajp.2017.17010100 PMID: 29145754.
59. Gordon J, Jack A, Pretzsch CM, Vander Wyk B, Leckman JF, Feldman R, et al. Intranasal Oxytocin Enhances Connectivity in the Neural Circuitry Supporting Social Motivation and Social Perception in Children with Autism. Scientific reports. 2016; 6:35954. https://doi.org/10.1038/srep35954 PMID: 27845765
60. Nacher J, Pham K, Gil-Fernandez V, McEwen BS. Chronic restraint stress and chronic corticosterone treatment modulate differentially the expression of molecules related to structural plasticity in the adult rat piriform cortex. Neuroscience. 2004; 126(2):503–9. https://doi.org/10.1016/j.neuroscience.2004.03. 038 PMID: 15207367.