Reduced Brain Volume and White Matter Alterations in *Shank3*-Deficient Rats

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Research

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Abstract

Background: Mutations and deletions in the *SHANK3* synaptic gene cause the major neurodevelopmental features of Phelan-McDermid syndrome (PMS). The *SHANK3* gene encodes a key structural component of excitatory synapses that is important for synaptogenesis. PMS is characterized by intellectual disability, autism spectrum disorder, cognitive deficits, physical dysmorphic features, sensory hyporeactivity, and alterations in the size of multiple brain regions. Clinical assessments and limited imaging studies have revealed a reduction in volume of multiple brain regions. They have also found white matter thinning and microstructural alterations to be persistent in patients with PMS. While many of these impairments have been replicated in mouse models of PMS, the brain structure of a rat model has not yet been studied.

Methods: We assessed the brain structure of haploinsufficient and homozygous *Shank3*-deficient rats that model the behavioral deficits of PMS with magnetic resonance and diffusion tensor imaging, and compared their brain structure to wild type littermates.

Results: Both gray and white matter structures were smaller in *Shank3*-deficient rats, leading to an overall reduction in brain size compared to wild type littermates. The largest region to be diminished in size was the neocortex. Some regions involved in sensory processing and white matter regions were also reduced in size. Lastly, the microstructure of two white matter tracts, the external capsule and fornix, was abnormal.

Conclusions: *Shank3*-deficient rats replicate the reduced brain volume and altered white matter phenotypes present in individuals with PMS. Therefore, the brain regions that were altered represent potential cross-species structural biomarkers that warrant further study.

Background

Phelan-McDermid syndrome (PMS) is a rare neurodevelopmental disorder caused by deletions of, or point mutations within, the terminal chromosomal region 22q13.3 that encompasses the *SHANK3* gene. The characteristics of PMS include intellectual disability, autism spectrum disorder (ASD), attention deficits, impaired communication, motor abnormalities, seizures, recurrent medical comorbidities, neonatal hypotonia, and macrocephaly (1). Additionally, alterations in reactivity to sensory stimuli is frequently observed in PMS (2, 3). The neuroanatomical abnormalities that could be underlying these physical and behavioral phenotypes have gone largely understudied. Understanding how the pathology that results from a *Shank3* deficiency affects brain structure in PMS pathogenesis could offer biomarkers to focus on in patients and rodent models. Neuroimaging studies on small cohorts have shown that individuals with PMS have reduced relative volumes of structures in the basal ganglia and cerebellum (4, 5), white matter thinning (6, 7), and alterations in fiber tracts (8, 9). However, these abnormalities have not yet been explored in a rat model. Rats offer larger brains than mice and thus finer imaging resolution as well as homology that is closer to humans compared to mice.

We used haploinsufficient and homozygous knockout (KO) rat models of PMS that we have previously demonstrated have social memory and attention deficits in addition to impaired synaptic plasticity (10) to investigate the effect of *Shank3* deficiency on brain structure with MRI and DTI.

Methods

Animal care and husbandry

This study used male wild type (WT), *Shank3*^{+/-}, and *Shank3*^{-/-} littermate three-month-old rats. *Shank3*-deficient rats were generated using zinc-finger nucleases on the outbred Sprague-Dawley background, as previously described (10). All rats were kept under veterinary supervision in a 12 h reverse light/dark cycle at 22±2°C. Animals were pair-caged with food and water available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

MRI

All imaging was performed by the BioMedical Molecular Imaging Institute using a Bruker Biospec 70/30 7 T scanner with a B-GA12S gradient insert (gradient strength 440 mT/m and slew rate 3444 T/m/s). A Bruker 4-channel rat brain phased array was used for all data acquisition in conjunction with a Bruker volume transmit 86-cm coil. All rats were imaged on a heated bed and respiration was monitored continuously until the end of the scan. The animals were anesthetized using isoflurane anesthesia (3% induction and 1.5% maintenance). After a three-plane localizer, a field map was acquired and the rat brain was shimmed using Mapshim software. A DTI protocol was acquired with a Pulsed Gradient Spin Echo – Echo-planar imaging (EPI) sequence with the following parameters: repetition time (TR) = 5000 ms, echo time (TE) = 22.6 ms, 4 segments, 30 gradient directions with b-value = 1000 s/mm² and 5 B0's, field of view (FOV) = 25 mm, matrix = 128x128, slice thickness = 1 mm, skip = 0, 6 averages, total acquisition time = 1 hr. The voxel size was 0.195 x 0.195 x 1 mm³ (1000 µm-thick). A high resolution T2 anatomical scan was obtained with a 3D Rapid Acquisition with Relaxation Enhancement (RARE) sequence with a RARE factor of 8, TR = 777 ms, effective TE = 52 ms, FOV = 30 mm x 27.25 mm x 30 mm, matrix size 256 x 256 x 128. The voxel size was 0.117 x 0.117 x 0.234 mm³ (234 µm-thick).

MRI region-based analytical pipeline with manual editing

A magnetic resonance imaging processing pipeline was used to perform semi-automated nonbiased brain segmentation, while blinded to genotype (WT: N = 6, *Shank3*^{+/-}: N = 10, *Shank3*^{-/-}: N = 10) (11). The pipeline is composed of six major steps: rigid registration of images to each other, generation of a whole-brain mask for each image, averaging of all images, creation of a whole-brain mask for this averaged image, segmentation of the average mask by regions of interest (ROIs), parcellation propagation of the segmented mask to individual subjects, and ROI-based statistics for the individual images. The deformation necessary to warp each subject's image to the average was used to calculate the volume of

the ROIs. After each mask was generated, it was improved manually in ITK-SNAP (www.itksnap.org). The segmentation into ROIs was determined by a template that was hand-segmented into 32 brain regions, listed in **Supplementary Table 1**.

Automatically generated segmented masks for the individual images that did not closely match the average segmented mask (two WT and one *Shank3*^{-/-} T2 mask) and individual data point outliers, defined as being outside 1.5 times the interquartile range, were excluded from the analysis. The whole-brain masks were used to determine whole-brain measures. Mean voxel intensity for each ROI and across the whole-brain was measured in both the T2 and DTI images and the volume of each ROI and whole-brain was calculated from the T2 images. Only white matter structures were included in the DTI analysis (see **Supplementary Table 1**). For each independent variable, if the distribution was nonparametric according to the Shapiro-Wilk's test, a Kruskal-Wallis test was performed, and if the distribution was normally distributed, a two-way ANOVA was used. Due to the fact that many comparisons were made across ROIs, the output was then assessed for the ability to survive a correction for multiple comparisons with a Bonferroni correction. Genotype was the only between-groups factor. If an effect had a significant nominal *p*-value (0.05), pairwise comparisons were made. If the data were parametric, a Tukey HSD test was used to compare the individual means. If the data were nonparametric, a Dunn's test was used for pairwise comparisons. An adjustment of the *p*-values was made to account for the additional comparisons. The effect size of each volumetric change was measured with a Cohen's *d*. Custom scripts written in the R statistical programming environment were used for the statistical analyses (R Development Core Team, 2006).

Results

Shank3 deficiency results in reduced brain volume

Structural T2-weighted MRI was used to assess both tissue density and volume in *Shank3*^{-/-}, *Shank3*^{+/-}, and WT littermates across the whole brain and in regions of interest (ROIs) (see all results in **Supplementary Table 2**). Notably, none of the nominal *p*-values from the pairwise comparisons survived a Bonferroni correction. Remarkably, we found a significant effect of genotype on the volume of the whole brain (Fig. 1A and B; ANOVA: *p* = 0.049), the brains of the *Shank3*^{-/-} rats showing a smaller size compared to their *Shank3*^{+/-} and WT littermates. This reduction in overall brain volume was driven by a decrease in the absolute volume of larger gray matter brain structures in the *Shank3*^{-/-} rats, including the neocortex, the piriform cortex, the thalamus, and the rest of forebrain (the regions of the forebrain that were not otherwise included in the list of ROIs) (Fig. 1A and B; ANOVA followed by Tukey HSD: *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.043; ANOVA followed by Tukey HSD: *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.0065; Kruskal-Wallis followed by Dunn's Test: WT vs. *Shank3*^{-/-}: *p* adj. = 0.0056, *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.02; Kruskal-Wallis followed by Dunn's Test: WT vs. *Shank3*^{-/-}: *p* adj. = 0.018, *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.012). The inferior and superior colliculi also had significantly smaller absolute volume in both *Shank3*^{-/-} and *Shank3*^{+/-} rats compared to WT littermates (Fig. 1A and B; ANOVA

followed by Tukey HSD: WT vs. *Shank3*^{-/-}: *p* adj. = 0.013, WT vs. *Shank3*^{+/-}: *p* adj. = 0.027 and ANOVA followed by Tukey HSD: WT vs. *Shank3*^{-/-}: *p* adj. = 0.0027, WT vs. *Shank3*^{+/-}: *p* adj. = 0.043, respectively). Finally, two white matter structures, the internal capsule and anterior commissure, were also reduced in absolute volume in the *Shank3*^{-/-} rats (Fig. 1A and B; ANOVA followed by Tukey HSD: WT vs. *Shank3*^{-/-}: *p* adj. = 0.065, *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.035 and WT vs. *Shank3*^{-/-}: *p* adj. = 0.099, *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.049) and trended to be decreased in the right external capsule (Fig. 1A and B; ANOVA: *p* = 0.085). The effect sizes of all changes were large (Cohen's *d* > |0.8|). Tissue density was not significantly altered and no alterations were identified in the volume of the ventricular system.

We next examined whether any ROIs were also altered in relative volume by calculating their percentage of the total brain volume. Due to the fact that the entire brain decreased in volume in *Shank3*^{-/-} rats, many of the brain regions that were decreased in absolute volume were not also decreased in relative volume. The only brain region that had a significantly lower relative volume in *Shank3*^{-/-} rats compared to WT littermates was the superior colliculus (Fig. 1A and C; ANOVA followed by Tukey HSD: WT vs. *Shank3*^{-/-}: *p* adj. = 0.041, WT vs. *Shank3*^{+/-}: *p* adj. = 0.089).

Loss of Shank3 expression leads to deficits in integrity of white matter regions

Lastly, we used DTI to assess the integrity of the white matter that connects these regions. Four indices were calculated from these images and used to describe the diffusion: axial, radial, and mean diffusion (AD, RD, and MD) and fractional anisotropy (FA). We examined diffusion in nine white matter pathways in *Shank3*-deficient rats and compared them to their WT littermates (see all results in **Supplementary Table 2**). We found that the nominal *p*-values for the DTI metrics of some of these regions were significant, however, none of them survived a Bonferroni correction. AD was significantly reduced in the left external capsule and FA was reduced in the right external capsule of *Shank3*^{-/-} rats (Fig. 2; ANOVA followed by Tukey HSD: *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.037; ANOVA followed by Tukey HSD: WT vs. *Shank3*^{-/-}: *p* adj. = 0.011, *Shank3*^{+/-} vs. *Shank3*^{-/-}: *p* adj. = 0.026). Similarly, MD was significantly increased in the fornix in *Shank3*^{-/-} rats compared to WT littermates (Fig. 2; ANOVA followed by Tukey HSD: WT vs. *Shank3*^{-/-}: *p* = 0.04).

Discussion

In this study, we discovered structural perturbations in the brains of *Shank3*-deficient rats, including an overall reduction in brain volume that was driven by a decrease in the volume of large, gray matter regions and alterations in the structural integrity of white matter domains.

Limited studies have shown that *Shank3* deficiency leads to decreased brain volumes in humans as well. A study of eleven individuals with PMS found reduced relative volumes of caudate nucleus, putamen, left pallidum, and cerebellar vermis (4). In a separate study, ten patients with a 22q13 terminal deletion had

cerebellar vermis hypoplasia (5). It would be worthwhile to explore the cause of this reduction in regional volumes in rodent models. At the cellular level, Shank3 protein deficiency in *Shank3*^{e4-9} and *Shank3B*^{-/-} mice has been shown to affect expression of synaptic proteins, spine head length, glutamatergic postsynaptic densities, dendritic arborizations and length, and spine density (12, 13). *Shank3B*^{-/-} mice have reduced levels of SAPAP3, Homer-1b/c, and PSD-93 in the postsynaptic densities of their striatum, a reduction in the glutamate receptor subunits GluR2, NR2A and NR2B, significantly reduced mean thickness and length of postsynaptic densities in glutamatergic neurons, and reduced spine densities of medium spiny neurons (13). *Shank3*^{e4-9} mice also have reduced expression of synaptic proteins (12). Future studies should examine how these cellular phenotypes affect the structure of these brain regions.

Many individuals with PMS are hyporeactive to sensory stimuli (2, 3). The superior and inferior colliculus are part of the visual and auditory pathways, respectively, and the thalamus plays a major role in relaying peripheric sensory information to the cerebral cortex. All three regions were smaller in *Shank3*-deficient rats. The response of the auditory cortex of *Shank3*^{+/-} rats to speech and non-speech sounds has been shown to be weaker than WT controls, with less spontaneous firing (14). Sensory sensitivities are a core feature of ASD (15). Interestingly, we recently reported comparable alterations in a rat model of another monogenic cause of ASD, Fragile X syndrome, using the same imaging techniques and the same parameters (16), indicating a commonality of sensory deficits between syndromes. The structure of these regions should be studied in relation to these functional consequences in rodent models of PMS.

The internal capsule and anterior commissure were decreased in absolute volume, AD was reduced in the external capsule, and MD was increased in the fornix of *Shank3*-deficient rats. These DTI findings suggest that white matter microstructure is not properly able to restrict diffusion along the axis of the axonal fibers, which may functionally affect major corticocortical and corticosubcortical projections. The fornix is one of the major output pathways of the hippocampus, where both long-term potentiation and mGluR-dependent long-term depression are impaired in these rats (10). Future studies should determine whether the deficits in white matter microstructure have any bearing on the impairments in activity.

These white matter deficits parallel those seen in humans and a mouse model. Clinical assessment and studies of individuals with *Shank3* deficiency, 22q13.3 deletion syndrome, and diagnosed PMS showed thinning or hypoplasia of the corpus callosum (6, 7) and reduced FA and increased MD in long association fiber tracts (8, 9), including the uncinate fasciculus and the inferior fronto-occipital fasciculus. Similarly, *Shank3* heterozygous KO mice have significantly reduced FA in long fiber tract systems compared to WT controls (8). Therefore, our study supports others in their conclusion that *Shank3* deficiency leads to white matter disease. It also lays the groundwork for future studies to examine the relationship between these anatomical impairments and the behavioral deficits we have previously identified in these *Shank3*-deficient rats (10).

Limitations

The generalizability of our findings is limited to males because we did not include female rat models of PMS. Future rodent studies should explore whether these impairments vary by sex. A further potential limitation of the current study is that whereas we had 10 animals per *Shank3*-deficient rat group, we only included imaging data from 6 WT rats. This was due to our main focus being on collecting data from littermates so that we could account for extraneous factors that were not relevant to the study, like parents, birth time, age, etc. We were therefore limited by the amount of variability in genotype in each litter. Lastly, many of our findings were not significant after correcting for multiple comparisons. For this reason, in addition to supplying the nominal p values, we have reported whether they survived this correction or not.

Conclusions

Our MRI and DTI findings indicate that *Shank3*-deficiency in rats leads to reduce brain volumes and impaired white matter pathways. The colliculi were among the regions that were affected, suggesting a potential locus for the sensory deficits often documented in PMS. Both reduced volume and white matter impairment have been identified in humans and mouse models with *Shank3*-deficiency, suggesting that they are potential cross-species structural biomarkers that warrant further study.

List Of Abbreviations

PMS = Phelan McDermid syndrome

ASD = Autism spectrum disorder

KO = Knock out

MRI = Magnetic resonance imaging

DTI = Diffusion tensor imaging

WT = Wild type

ROI = Regions of interest

EPI = Echo-planar imaging

TR = Repetition time

TE = Echo time

FOV = Field of view

RARE = Rapid acquisition with relaxation enhancement

AD = Axial diffusion

RD = Radial diffusion

MD = Mean diffusion

FA = Fractional anisotropy

Declarations

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author's contributions

CEMG and JDB designed the study. VXW acquired the imaging data. CEMG analyzed and interpreted the data, and wrote the paper. PRH and HHN helped interpret the data and revise the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Tissue density and absolute and relative volume of brain regions of WT and Shank3-deficient littermates using T2 MRI. (A) Heatmap of p-values from ANOVA and Kruskal-Wallis tests for an effect of genotype on tissue density and absolute and relative volumes where an increase in red denotes an increase in significance of nominal p-values. Boxplots of the brain regions that significantly changed in (C) absolute and (D) relative volume. Effect sizes for the differences between WT and Shank3^{-/-} littermates were reported as Cohen's d below the graphs and are considered large if they are > |0.8|. The pie charts for each absolute volume represent the percent of the total volume each region comprises in the WT rats. Significance bars represent the effect of genotype or pair-wise comparisons from a Tukey HSD or Dunn's test if they were significant after an effect of genotype was significant, (WT: N = 6, Shank3^{+/-}: N = 10, Shank3^{-/-}: N = 10), **p < 0.01, *p < 0.05.

Figure 2

Changes in diffusion indices of WT and Shank3-deficient littermates using DTI. (A) Heatmap of *p*-values from ANOVA and Kruskal-Wallis tests for the effect of genotype on AD, FA, MD, and RD where an increase in red denotes increased significance. (B) Boxplots of mean intensity of voxels across altered regions in Shank3-deficient rats. Significance bars represent pair-wise comparisons from a Tukey HSD test after an effect of genotype was significant, (WT: N = 6, Shank3^{+/-}: N = 10, Shank3^{-/-}: N = 10), **p* < 0.05, #*p* < 0.1.

Supplementary Files

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