Proteomic Analysis of Post-synaptic Density Fractions from Shank3 Mutant Mice Reveals Brain Region Specific Changes Relevant to Autism Spectrum Disorder

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Disruption of the human SHANK3 gene can cause several neuropsychiatric disease entities including Phelan-McDermid syndrome, autism spectrum disorder (ASD), and intellectual disability. Although, a wide array of neurobiological studies strongly support a major role for SHANK3 in organizing the post-synaptic protein scaffold, the molecular processes at synapses of individuals harboring SHANK3 mutations are still far from being understood. In this study, we biochemically isolated the post-synaptic density (PSD) fraction from striatum and hippocampus of adult Shank3Δ11−/− mutant mice and performed ion-mobility enhanced data-independent label-free LC–MS/MS to obtain the corresponding PSD proteomes (Data are available via ProteomeXchange with identifier PXD005192). This unbiased approach to identify molecular disturbances at Shank3 mutant PSDs revealed hitherto unknown brain region specific alterations including a striatal decrease of several molecules encoded by ASD susceptibility genes such as the serine/threonine kinase Cdkl5 and the potassium channel KCa1.1. Being the first comprehensive analysis of brain region specific PSD proteomes from a Shank3 mutant line, our study provides crucial information on molecular alterations that could foster translational treatment studies for SHANK3 mutation-associated synaptopathies and possibly also ASD in general.

Keywords: Shank3, autism spectrum disorder, synapse, proteome, striatum, Homer1

Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ASD, autism spectrum disorder; DIA, data-independent acquisition; GO-term, gene ontology-term; Ho, homogenate; ID, intellectual disability; KO, “Knockout” (Shank3Δ11−/− mutants); P2 Crude, membrane fraction; PMS, Phelan McDermid syndrome; PSD, post-synaptic density; S1, supernatant 1 (purified homogenate); S2, supernatant 2 (cytosol); S3, supernatant 3 (synaptic cytosol); Syn, synaptosomes; WT, wild type.
INTRODUCTION

Studies from the last decade have repeatedly outlined that genetic disruptions of SHANK3 in humans are of upmost clinical relevance as they can lead to various neuropsychiatric disorders including the PMS, a complex neurodevelopmental condition and syndromic autism variant, non-syndromic ASD and ID (Durand et al., 2007; Betancur and Buxbaum, 2013; Kleijer et al., 2014; Leblond et al., 2014). As SHANK3 encodes a large scaffold protein organizing the PSD of excitatory glutamatergic synapses (Boeckers, 2006) it is hypothesized that SHANK3 mutations perturb synaptic transmission in neural circuits throughout the brain and thereby cause diverse neuropsychiatric phenotypes (Grabrucker et al., 2011). To identify the respective circuits and the underlying molecular pathomechanisms, several Shank3 mutant mouse lines have been engineered up to this date (Rozdagi et al., 2010; Peca et al., 2011; Wang et al., 2011, 2016; Schmeisser et al., 2012; Han et al., 2013; Kouser et al., 2013; Lee J. et al., 2015; Speed et al., 2015; Bidinosti et al., 2016; Jaramillo et al., 2016a,b; Mei et al., 2016; Zhou et al., 2016). Importantly, mutants from each line exhibit behavioral traits related to neuropsychiatric diseases and neurobiological alterations at the synaptic level that could be of further use for the development of targeted therapies (Jiang and Ehlers, 2013; Schmeisser, 2015). However, most of the studies have thus far included the molecular analysis of a biased selection of synaptic proteins that had previously been identified in Shank3 interaction studies, i.e., direct binding partners such as GKAP/SAPAP or Homer and indirect binding partners such as MAGUKs or glutamate receptors (Boeckers, 2006). Additionally, previous studies mainly focused on a single brain region and most of them analyzed crude synaptosomes rather than purified PSD fractions. However, for a better understanding of the synaptic pathology of SHANK3 mutations in neuropsychiatric diseases, we need an unbiased and more comprehensive insight into the molecular PSD composition of Shank3 mutant mice in ASD-associated brain regions. For this reason, we biochemically isolated the PSD fraction from brain and thereby cause diverse neuropsychiatric disorders (Schmeisser et al., 2012). Mice were bred on a 12 h rhythm. All homozygous Shank3 Δ11−/− mutants and WT littermates used for this study were from hetero-hetero

MATERIALS AND METHODS

Mice

Generation of Shank3 Δ11−/− mutant mice has been described previously (Schmeisser et al., 2012). Mice were bred on a C57BL/6J background and housed under standard laboratory conditions (average temperature of 22°C, food and water available ad libitum). Lights were automatically turned on/off in a 12 h rhythm. All homozygous Shank3 Δ11−/− mutants and WT littermates used for this study were from hetero-hetero

breedings, from both sexes and between P56 and P84. Animal experiments were approved by the review board of the Land Baden-Wuerttemberg, Permit Number 0.103 and performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany.

Antibodies

The anti-Shank3 antibody has been described previously (Schmeisser et al., 2012). The other antibodies have been obtained from commercial suppliers as it follows: anti-PSD95 (dilution: 1:2 000, Synaptic Systems, #124011, RRID:AB_10804286), anti-Synaptophysin (dilution: 1:20 000, Abcam, Cambridge, UK, #ab14692, RRID:AB_3014177), anti-rabbit HRP (dilution: 1:1 000, Dako, Hamburg, Germany, #P0399, RRID:AB_2617141), and anti-mouse HRP (dilution: 1:3 000, Dako, Hamburg, Germany, #P0260, RRID not available).

Subcellular Fractionation and Western Blot

Subcellular fractionation of brain tissue was performed as previously described with minor modifications (Distler et al., 2014b). For each of the n = 5 samples per genotype used for proteomic analysis, striata or hippocampi of five individual mice were combined. All steps were performed at 4°C. Tissue was homogenized (Ho) in buffer A (0.32 M sucrose, 5 mM HEPES, pH 7.4) and centrifuged at 1 000 × g. The supernatant (S1) was further centrifuged at 12 000 × g and the pellet containing the crude membrane fraction (P2) was obtained. This fraction was solubilized in buffer B (0.32 M sucrose, 5 mM Tris-HCl, pH 8.1) and loaded on a discontinuous sucrose step gradient (0.85 M/1.0 M/1.2 M). After centrifugation at 85 000 × g the synaptosomes (Syn) were collected from the 1.0 M/1.2 M interface and incubated with buffer C (0.32 M sucrose, 12 mM Tris, pH 8.1, 1% Triton X-100). After centrifugation at 32 800 × g, the PSD pellet was collected (PSD) and solubilized in H2O.

Equal amounts of 10 µg from each sample were separated via SDS-PAGE and subsequently blotted on Nitrocellulose membranes according to standard protocols. Incubation with the primary antibody was followed by incubation with an HRP-conjugated secondary antibody (Dako). Signals were visualized with the Pierce ECL Western Blotting Substrate and further detected with the MicroChemi 4.2 machine. Signals were quantified with Gelanalyzer software.

Tryptic Digestion

Aliquots corresponding to 20 µg PSD protein were digested using a modified filter-aided sample preparation (FASP) protocol, which has been previously described in detail (Wisniewski et al., 2009; Distler et al., 2016). Prior to LC–MS analysis, the resulting tryptic digest solutions were diluted to a concentration of 500 ng/µL using aqueous 0.1% formic acid and spiked with 25 fmol/µL of enolase 1 (Saccharomyces cerevisiae) tryptic digest standard (Waters GmbH, Eschborn, Germany).

1www.gelanalyzer.com/
Nanoscale Liquid Chromatography Mass Spectrometry (nanoLC–MS) of Tryptic Digests

NanoLC–MS analysis of tryptic peptides was performed as described before using a nanoAcquity UPLC system (Waters) coupled to a Waters Synapt G2-S HDMS mass spectrometer (Waters) (Distler et al., 2014b, 2016). In brief, peptides were separated using a HSS-T3 C18 1.8 µm, 75 µm × 250 mm reversed phase column. Mobile phase A was water containing 0.1% formic acid and 3% DMSO. Mobile phase B was ACN containing 0.1% formic acid and 3% DMSO. Samples were loaded onto the column in direct injection mode with 1% mobile phase B as described before (Distler et al., 2014b, 2016). Peptides were separated using a gradient from 1 to 35% mobile phase B over 90 min at a flow rate of 300 nL/min. After separation of peptides, the column was rinsed with 90% mobile phase B, followed by a re-equilibration step at initial conditions (1% mobile phase B) resulting in a total run time of 120 min. [Glu1]-fibrinopeptide was used as lock mass at 100 fmol/µl. All samples were analyzed in three technical replicates.

NanoESI-MS analysis of tryptic peptides on the Waters Synapt G2-S system was performed in positive V-mode with a resolving power of at least 25 000 FWHM (full width half maximum). The instrument was equipped with a NanoLockSpray source and the lock mass channel was sampled every 30 s. LC–MS data were collected using ion mobility enhanced MS (Silva et al., 2015; Geromanos et al., 2012). Precursor ion information was collected in low-energy MS mode applying a constant collision energy of 4 eV. Fragment ion information was obtained in the elevated energy scan using drift-time specific collision energies as detailed before (UDMS) (Distler et al., 2014a, 2016). The spectral acquisition time in each mode was 0.6 s with a 0.05 s-interscan delay resulting in an overall cycle time of 1.3 s for the acquisition of one cycle of low and elevated energy data.

Raw Data Processing and Data Analysis

Initial signal processing of continuum LC–IMS–MS data and subsequent database search were performed using vendor software ProteinLynx Global SERVER (PLGS, version 3.02, Waters). For protein and peptide identification, data were searched against a custom compiled database containing UniProtKB/Swiss-Prot entries of the mouse reference proteome (UniProtKB release 2014_02, 16 780 entries). Sequence information for enolase 1 (Saccharomyces cerevisiae) as well as for common contaminants (i.e., human keratins, porcine trypsin) was added to the databases. Following criteria were applied for database search: (i) trypsin as digestion enzyme, (ii) up to two missed cleavages per peptide, (iii) a minimum peptide length of six amino acids, (iv) carbamidomethyl cysteine as fixed and (v) methionine oxidation as variable modification. The false discovery rate (FDR) for peptide and protein identification was assessed searching a reverse database and set to 0.01 for database search in PLGS.

Label-free quantification analysis was performed using the ISOQuant software tool (Distler et al., 2014a, 2016). Briefly, the analysis included retention time alignment, exact mass retention time (EMRT) and IMS clustering as well as data normalization and protein homology filtering. Settings and algorithms have been described in detail in previous protocols (Distler et al., 2014a, 2016). The peptide-level FDR for cluster annotation was set to 0.01 within ISOQuant. For further quantitative analysis, only proteins identified by at least two peptides with a minimum length of six amino acids were considered. Additionally, to be included in the final dataset, a protein had to be identified in at least four biological replicates in at least one condition (i.e., either in WT striatum, Shank3Δ11−/− mutant striatum, WT hippocampus or Shank3Δ11−/− mutant hippocampus). For each protein, absolute in-sample amounts were estimated using TOP3 quantification (Silva et al., 2006). Based on the quantitative information derived from the TOP3 approach, we calculated the relative amount of each protein with respect to the sum over all detected proteins [ppm: parts per million (w/w) of total protein].

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2014, 2016) with the dataset identifier PXD005192.

Statistical analysis of the data was conducted using Student's t-test with Bonferroni correction for multiple hypothesis testing to largely exclude potential false-positive hits. As this is a very conservative and stringent approach, Bonferroni adjusted p-values of p ≤ 0.05 were considered statistically significant to not exclude proteins that are actually displaying differences between WT and Shank3Δ11−/− mutants. In addition, for each protein we calculated the log-transformed ratio of its average amount in WT mice divided by its average amount in Shank3Δ11−/− mutant mice for hippocampus and striatum. To be included in the list of regulated proteins, proteins had to be statistically significant (p ≤ 0.05) and display a log2 ratio of at least ±0.24 between WT and Shank3Δ11−/− mutant mice.

Functional annotation and analysis of proteins that displayed significant changes between WT and Shank3Δ11−/− mutant mice was performed using DAVID Bioinformatics Resources (version 6.7) (Huang da et al., 2009a,b). To identify autism-associated gene products among the pool of changed proteins in the Shank3Δ11−/− mutant PSD from either brain region, proteins were individually matched with the SFARI (Simons Foundation Autism Research Initiative) autism gene database (Banerjee-Basu and Packer, 2010). For the evaluation of known and predicted protein–protein interactions among these proteins, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v10.0 was used (Szklarczyk et al., 2015). Protein–protein interactions were visualized with the Gephi software v0.9.1 (Bastian et al., 2009).

http://proteomecentral.proteomexchange.org
https://david.ncifcrf.gov
https://gene.sfari.org/autdb/Welcome.do
http://string-db.org
https://gephi.org
RESULTS

Proteomic Characterization of the Striatal and Hippocampal PSD from Shank3Δ11−/− Mutants

After subcellular fractionation, we performed Western Blot analyses to confirm the purity of the isolated PSD fractions. As expected, the anti-Shank3 antibody differentiated between material from WT and Shank3Δ11−/− mutant (KO) samples. Our analysis confirmed that the post-synaptic scaffold protein PSD95 was increased and the presynaptic vesicle protein Synaptophysin was decreased in the PSD fraction from each brain region of both genotypes when compared to the corresponding region of both genotypes when compared to the corresponding

PSD fractions from Shank3Δ11−/− mutant animals (p ≤ 0.05). In both brain regions, PSDs derived from Shank3Δ11−/− mutant animals displayed higher numbers of down- than up-regulated proteins (Figures 1B,C). All regulated proteins including KO/WT abundance ratios are listed in detail in Supplementary Table S2. Notably, Shank3 was still detectable in the Shank3Δ11−/− mutant in our proteomic analysis at ~41% of WT levels in the striatal and ~24% of WT levels in the hippocampal Shank3Δ11−/− mutant PSD. This result served as internal control due to the fact that the Shank3Δ11−/− mutants are devoid of major, but not all Shank3 isoforms (Figure 1A; Supplementary Tables S1 and S2; Supplementary Figure S1) (Schneisser et al., 2012; Vicedomini et al., 2016; Wang et al., 2016). Biochemical analysis further revealed that the protein levels of the remaining isoforms, Shank3e and Shank3f, were not altered in the PSD fraction from either brain region (Supplementary Figure S4). We next analyzed if there were converging changes among the regulated proteins in the Shank3Δ11−/− mutant PSD from both brain areas when compared with the Shank3 in vivo interactome from murine synaptosomes (Han et al., 2013). Intriguingly, only one protein was identified (Figure 1D): Homer1, which was decreased in both

![Graphical representation](https://www.frontiersin.org/article/10.3389/fnmol.2017.00026)
the striatal and hippocampal PSD of Shank3Δ11−/− mutants (Supplementary Table S2).

**Gene Ontology Enrichment Analysis Reveals Distinct Changes of Biological Processes and Molecular Functions in Striatal and Hippocampal PSD Proteomes of Shank3Δ11−/− Mutants**

We next performed GO-term based protein enrichment analysis to gain more insight into the functionality of the molecular changes in the striatal and hippocampal PSD proteome of Shank3Δ11−/− mutant animals. Statistical enrichment analysis of the three categories “cellular compartment,” “biological process” and “molecular function” yielded distinct results among the two brain regions. In the striatal Shank3Δ11−/− mutant PSD, the top five enriched GO-terms from all three categories pointed toward altered levels of proteins exhibiting ionotropic glutamate receptor activity and involved in cell–cell signaling at the PSD as the most prominent molecular consequences of Shank3 deficiency (Figures 2A,B). For example, this included a decrease of the AMPAR subunits GluA1 and GluA2, the NMDAR subunits GluN1 and GluN2B and the kainate receptor GluK5 (Supplementary Table S2). Contrary to that, the same type of analysis revealed that in the hippocampal Shank3Δ11−/− mutant PSD proteins involved in cytoskeleton organization such as Abi1, Gelsolin or Profilin2 were predominantly changed following loss of Shank3 (Figures 2C,D; Supplementary Table S2).

**The Striatal PSD Proteome of Shank3Δ11−/− Mutants Comprises More Altered Proteins Encoded by ASD Susceptibility Genes than the Hippocampal One**

Similar to the GO-term based protein enrichment analysis, comparison of the quantitative datasets of altered proteins in the striatal or hippocampal PSD proteome of Shank3Δ11−/− mutant animals with the 826 autism-associated primary target genes presently listed in the SFARI autism gene database also rendered distinct results: 14 of the 61 proteins (23%) altered in the striatal and 8 of the 55 proteins (15%) altered in the hippocampal Shank3Δ11−/− mutant PSD matched with proteins encoded by SFARI autism genes (Figures 3A,B). The only converging molecular alteration in this context was a decrease of Homer1 in both Shank3Δ11−/− mutant PSD proteomes, while all other changes were unique to each brain region. Importantly, all molecules changed in the striatal Shank3Δ11−/− mutant PSD

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**FIGURE 2 | Gene ontology analysis of significantly altered proteins in the Shank3Δ11−/− mutant PSD from striatum and hippocampus.** Functional annotation of proteins with significantly altered expression levels in the striatal (A,B) and hippocampal (C,D) Shank3Δ11−/− mutant PSD was performed using DAVID (v6.7, https://david.ncifcrf.gov) with the data in Supplementary Table S2 used as input. Bar diagrams visualize the top five significantly enriched GO terms for ‘cellular compartment’ for molecular alterations in the striatal (A) or hippocampal (C) PSD. The top five significantly enriched GO terms for ‘biological process’ and ‘molecular function’ for molecular alterations in the striatal (B) or hippocampal (D) PSD are listed in table-like diagrams.
FIGURE 3 | A relevant number of the significantly altered proteins in the Shank3Δ11−/− mutant PSD from striatum is encoded by ASD susceptibility genes. Significantly altered proteins in the striatal (A) and hippocampal (B) Shank3Δ11−/− mutant KO PSD match with entries of the SFARI autism gene database. Proteins further encoded by high-risk TADA genes are highlighted in bold. The KO/WT ratios are marked in blue for down- and in orange for up-regulated proteins. (C, D) Protein–protein interactions (lines) among the significantly altered proteins in the striatal (C) and hippocampal (D) Shank3Δ11−/− mutant PSD that match with entries in the SFARI autism gene database. Proteins, which are further encoded by TADA genes, are marked in bold and are in boxes. Proteins significantly down-regulated in Shank3Δ11−/− mutant mice are marked in blue, significantly up-regulated proteins in orange.
that matched with the SFARI autism gene database were reduced and comprised the murine homologs of proteins encoded by several major ASD candidates (Figure 3A) including NCKAP1, GRIN2B and TRIO, 3 of the 65 high-risk ASD TADA genes (De Rubeis et al., 2014; Sanders et al., 2015). Based on these results, we generated minimal distance interaction networks that showed a high degree of interactions among these molecules almost exclusively in the striatal Shank3Δ11−/− mutant PSD (Figures 3C,D). The core of this network comprises known direct and indirect Shank interactors (Han et al., 2013) and is extended by several other intriguing candidates that have not yet been associated with Shank3 or Shankopathies including the serine/threonine kinase Cdkl5 and the potassium channels Kir2.1 and KcN1.1.

**DISCUSSION**

In this study, we used ion-mobility enhanced data-independent label-free LC–MS/MS to generate an unbiased dataset of in vivo changes in the PSD fraction from both striatum and hippocampus of adult Shank3Δ11−/− mutant mice lacking major isoforms of Shank3 (Schmeisser et al., 2012; Vicidomini et al., 2016). We have previously shown by the same methodological approach (biochemical isolation followed by LC–MS/MS) that in this fraction, PSD-specific proteins were highly enriched whereas contaminating proteins (i.e., mitochondrial or presynaptic ones) were efficiently decreased (Distler et al., 2014b).

Our comprehensive analysis quantified approximately 2,500 proteins in the striatal and 2,400 proteins in the hippocampal Shank3Δ11−/− mutant PSD and identified several significantly regulated proteins largely distinct for either brain region. These findings are intriguing as they point toward a specific function of Shank3 in organizing the molecular anatomy of the PSD in a brain region specific manner. Interestingly, our GO-term enrichment analysis revealed that deficiency of Shank3 in the striatal PSD primarily results in changes of proteins involved in glutamatergic synaptic transmission, while cytoskeleton-associated proteins were mainly affected in the Shank3Δ11−/− mutant hippocampal PSD. This is supported by previous studies on impaired striatal glutamatergic synaptic transmission in the same (Vicidomini et al., 2016) and other Shank3 mutants (Peca et al., 2011; Jaramillo et al., 2016a,b; Peixoto et al., 2016; Wang et al., 2016; Zhou et al., 2016) and on impaired cytoskeletal organization in hippocampal neurons with altered gene dosage or protein structure of Shank3 (Durand et al., 2012; Han et al., 2013). In addition, loss of differential Shank3 isoforms with distinct functions from striatal or hippocampal PSDs in a varying degree depending on their physiological expression pattern and levels throughout the brain might play a role in this context (Wang et al., 2014).

Based on the fact that approximately 0.7% of individuals with ASD exhibit a mutation in SHANK3 (Leblond et al., 2014), we further compared our datasets with the SFARI autism gene database to identify molecular patterns that could be of relevance for a better understanding of ASD-associated pathomechanisms in our model. Interestingly, we only found one protein to be altered in the Shank3Δ11−/− mutant PSD of both brain regions: Homer1. This post-synaptic scaffold protein and C-terminal Shank interactor, which interconnects the latter with group I metabotropic glutamate receptors and regulators of post-synaptic calcium signaling (Tu et al., 1999; Sala et al., 2001, 2005; Hayashi et al., 2009) was decreased predominantly in the striatal PSD. These data support previous biochemical findings in the same (Vicidomini et al., 2016) and other Shank3 mutants (Peca et al., 2011; Wang et al., 2011, 2016; Jaramillo et al., 2016a,b; Zhou et al., 2016) pointing toward a common molecular phenotype at synapses caused by any genetic disruption of Shank3. Together with the fact that several rare variants of the HOMER1 gene have been found in individuals with ASD (Kelleher et al., 2012) our data strongly underline the central role of HOMER1 gene dosage and – as we have previously demonstrated – to the associated mGlu5 altered signaling (Vicidomini et al., 2016) to better understand the molecular underpinnings of SHANK3 mutation-associated ASDs. This finding subsequently calls for a detailed analysis of ASD-like behaviors and the underlying molecular pathomechanisms in Homer1 mutants. Intriguingly, we also found that almost 25% of the altered proteins in the striatal Shank3Δ11−/− mutant PSD matched with the SFARI autism gene database. These molecules were all reduced and comprised several major ASD candidates including three proteins encoded by the murine homologs of the TADA genes NCKAP1, GRIN2B and TRIO, which are highly relevant for ASD formation in humans (De Rubeis et al., 2014; Sanders et al., 2015). Minimal distance interaction network analysis further showed that the majority of these proteins interact with each other pointing toward a defined ASD-associated molecular network that is disrupted specifically at the PSD of Shank3Δ11−/− mutant corticostriatal synapses. The core of this network includes known direct and indirect Shank interactors including Homer1, IRSp53, members of the GKAP/SAPAP family and the NMDA receptor, a well-known target of translational pharmacotherapy of ASD-like phenotypes in mice (Graberucker et al., 2011; Han et al., 2013; Jiang and Ehlers, 2013; Lee E.J. et al., 2015). In addition, the network comprises several other intriguing ASD-related molecules that have not yet been associated with Shank or Shankopathies and could well serve as targets for future treatment studies. Among these is Cdkl5, a serine/threonine kinase involved in Akt-mTOR signaling at the synapse, genetically related to several neurodevelopmental disorders in humans including ASD, Rett syndrome and epileptic encephalopathies and whose disruption leads to ASD-like behavior and impaired neural circuitry in mice (Wang et al., 2012; Sivilia et al., 2016). Other intriguing candidates are the potassium channels Kir2.1 and KcN1.1. Especially the latter is of high interest as genetic disruption is not only found in individuals with ASD and epilepsy, but also impairs network properties in ASD-related brain regions in mice including Purkinje cell circuits (Guglielmi et al., 2015). Importantly, our data on the exclusive reduction of a significant number of ASD-associated molecules in the striatal PSD of Shank3Δ11−/− mutants mirror findings from previous studies on two different gene targeted mouse models of ASD: Ptenm3mt mutants expressing the mistargeted tumor suppressor Pten and Fmr1−/− mutants lacking the fragile X. 
mental retardation protein (Fmrp). Transcriptome analysis of Pten<sup>m304v</sup> mutant cortices revealed broad down- and proteomic analysis of Fmr1<sup>−/−</sup> mutant cortical synaptosomes broad up-regulation of many human ASD-susceptibility genes (Tang et al., 2015; Tilot et al., 2016). We therefore again emphasize the need of unbiased and comprehensive screening of both gene expression and molecular synapse anatomy in ASD-associated brain regions of genetically based model systems to not only understand the molecular consequences of the corresponding mutation, but also the molecular pathology of ASD in a broader fashion.

**AUTHOR CONTRIBUTIONS**

MS, TB, and ST designed research. JB supervised mouse breeding. DR, UD, and SH carried out experiments; DR, UD, CV, CS, and ST performed data analysis. DR, UD, ST, and MS designed all figures. MS jointly wrote the manuscript with all other authors.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2017.00026/full#supplementary-material

**REFERENCES**

Banerjee-Basu, S., and Packer, A. (2010). SFARI Gene: an evolving database for the autism research community. *Dis. Model. Mech.* 3, 133–135. doi: 10.1242/dmm.005439

Bastian, M., Heymann, S., and Jacomy, M. (2009). "Gephi: an open source software for exploring and manipulating networks," in *Proceedings of the International AAAI Conference on Weblogs and Social Media*, Boston, MA.

Betancur, C., and Buxbaum, J. D. (2013). SHANK3 haploinsufficiency: a “common” but underdiagnosed highly penetrant monogenic cause of autism spectrum disorders. *Mol. Autism* 4, 17. doi: 10.1186/2040-2392-4-17

Bidinosti, M., Botta, P., Kruttner, S., Proenca, C. C., Stoehr, N., Bernhard, M., et al. (2016). CLK2 inhibition ameliorates autistic features associated with SHANK3 deficiency. *Science* 351, 1199–1203. doi: 10.1126/science.aad5487

Boeckers, T. M. (2006). The postsynaptic density. *Cell Tissue Res.* 326, 409–422. doi: 10.1007/s00441-006-0274-5

Bozdagi, O., Sakurai, T., Papapetrou, D., Wang, X., Dickstein, D. L., Takahashi, N., et al. (2010). Haploinsufficiency of the autism-associated Shank3 gene leads to
deficits in synaptic function, social interaction, and social communication. **Mol. Autism** 1, 15. doi: 10.1186/2040-2392-1-15

De Rubeis, S., He, X., Goldberg, A. P., Poutnley, C. S., Samocha, K., Cicic, A. E., et al. (2014). Synaptic, transcriptional and chromatin genes disrupted in autism. *Nature* 515, 209–215. doi: 10.1038/nature13772

Dister, U., Kuharev, J., Navarro, P., Levin, Y., Schild, H., and Tenzer, S. (2014a). Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nat. Methods* 11, 167–170. doi: 10.1038/nmeth.2767

Dister, U., Schmeisser, M. J., Pelosi, A., Reim, D., Kuharev, J., Weiczner, R., et al. (2014b). In-depth protein profiling of the postsynaptic density from mouse hippocampus using data-independent acquisition proteomics. *Proteomics* 14, 2607–2613. doi: 10.1002/pmc.201300520

Dister, U., Kuharev, J., Navarro, P., and Tenzer, S. (2016). Label-free quantification in ion mobility-enhanced data-independent acquisition proteomics. *Nat. Protoc.* 11, 795–812. doi: 10.1038/nprot.2016.042

Durand, C. M., Betancur, C., Boeckers, T. M., Bockmann, J., Chaste, P., Distler, U., Kuharev, J., Navarro, P., and Tenzer, S. (2016). Label-free quantification of predominant Shank3 isoforms results in hippocampus-dependent impairments in behavior and synaptic transmission. *J. Neurosci.* 33, 18448–18468. doi: 10.1523/JNEUROSCI.3017-13.2013

Leblond, C. S., Nava, C., Polge, A., Gauthier, J., Huguet, G., Lumbroso, S., et al. (2014). Meta-analysis of SHANK Mutations in Autism Spectrum Disorders: a gradient of severity in cognitive impairments. *PLoS Genet.* 10:e1004580. doi: 10.1371/journal.pgen.1004580

Lee, E. J., Choi, S. Y., and Kim, E. (2015). NMDA receptor dysfunction in autism spectrum disorders. *Curr. Opin. Pharmacol.* 20, 8–13. doi: 10.1016/j.coph.2014.10.007

Lee, J., Chung, C., Ha, S., Lee, D., Kim, D. Y., Kim, H., et al. (2015). Shank3-mutant mice lacking exon 9 show altered excitation/inhibition balance, enhanced rearing, and spatial memory deficit. *Front. Cell Neurosci.* 9:94. doi: 10.3389/fncel.2015.00094

Mei, Y., Monteiro, P., Zhou, Y., Kim, J. A., Gao, X., Fu, Z., et al. (2016). Adult restoration of Shank3 expression rescues selective autistic-like phenotypes. *Nature* 530, 481–484. doi: 10.1038/nature16971

Peca, J., Feliciano, C., Ting, I. T., Wang, W., Wells, M. F., Venkatraman, T. N., et al. (2011). Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. *Nature* 472, 437–442. doi: 10.1038/nature09965

Peixoto, R. T., Wang, W., Croney, D. M., Kozorovitsky, S., and Sabatini, B. L. (2016). Early hyperactivity and precocious maturation of corticostriatal circuits in Shank3B(-/-) mice. *Nat. Neurosci.* 19, 716–724. doi: 10.1038/nn.4260

Sala, C., Piech, V., Wilson, N. R., Passafaro, M., Liu, G., and Sheng, M. (2001). Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31, 115–130. doi: 10.1016/S0896-6771(01)00339-7

Sala, C., Roussignol, G., Meldolesi, J., and Fagni, L. (2005). Key role of the postsynaptic density scaffold proteins Shank and Homer in the functional architecture of Ca2+ homeostasis at dendritic spines in hippocampal neurons. *J. Neurosci.* 25, 4587–4592. doi: 10.1523/JNEUROSCI.4822-04.2005

Sanders, S. J., He, X., Willsey, A. J., Erkan-Sencicke, A. G., Samocha, K. E., Cicic, A. E., et al. (2015). Insights into autism spectrum disorder genomic architecture and biology from 71 Risk Locis. *Neuron* 87, 1215–1233. doi: 10.1016/j.neuron.2015.09.016

Schmeisser, M. J. (2015). Translational neurobiology in Shank mutant mice–model systems for neuropsychiatric disorders. *Annu. Anat.* 20. 115–117. doi: 10.1016/j.anaat.2015.03.006

Schmeisser, M. J., Ev, E., Wegener, S., Bockmann, J., Stempel, A. V., Kuebler, A., et al. (2012). Autistic-like behaviours and hyperactivity in mice lacking ProSAP/Shank2. *Nature* 486, 256–260. doi: 10.1038/nature11015

Silva, J. C., Denny, R., Dorschel, C. A., Gorenstein, M., Kass, J. I., Li, G. Z., et al. (2005). Quantitative proteomic analysis by accurate mass retention time pairs. *Anal. Chem.* 77, 2187–2200. doi: 10.1021/ac048455k

Silva, J. C., Gorenstein, M. V., Li, G. Z., Vissers, J. P., and Geromanos, S. J. (2006). Absolute quantification of proteins by LC/MS: a virtue of parallel MS acquisition. *Mol. Cell. Proteom.* 5, 144–156. doi: 10.1074/mcp.M500230-MCP200

Sivilla, S., Mangano, C., Beggiato, S., Guiuliani, A., Torricella, R., Baldassarvo, V. A., et al. (2016). CDKL5 knockout leads to altered inhibitory transmission in the cerebellum of adult mice. *Genes Brain Behav.* 15, 491–502. doi: 10.1111/gbb.12292

Speed, H. E., Kouser, M., Xuan, Z., Reimers, J. M., Ochoa, C. F., Gupta, N., et al. (2015). Autism-associated insertion mutation (InsG) of Shank3 Exon 21 causes impaired synaptic transmission and behavioral deficits. *J. Neurosci.* 35, 9648–9665. doi: 10.1523/JNEUROSCI.3125-14.2015

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., et al. (2015). STRING v10: protein-protein interaction networks, systems for neuropsychiatric disorders. *Mol. Syst. Biol.* 11, 795–812. doi: 10.1038/ncomms12292

Tilot, A. K., Bebek, G., Niazi, F., Altemus, J. B., Romigh, T., Frazier, T. W., et al. (2014). Neurobehavioral phenotype of constitutive Pten dysfunction in mice and its relevance to human idiopathic autism spectrum disorder. *Mol. Psychiatry* 21, 118–125. doi: 10.1038/mp.2015.17

Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakeman, P., et al. (2015). Insights into autism spectrum disorder genomic architecture and biology from 71 Risk Locis. *Neuron* 87, 1215–1233. doi: 10.1016/j.neuron.2015.09.016

Tu et al. (2015). Shank3 mutant PSD Proteomes.
of postsynaptic density proteins. *Neuron* 23, 583–592. doi: 10.1016/S0896-6273(00)80810-7

Vicidomini, C., Ponzoni, L., Lim, D., Schmeisser, M. J., Reim, D., Morello, N., et al. (2016). Pharmacological enhancement of mGlu5 receptors rescues behavioral deficits in SHANK3 knock-out mice. *Mol. Psychiatry* doi: 10.1038/mp.2016.70 [Epub ahead of print].

Vitcaino, J. A., Cordas, A., del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., et al. (2016). 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 44, D447–D456. doi: 10.1093/nar/gkv1145

Vitcaino, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Rios, D., et al. (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* 32, 223–226. doi: 10.1038/nbt.2839

Wang, I. T., Allen, M., Goffin, D., Zhu, X., Fairless, A. H., Brodkin, E. S., et al. (2012). Loss of CDKL5 disrupts kinase profile and event-related potentials leading to autistic-like phenotypes in mice. *Proc. Natl. Acad. Sci. U.S.A.* 109, 21516–21521. doi: 10.1073/pnas.1216988110

Wang, X., Bey, A. L., Katz, B. M., Badea, A., Kim, N., David, L. K., et al. (2016). Altered mGluR5-Homer scaffolds and corticostriatal connectivity in a Shank3 complete knockout model of autism. *Nat. Commun.* 7, 11459. doi: 10.1038/ncomms11459

Wang, X., McCoy, P. A., Rodriguez, R. M., Pan, Y., Je, H. S., Roberts, A. C., et al. (2011). Synaptic dysfunction and abnormal behaviors in mice lacking major isoforms of Shank3. *Hum. Mol. Genet.* 20, 3093–3108. doi: 10.1093/hmg/ddr212

Wang, X., Xu, Q., Bey, A. L., Lee, Y., and Jiang, Y. H. (2014). Transcriptional and functional complexity of Shank3 provides a molecular framework to understand the phenotypic heterogeneity of SHANK3 causing autism and Shank3 mutant mice. *Mol. Autism* 5, 30. doi: 10.1186/2040-2392-5-30

Wisniewski, J. R., Zougman, A., Nagaraj, N., and Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nat. Methods* 6, 359–362. doi: 10.1038/nmeth.1322

Zhou, Y., Kaiser, T., Monteiro, P., Zhang, X., Van der Goes, M. S., Wang, D., et al. (2016). Mice with Shank3 mutations associated with ASD and schizophrenia display both shared and distinct defects. *Neuron* 89, 147–162. doi: 10.1016/j.neuron.2015.11.023

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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