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Heterozygote Shank3B mice exhibit sex-dependent changes in proteins required for excitatory/inhibitory balance

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Abstract

Altered expression of SHANK3 proteins has been implicated in multiple neurodevelopmental disorders, including Phelan-McDermid syndrome (PMS), schizophrenia (SZ), and autism spectrum disorders (ASDs). SHANK3B is a postsynaptic density protein that physically links ionotropic NMDA receptors to metabotropic mGlu5 receptors through interactions with scaffolding proteins, a connection essential for long-term potentiation. Previous studies have demonstrated that endosomal trafficking within dendritic spines regulates receptor insertion and recycling, processes fundamental to maintaining excitatory/inhibitory (E/I) balance. Here, we investigated endosomal protein expression and E/I regulatory proteins in a SHANK3B mouse model to better understand sex-dependent contributions to neurodevelopment. Using immunoblotting and immunohistochemistry, we identified sex-specific alterations in hippocampal expression of endosomal trafficking proteins and E/I markers. These findings represent a novel dimension of SHANK3B-related pathology. To assess behavioral relevance, we paired these molecular analyses with the open field test, the forced swim test, and seizure induction assays. Notably, we observed no significant behavioral differences between groups. Together, these results suggest that sex-dependent molecular alterations in endosomal and E/I pathways occur in SHANK3B mice in the absence of overt behavioral phenotypes. These findings advance our understanding of how SHANK3B deficiency shapes neurodevelopmental processes and highlight the importance of incorporating sex as a biological variable in studies of synaptic dysfunction.

Keywords: SHANK3 proteins • Neurodevelopmental • Psychiatric disorders • Phelan-McDermid Syndrome (PMS) • Schizophrenia (SZ) • Autism Spectrum Disorder (ASD).

Introduction

SHANK3B codes for a key postsynaptic density (PSD) protein that interacts with PSD95 and Homer to physically link ionotropic NMDA receptors to metabotropic mGlu5 receptors, a linkage necessary for inducing synaptic plasticity [1-5]. Mutations in SHANK3 have been reported in neurodevelopmental disorders schizophrenia (SZ), autism spectrum disorder (ASD), and Phelan-McDermid syndrome (PMS), which are also associated with learning deficits, increased anxiety, and developmental delay [6-10].

SHANK3B mutant mice demonstrate altered dendritic spine morphology and dendritic spine composition. In previous studies that examined SHANK3 mutations associated with ASD and SZ, there was a significant decrease in spine density, a significant decrease in spine length, and a significant increase in spine head width [11,12]. Reduced levels of the glutamate receptor subunits GluR2, NR2A, and NR2B have been reported in shank3b -/- mice [8]. SHANK3 regulates AMPA receptor insertion [13], which requires endosomal trafficking for clustering and insertion of the AMPARs [14-19].

Each of the subcompartments within the endosomal system is regulated by specific Arfs, Arf GTPase activating proteins (GAPs), Arf guanine exchange factors (GEFS), coat proteins, and coat-associated proteins [20,21]. AGAP1 is an Arf1 GAP that is localized to endosomes in the dendrites and dendritic spines Altered expression of AGAP1 in the hippocampus alters the endosomal trafficking kinetics and shifts dendritic spines morphology from a mature, stubby spine to an immature, thin spine [19]. AGAP1 interacts with the heterotetrameric coat protein adaptor protein 3 (AP3). AP3 regulates coat proteins and vesicle formation specifically at the level of the early endosome and is necessary for transport of specific cargo to the axon terminal [19,22-26] AGAP1 and AP3 have been implicated in neurodevelopment disorders including ASD, Rett Syndrome (RTT), and SZ, which indicates potential role of endosomal trafficking in proper neuronal development [19,23,27-38] The mRNA levels of AGAP1 and AP3 are decreased in the hippocampus of the SHANK3B heterozygote mice but the protein expression of these endosomal proteins in the SHANK3B mice has not yet been described.

Reduction of SHANK3B protein results in spatial memory deficits, increased anxiety, and alterations in excitatory neurotransmission [39-43]. Previous work in the open field test (OFT) demonstrated shank3b–/– mice showed similar levels of activity and thigmotaxis. However, rearing, a form of vertical exploration considered to be anxiogenic for mice, was significantly reduced in the shank3b–/– mice [8]. Additional studies in shank3b+/– mice demonstrated no observational change in the time in center and time in the perimeter in the OFT; however, mouse sex was not considered [44]. Finally, shank3–/– mice carrying the human Q321R mutation on both alleles demonstrate decreased susceptibility to PTZ-induced seizures [45].

Based on the altered spine morphology, dendritic spine composition, the genetic disruptions observed in human neurodevelopmental disorders, and behavioral characteristics, SHANK3B mice are a highly impactful model to further understand neurodevelopment, specifically, learning and memory. As human development has a sex component, we wanted to analyze these questions in a sex dependent manner. Because endosomal trafficking regulates spine morphology and the endosomal proteins AGAP1 and AP3 have reduced mRNA in the hippocampus of heterozygote SHANK3B mice, we wanted to measure the protein levels of AGAP1 and AP3 in the hippocampus of SHANK3B heterozygote mice in a sex-dependent manner. We wanted to measure the protein levels of E/I markers in the hippocampus because AGAP1 regulates, in part, the dendritic morphology that contributes to E/I balance. Because E/I

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balance impacts animal behavior, we wanted to measure animal behavior. As the previous OFT with the heterozygote mice showed no difference in time in the center, we wanted to examine if there was any sex difference for OFT. Additionally, we want to determine if there is any sex difference for the forced swim test (FST) and seizure induction. Investigating the molecular mechanisms and behavioral characteristics of the SHANK3B mice will further our greater understanding of how neurodevelopment is impacted by sex.

Materials and Methods

Mice

B6.129-Shank3tm2Gfng/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were maintained according to an approved IACUC protocol. The Shank3B- knockout allele has a neo cassette replacing the PDZ domain (exons 13-16) of the Shank3 gene, resulting in altered expression of the Shank3b isoform. For this study, we utilized 12-week-old heterozygote mice for the mutation, noted as shank3b +/-, and littermate controls. Genotype PCR was performed according to the protocols defined by Jackson Laboratories. The common primer was GAGACTGATCAGCGCAGTTG, the wild-type primer was TGACATAATCGCTGGCAAAG, and the mutant primer was GCTATACGAAGTTATGTCGACTAGG

Antibodies

The following antibodies were used for immunoblotting and immunohistochemistry during these experiments: Mouse anti-actin (DHSB, The University of Iowa), mouse anti AP3 (DHSB, The University of Iowa), rabbit anti-SCN1A (DHSB, The University of Iowa), mouse anti-SCN1A (Almone Labs, Jerusalem, Israel), rabbit anti-CAV1.2 (EMD Millipore, Burlington MA), mouse anti-beta tubulin (DHSB, The University of Iowa), rabbit anti-NKCC1 (Cell Signaling Technology, Attleboro, MA), rabbit anti-AGAP1 (Sigma, St. Louis, Missouri), mouse anti-PARV (DHSB, The University of Iowa), rabbit anti-VGLUT1 (Synaptic Systems, Gottingen, Germany), goat anti-mouse HRP (EMD Millipore, Burlington MA), goat anti-rabbit HRP (EMD Millipore, Burlington MA), donkey anti-mouse HRP EMD Millipore, Burlington MA), goat anti-mouse 488 (EMD Millipore, Burlington MA), goat anti-rabbit 488 (EMD Millipore, Burlington MA), donkey anti-goat 488 (EMD Millipore, Burlington MA) donkey anti-rabbit 555 (EMD Millipore, Burlington MA), donkey anti-mouse 555 (EMD Millipore, Burlington MA), donkey anti-goat 555 (EMD Millipore, Burlington MA).

Immunoblotting

Detailed procedures for mouse tissue preparation, indirect immunofluorescence microscopy, and quantification procedures were described in our previous work [26,27]. Briefly, at 12 weeks postnatal, animals were anesthetized using CO2 and rapidly decapitated. The hippocampus was dissected and transferred to ice cold phosphate buffered saline (PBS) with a protease inhibitor (Sigma Aldrich). Hippocampal lysates were separated for SDS-PAGE and transferred to PVDF membranes (BioRad, Hercules, CA, USA). Membranes were probed with primary antibodies, followed by incubation with secondary antibodies, HRP-conjugated secondary antibodies. Secondary antibodies were detected using Supersignal West Dura Extended Duration substrate (Pierce Chemical, Rockford, IL, USA) and developed on film. Relative intensities of the protein bands were quantified by scanning densitometry via the NIH ImageJ Software. Background-corrected values of each sample were normalized to their respective background-corrected β-actin value. Relative protein levels are expressed as a ratio of the normalized value obtained, reported as means ± SEM of 3-4 samples of shank3b +/- and shank3b +/+ mice per sex, each run on 3-4 blots.

Immunohistochemistry

60 micron coronal brain slices were prepared from 12-week-old mice. Mice were exposed to CO_2 until loss of consciousness, then transcardially perfused with Ringer's solution, followed by perfusion of fixative (4% paraformaldehyde with 0.1% glutaraldehyde in PBS). After fixation, brains were post-fixed in 4% paraformaldehyde. Following post-fixation, the brains were sectioned into 60 µm slices using a vibrating microtome. Brain sections containing the hippocampal formation were incubated for 20 min in 1% sodium borohydride. Sections were rinsed with PBS, then incubated for 60 min in pre-incubation. Pre-incubation was removed, and primary antibodies were added and incubated overnight. Tissues were washed 3 x 5 min with 1X PBS and then incubated in secondary antibodies for 60 min. Tissues were then incubated in cupric sulfate (for 100mL, 0.3854g ammonium acetate, 0.1596 cupric sulfate, in dH2O, pH 5.0) and then washed 3 x 5 min with 1X PBS. Finally, tissues were mounted onto glass slides with Vectashield (Vector Laboratories) and sealed. Relative protein levels are expressed as a ratio of the normalized value obtained, reported as means ± SEM of 3-4 samples of shank3b +/- and shank3b +/+ mice per sex, examined in triplicate experiments.

Open Field Test

The open field consisted of a 44.45 x 44.45 cm2 arena enclosed with opaque Plexiglas. The center zone was defined as a 14.8 x 14.8 cm2 area in the center of the arena. At the beginning of the test, each mouse was placed along one side of the chamber and allowed to explore for 10 minutes. A total of 20 shank3b +/- (13 males and 7 females) and 10 shank3b +/+ littermates (4 males and 6 females) were evaluated. Mice were videotaped and scored manually, with the experimenters blind to the genotype and gender. Behaviors scored included the time spent in the center zone, the time spent in the perimeter, the number of vertical jumps, and the number of rears.

Forced Swim Test

The forced swim test utilizes a 25.4 cm x 13.9 cm2 cylindrical tank filled with room temperature water approximately three-quarters of the way full. Mice were placed individually into each tank and allowed to swim for 10 minutes. Mice were videotaped and scored manually for the time spent immobile and the time mobile for a total of 20 shank3b +/- (13 males and 7 females) and 10 shank3b +/+ littermates (4 males and 6 females). The first 2 minutes of each test were not scored as mice were acclimatizing to the water. The next six minutes were evaluated for our data points. Immediately after each session, each mouse was removed from the water and placed on a heating pad to regulate body temperature for 10 minutes before returning to their home cage.

Seizure Induction

Picrotoxin seizure induction was done to determine the seizure susceptibility of the shank3b +/- mice. The chemoconvulsant model was employed using intraperitoneal (IP) administration of picrotoxin (10 mg/kg, dissolved in 0.9% sterile saline; Sigma Aldrich), a noncompetitive antagonist of GABAA receptors. Mice were weighed immediately prior to injection to ensure accurate dose delivery. Mice were continuously observed for 20 minutes post-injection, and latencies to each seizure stage were recorded. Mice that did not exhibit full seizure activity were monitored for over 20 minutes. Seizure behavior was scored using a modified six-point Racine scale to assess progression and severity. Stage-1 was defined as behavioral arrest, characterized by prolonged staring or immobility with reduced exploratory activity. Stage-2 involved myoclonic jerks, consisting of isolated, sudden movements of the head or upper body. Stage-3 was marked by forelimb clonus, represented by repetitive paw waving or rhythmic forelimb movements without rearing. Stage -4 included rearing and falling, with sustained forelimb clonus accompanied by loss of balance. Stage-5 corresponded to generalized tonic-clonic seizures (GTCS), featuring full-body convulsions and loss of postural control. Stage -6 indicated mortality through hind-limb extension as a direct result of seizure activity. A total of 16 shank3b +/- (11 males and 5 females) and 8 shank3b +/+ littermates (4 males and 4 females) were evaluated.

Statistical analysis for the molecular data

For the immunoblotting and immunohistochemistry, relative protein levels are expressed as a ratio of the normalized value obtained, reported as means ± standard error of the mean (SEM) of 3-4 samples of shank3b +/- and shank3b +/+ mice per sex. A two-way ANOVA was used to identify statistically significant differences between genotypes and sex. Statistical significance was defined as $p < 0.05$.

Statistical analysis for the behavioral data

For the open field, forced swim, and seizure induction tests, A One-Way Analysis Of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to identify statistically significant differences between genotype and sex. No statistically significant differences were found. Therefore, all data presented represents the combined sex.

To determine statistically significant differences between genotypes in the open field, forced swim tests and seizure induction, the Student T-test was used. Data are represented as mean ± standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

Results

Endosomal pathways regulate the trafficking of neurotransmitters and receptors throughout neurons as well as regulating proper dendritic morphology. AGAP1 and AP3 are part of the endosomal machinery that regulate proper dendritic spine morphology. To gain a deeper understanding of the role of endosomal trafficking in neurodevelopment in a sex-dependent manner, we analyzed the protein expression using immunoblotting and immunohistochemistry of the hippocampus in a sex-dependent manner using a Shank3B heterozygote mouse model, which better aligns with clinical observations.

Sex dependent changes in endosomal trafficking proteins

First, we analyzed protein levels of endosomal proteins in the hippocampus of 12-week-old shank3b+/+ and shank3b+/- mice using immunoblotting (Figure 1A and B). Using a two-way ANOVA, we analyzed a minimum of triplicate blots, which analyzed a minimum of 3 hippocampi per genotype per sex.

From the immunoblotting, we determined that sex had no significant effect on AGAP1 levels ($p = 0.3750$), however, genotype demonstrated a significant difference ($p = 0.0321$), meaning that loss of one allele of SHANK3B results in a loss of AGAP1 levels. This matches the mRNA data reported by For AP3, sex ($p = 0.1248$) and genotype (0.6696) did not have an alter protein levels in the hippocampus.

As the immunoblot analyzes the total hippocampus, we next analyzed endosomal protein levels in a region-specific manner using immunohistochemistry in the hippocampus of 12-week-old shank3b+/+ mice and shank3b+/- mice (Figure 1 C - L). To quantify changes observed in staining, we determined the fluorescent intensity of our protein of interest relative to that of synaptophysin to create a ratio. For AGAP1, sex has a statically significant effect on protein levels in the DG ($p < 0.0001$), the CA1 ($p < 0.0001$), and the CA3 ($p < 0.0001$) with a higher level of AGAP1 protein in the females than the males, but genotype did not have an effect in the DG ($p = 0.5$) or CA1 ($p=0.7034$). shank3b+/- did have a significant decrease in protein levels of AGAP1 in the CA3($p = 0.0172$) compared to control.

For AP3, sex has a statistically significant effect on protein levels, with a higher level of AP3 in the females than in the males in the DG ($p = 0.0143$) and the CA1 ($p = 0.0409$), but not in the CA3 ($p = 0.3173$). For AP3, there was no significant effect on protein levels based on genotype in the DG ($p = 0.5725$), CA1 ($p = 0.3398$), or CA3 ($p = 0.5618$). For the endosomal proteins, the immunohistochemistry results are novel. The fact that AGAP1 has a genotype result in the CA3 only is an interesting find. It is also important to note that there were sex differences noted between the groups in the DG, CA1 for AP3, and for all regions of the hippocampus for AGAP1. The immunoblotting and immunohistochemistry data in shank3b+/- mice demonstrate a sex-dependent and region-dependent protein expression pattern of endosomal trafficking proteins that regulate dendritic spine morphology.

Sex dependent changes in E/I proteins

As the SHANK3B protein regulates excitatory synapses and endosomal trafficking regulates E/I balance in the hippocampus, we next wanted to analyze key E/I protein markers in the hippocampus. We measured protein levels of key markers involved in E/I balance in the hippocampus of 12-week-old shank3b +/+ and shank3b +/- mice (Figure 2A and B). Using a two-way ANOVA, we analyzed a minimum of triplicate blots, which analyzed a minimum of 3 hippocampi per genotype per sex.

For the calcium channel CAV1.2, sex ($p = 0.5349$) and genotype (0.1172) did not have a significant effect on protein levels in the hippocampus. For the chloride channel NKCC1, sex ($p = 0.5340$) and genotype ($p = 0.6376$) did not have a significant effect on protein levels in the hippocampus. Sex has a significant effect on parvalbumin (PARV) protein levels in the hippocampus ($p = 0.0009$), with higher levels of PARV in females compared to males, whereas genotype has no significant effect ($p = 0.4056$). For the sodium channel SCN1A, sex ($p = 0.5155$) and genotype (0.1304) did not have a significant effect on protein levels in the hippocampus. For vesicular glutamate transporter VGLUT, sex ($p = 0.2615$) and genotype (0.8983) did not have a significant effect on protein levels in the hippocampus. For the immunoblotting, the only significant result observed was a difference between sexes for parvalbumin.

Next, we analyzed E/I protein levels in a region-specific manner using immunohistochemistry in the hippocampus of 12-week-old shank3b+/+ and shank3b+/- mice (Figure 2C-L). To quantify changes observed in staining, we determined the fluorescent intensity of our protein of interest relative to that of synaptophysin to create a ratio. For CAV1.2, sex has a statistically significant effect on protein levels in the DG ($p = 0.05$) and CA3 (0.0078), where CAV1.2 was higher (on average) in the females in the DG and in the males in the CA3, but not in CA1 ($p = 0.08$). For CAV1.2, there was no significant effect on protein levels based on genotype in the DG ($p = 0.079$), CA1 ($p = 0.235$), or CA3 ($p = 0.836$). For NKCC1, sex has a statistically significant effect on protein levels in the DG ($p = 0.005$), where NKCC1 protein levels were higher in the females, but not in CA1 ($p = 0.325$) or CA3 (0.326. For NKCC1, there was no significant effect on protein levels based on genotype in the DG ($p = 0.2564$), CA1 ($p = 0.926$), or CA3 ($p = 0.829$). For PARV, sex has a statistically significant effect on protein levels in the CA3($p < 0.0001$) where PARV protein levels were higher in the females, but not DG ($p = 0.08$), or CA1 ($p = 0.5222$). For PARV, there was no significant effect on protein levels based on genotype in the DG ($p = 0.95$), CA1 (0.2393), or CA3 ($p = 0.2269$).

For SCN1A, sex has a statistically significant effect on protein levels in the CA3 ($p = 0.0421$) where SCN1A protein levels were higher in the females, but not DG ($p = 0.617$), or CA1 ($p = 0.3132$). For SCN1A, there was no significant effect on protein levels based on genotype in the DG ($p = 0.41$), CA1 ($p = 0.0932$), or CA3 ($p=0.7707$). For VGLUT1, sex has a statistically significant effect on protein levels in the DG ($p < 0.0001$), where protein levels were higher in females, and CA3 ($p < 0.0001$) where protein levels were higher in males, and in CA1 ($p = 0.0439$) where protein levels were higher in males. For VGLUT1, there was no significant effect on protein levels based on genotype in the DG ($p = 0.529$), CA1 (0.9370), or CA3 (0.8203). The E/I immunohistochemistry results were fascinating. There were no results that were significant based on genotype. However, there were several findings that were significant based on sex in sub-regions of the hippocampus. CAV1.2 and VGLUT1 had a significant difference between the sexes in CA3 and the DG, but in CA1. For NKCC1 and SCN1A, there was a difference in the DG only. For PARV, the difference was only in the CA3. These sub-region, sex-dependent changes are novel and enhance our understanding of the complexity of neurodevelopment.

Shank3B+/- mice display normal behavior

Based on our molecular findings indicating alterations in proteins involved in regulating E/I balance, we sought to determine whether this imbalance impacts behavior in Shank3B+/- mice. To assess these potential behavioral effects, we conducted both the open field and forced swim tests. Previous studies using the Shank3B+/– mice demonstrated no change in the time in center and time in the perimeter in the OFT [44]. Previous studies with the Shank3B–/– mice showed similar levels of activity and thigmotaxis in the OFT but with an observed decrease in rearing [5].

In the open field test (Figure 3A–D), we assessed several anxiety-related behaviors, including time spent in the center (Figure 3A: +/+: 55.1 ± 8.4 s vs. +/-: 42.1 ± 4.7 s, $p = 0.08$), time spent in the perimeter (Figure 3B: +/+: 541.9 ± 8.4 s vs. +/-: 557.8 ± 4.7 s, $p = 0.08$), the average number of vertical jumps (Figure 3C: +/+: 1.1 ± 0.7 vs. +/-: 1.4 ± 0.7, $p = 0.8$), and the average

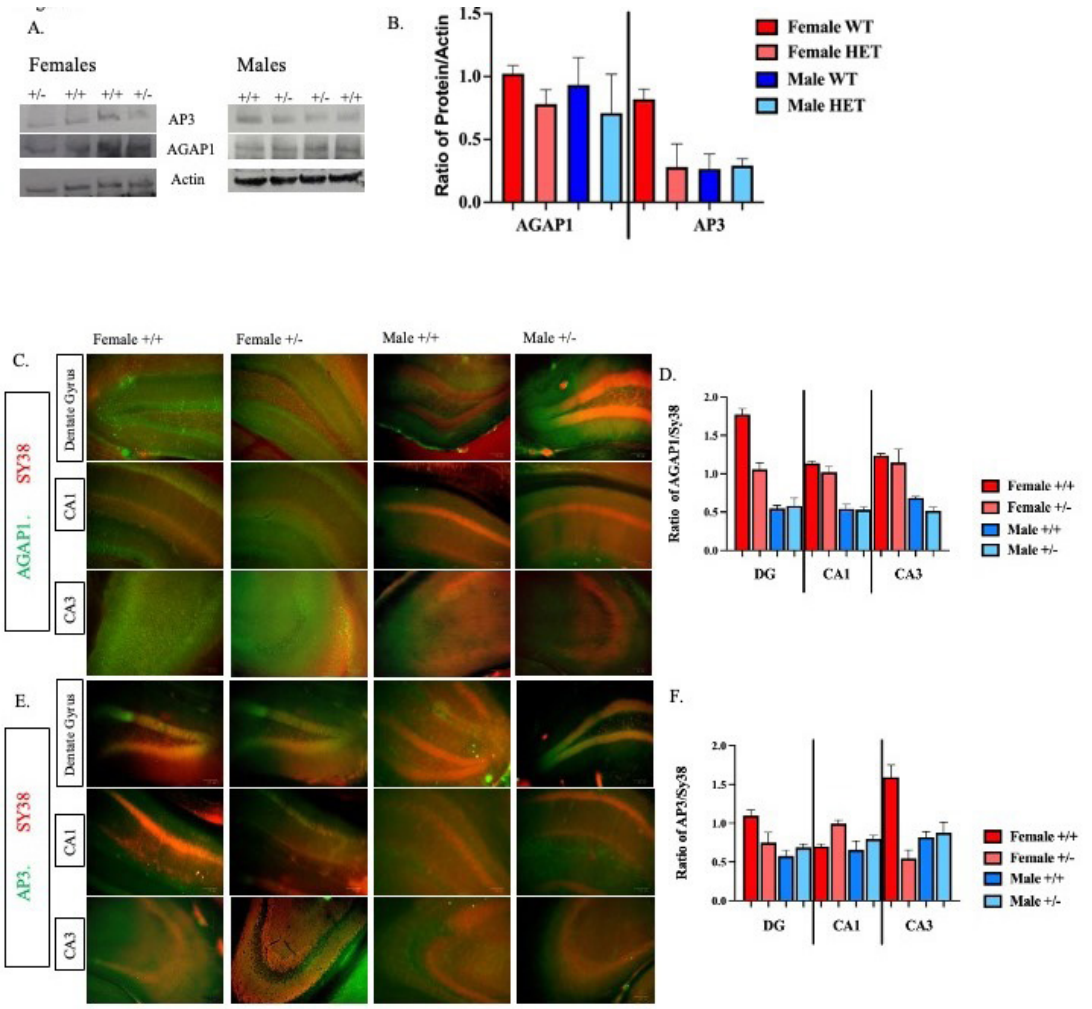

Figure 1. Alterations of protein expression of endosomal trafficking proteins in Shank3B$^{+/+}$ and Shank3B$^{+/-}$ mice observed by immunoblotting and immunohistochemistry. **A).** Representative immunoblots from the hippocampus of 12-week-old Shank3B$^{+/+}$ and Shank3B$^{+/-}$ mice using immunoblotting with 3-5 Shank3B$^{+/+}$ mice and 4 - 5 Shank3B$^{+/-}$ mice per blot. Each series of blots was completed in triplicate at minimum. **B).** Bar graph of the mean of each ratio of the endosomal protein of interest to the loading control, actin. Two-way ANOVA measured that sex had no significant effect on AGAP1 levels (p= 0.3750). Genotype demonstrated a significant decrease (p = 0.0321) in AGAP1 levels. For AP3, sex (p = 0.1248) and genotype (0.6696) did not have an alter protein levels in the hippocampus. **C).** Immunohistochemistry of AGAP1 in green with SY38 in red in the DG, CA1 and CA3 in the hippocampus of 12-week-old Shank3B$^{+/+}$ mice in 3 control mice, and 3-4 Shank3B$^{+/-}$ mice. **D).** Bar graph of the mean of each ratio of the AGAP1 to the staining control, Sy38. These results were analyzed by a two-way ANOVA. For AGAP1, sex has a statically significant effect on protein levels in the DG (p < 0.0001), the CA1 (p < 0.0001), and the CA3 (p < 0.0001) but genotype not in the DG (p = 0.5) or CA1 (p=0.7034). Shank3B$^{+/-}$ did have a significant decrease in protein levels of AGAP1 in the CA3(p = 0.0172) compared to control. **E).** Immunohistochemistry of AP3 in green with SY38 in red in the DG, CA1 and CA3 in the hippocampus of 12- week-old Shank3B$^{+/+}$ mice in 3 control mice, and 3-4 Shank3B$^{+/-}$ mice. **F).** Bar graph of the mean of each ratio of the AP3 to the staining control, Sy38. This was analyzed by a two-way ANOVA. For AP3, sex has a statically significant effect on protein levels in the DG (p = 0.0143) and the CA1 (p = 0.0409), but no in the CA3 (p = 0.3173). For AP3, there was no significant effect on protein levels based on genotype in the DG(p = 0.5725), CA1 (p = 0.3398), or CA3 (p = 0.5618).

number of rears (Figure 3D: +/+: 71.5 ± 6.3 vs. +/-: 72.7 ± 3, p = 0.9). Across all measures, the Shank3B+/- mice did not exhibit significant differences compared to wild-type controls, suggesting normal anxiety-like behavior.

In the forced swim test (Figure 3E–F), we evaluated depressive-like behaviors by measuring time spent mobile (Figure 3E: +/+: 26.2 ± 6.3 s vs. +/-: 19.4 ± 5.1 s, p = 0.42) and time spent immobile (Figure 3F: +/+: 343.2 ± 5.7 s vs. +/-: 338.6 ± 6.2 s, p = 0.64). Similarly, no significant differences were observed between groups, indicating that Shank3B+/- mice do not display depressive-like phenotypes.

Shank3B+/- mice have normal picrotoxin-induced seizure thresholds

Given the role of Shank3B in regulating excitatory synapses and our observed alterations in proteins associated with increased seizure susceptibility (e.g., SCN1A), we aimed to assess seizure thresholds in Shank3B+/- mice. In addition, previous studies reported that Shank3–/– mice carrying the human Q321R mutation on both alleles demonstrate decreased susceptibility to PTZ-induced seizures [45]. To explore seizure induction in heterozygote mice, we utilized the picrotoxin (a GABAA receptor antagonist) seizure induction paradigm.

Using the chemoconvulsant picrotoxin, we induced seizures in Shank3B+/- mice and their wild-type littermates (Figure 4A–C). We evaluated the latency to the first myoclonic jerk (Figure 4A: +/+: 323 ± 71 s vs. +/-: 291 ± 40.3 s, p = 0.68), latency to the first generalized tonic-clonic seizure (GTCS) (Figure 4B: +/+: 411.1 ± 110.2 s vs. +/-: 499.4 ± 80.9 s, p = 0.53), and the average seizure duration (Figure 4C: +/+: 425.1 ± 115.3 s vs. +/-: 442.4 ± 88.1 s, p = 0.90). Across all measures, Shank3B+/- mice did not exhibit increased susceptibility to picrotoxin-induced seizures, indicating normal neuronal excitability.

Discussion

In this study, we wanted to explore potential sex-dependent differences in a mouse model of neurodevelopmental disorders, SHANK3B. We utilized immunoblotting, immunohistochemistry, and behavioral analysis to analyze the mice. From the immunoblotting, we determined that the genotype altered AGAP1 protein levels significantly. The genotype difference was predicted

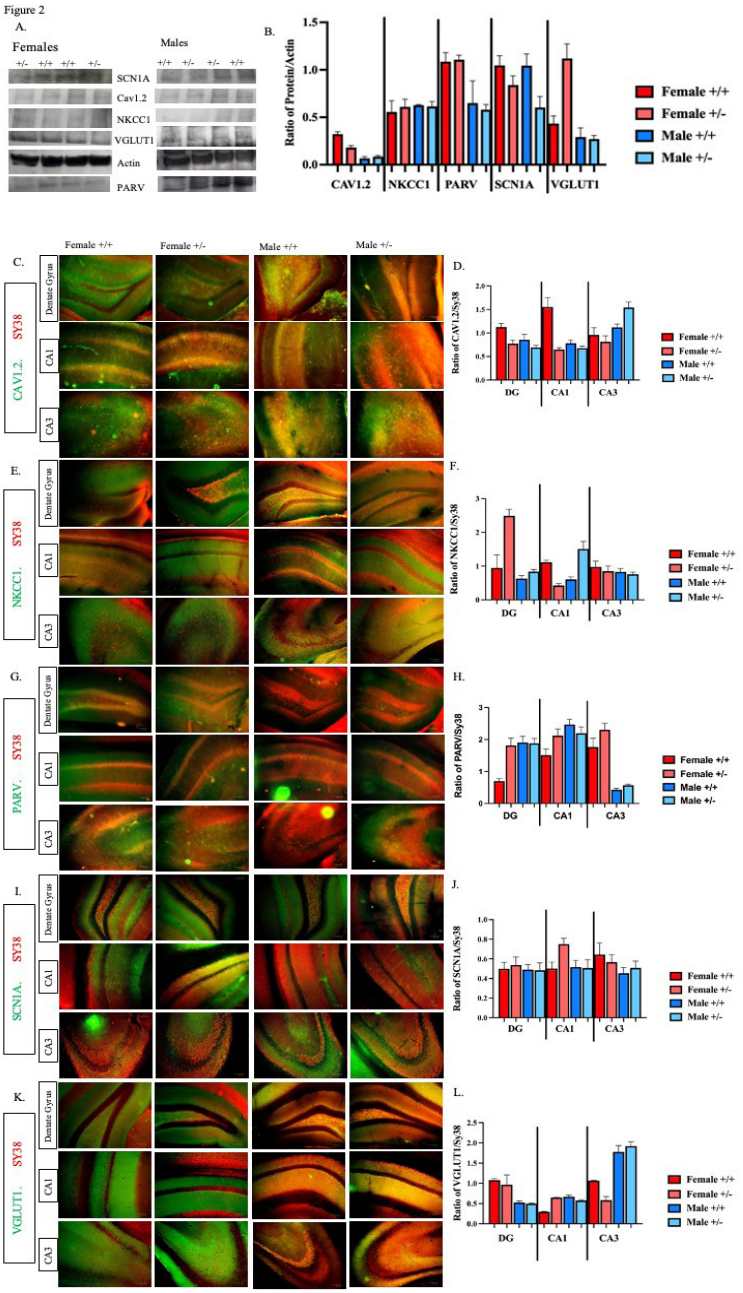

Figure2. Alteration of protein expression of E/I balance proteins in Shank3B+/+ and Shank3B+/- mice observed by immunoblotting and immunohistochemistry. **A).** Representative immunoblots from the hippocampus of 12-week-old Shank3B+/+ and Shank3B+/- mice using immunoblotting with 3-5 Shank3B+/+ mice and 4 - 5 Shank3B+/- mice per blot. Each series of blots was completed in triplicate at minimum. **B).** Bar graph of the mean of each ratio of the endosomal protein of interest to the loading control, actin. The data was analyzed by a two-way ANOVA. For the calcium channel CAV1.2, sex ($p = 0.5349$) and genotype (0.1172) did not have a significant effect on protein levels in the hippocampus. For the chloride channel NKCC1, sex ($p = 0.5340$) and genotype ($p = 0.6376$) did not have a significant effect on protein levels in the hippocampus). Sex has a significant effect on parvalbumin (parv) protein levels in the hippocampus ($p = 0.0009$), whereas genotype has no significant effect ($p = 0.4056$). For the sodium channel SCN1A, sex ($p = 0.5155$) and genotype (0.1304) did not have a significant effect on protein levels in the hippocampus. For vesicular glutamate transporter VGLUT, sex ($p = 0.2615$) and genotype (0.8983) did not have a significant effect on protein levels in the hippocampus. **C).** Immunohistochemistry of CAV1.2 in green with SY38 in red in the DG, CA1, and CA3 in the hippocampus of 12-week-old Shank3B+/+ mice in 3 control mice, and 3-4 Shank3B+/- mice. **D).** Bar graph of the mean of each ratio of the CAV1.2 to the staining control, Sy38. We analyzed the data by two-way ANOVA. For CAV1.2, sex has a statically significant effect on protein levels in the DG ($p = 0.05$) and CA3 (0.0078), but not in CA1 ($p = 0.08$). For CAV1.2, there was no significant effect on protein levels based on genotype in the DG ($p = 0.079$), CA1 ($p = 0.235$), or CA3 ($p = 0.836$). **E).** Immunohistochemistry of NKCC1 in green with SY38 in red in the DG, CA1, and CA3 in the hippocampus of 12-week-old Shank3B+/+ mice in 3 control mice, and 3-4 Shank3B+/- mice. **F).** Bar graph of the mean of each ratio of the NKCC1 to the staining control, Sy38. We analyzed this data with a two-way ANOVA. For NKCC1, sex has a statically significant effect on protein levels in the DG ($p = 0.005$), but not in CA1 ($p = 0.325$) or CA3 (0.326. For NKCC1, there was no significant effect on protein levels based on genotype in the DG ($p = 0.2564$), CA1 ($p = 0.926$), or CA3 ($p = 0.829$). **G).** Immunohistochemistry of PARV in green with SY38 in red in the DG, CA,1 and CA3 in the hippocampus of 12-week-old Shank3B+/+ mice in 3 control mice, and 3-4 Shank3B+/- mice. **H).** Bar graph of the mean of each ratio of the PARV to the staining control, Sy38. We analyzed this data with a two-way ANOVA. For PARV, sex has a statically significant effect on protein levels in the CA3($p < 0.0001$) but not DG ($p = 0.08$), or CA1 ($p = 0.5222$). For PARV, there was no significant effect on protein levels based on genotype in the DG ($p = 0.95$), CA1 (0.2393), or CA3 ($p = 0.2269$). **I).** Immunohistochemistry of SCN1A in green with SY38 in red in the DG, CA1, and CA3 in the hippocampus of 12-week-old Shank3B+/+ mice in 3 control mice, and 3-4 Shank3B+/- mice. **J).** Bar graph of the mean of each ratio of the SCN1A to the staining control, Sy38. We analyzed the data by two-way ANOVA. For SCN1A, sex has a statically significant effect on protein levels in the CA3(0.0421) but not DG ($p = 0.617$), or CA1 ($p = 0.3132$). For SCN1A, there was no significant effect on protein levels based on genotype in the DG ($p = 0.41$), CA1 ($p = 0.0932$), or CA3 ($p=0.7707$). **K).** Immunohistochemistry of VGLUT1 in green with SY38 in red in the DG, CA1, and CA3 in the hippocampus of 12-week-old Shank3B+/+ mice in 3 control mice, and 3-4 Shank3B+/- mice. **L).** Bar graph of the mean of each ratio of the VGLUT1 to the staining control, Sy38. We analyzed this by two-way ANOVA. For VGLUT1, sex has a statically significant effect on protein levels in the DG ($p < 0.0001$) and CA3 ($p < 0.0001$), and in CA1 ($p = 0.0439$). For VGLUT1, there was no significant effect on protein levels based on genotype in the DG ($p = 0.529$), CA1 (0.9370), or CA3 (0.8203).

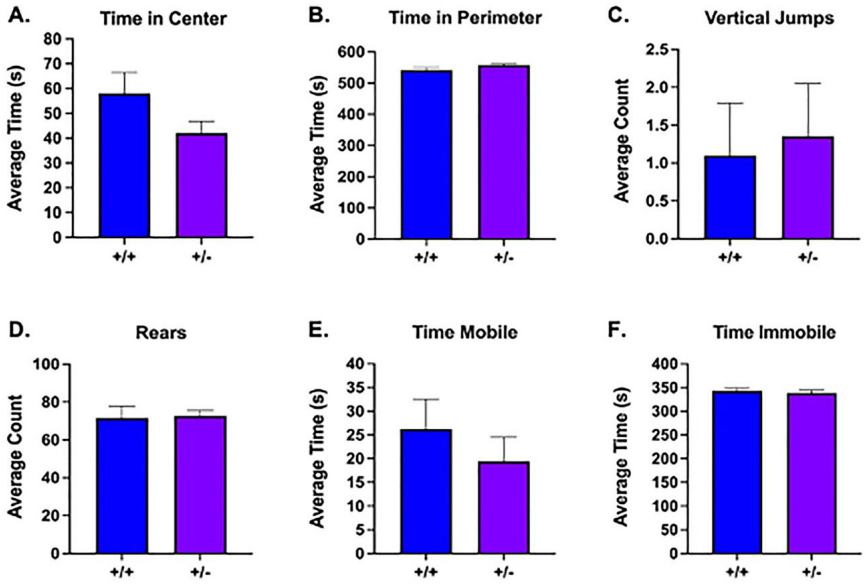

Figure 3. No Alterations in Anxiety or Depression-like Behaviors Observed in the Shank3B+/- mice. Shank3B+/- mice and their Shank3B+/+ littermates were evaluated for (A-D) anxiety-like and (E-F) depression- like behaviors in the open field and forced swim test, respectively. There were no differences in the time spent in **A).** center, **B).** perimeter, **C).** the number of vertical jumps, and **D).** rears in the Shank3B+/- compared to their Shank3B+/+ littermates. There were no differences in **E).** the amount of time spent mobile and **F).** immobile in the Shank3B+/- mice compared to the Shank3B+/+ littermates. Error bars represent SEM. * indicates significance of $p < 0.05$ compared with controls.

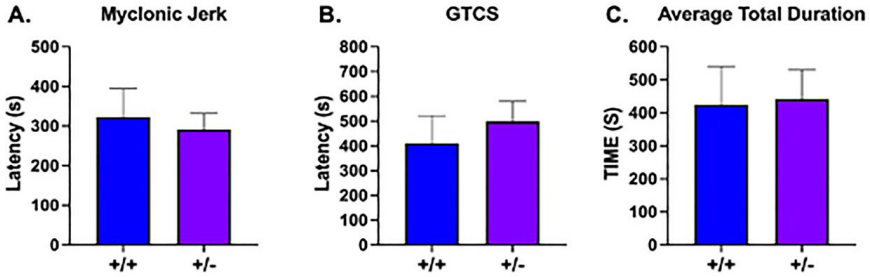

Figure 4. Shank3B+/- mice do not exhibit reduced susceptibility to picrotoxin-induced seizures. Shank3B+/- mice and their Shank3B+/+ littermates were evaluated for their susceptibility to picrotoxin-induced seizures. Their latency to the 1st myoclonic jerk **A).** Latency to the 1st GTCS **B).** Average total seizure duration were evaluated **C).** Compared to their Shank3B+/+ littermates. No differences were identified in any of the seizure measurements. Error bars represent SEM. * indicates significance of $p < 0.05$ compared with controls.

based on previous mRNA data reported. For E/I proteins, sex altered parvalbumin levels significantly. The sex difference for Parvalbumin was not predicted and is a novel finding. Understanding sex dependent expression of E/I proteins in neurodevelopmental models is essential.

For the immunohistochemistry, we determined that AGAP1 has a significant loss in the CA3 of the heterozygote mice is an interesting finding. It is also important to note that there were sex differences observed in the DG, CA1 for AP3, and for all regions of the hippocampus for AGAP1. Again, these sex differences were not predicted and are novel. For the E/I, there were no results that were significant based on genotype. However, for the E/I there were findings that were significant based on sex, which provides an interesting basis for sex-based studies in the future. CAV1.2 protein levels were increased in the female DG and in the male CA3. NKCC1 protein levels were increased in the female DG. PARV protein levels were increased in female CA3. For SCN1A, protein levels in the CA3 were higher in the females. Finally, for VGLUT1 protein levels in the DG were higher in females and in males in CA3 and CA1.

These sex-dependent differences reported here are novel and underscore the need to measure and report any sex differences in neurodevelopmental models. These differences are in the control mice and are not based on genotype, which is fascinating. We report sex differences in key E/I proteins in the hippocampus that were not dependent on genotype. This data should inform how we frame experimental design in neurodevelopment.

As we analyze our behavioral data, we conclude that there is more to know. To explore additional behavioral paradigms that look at detailed aspects of mouse behavior using 3D capture technology or very detailed cage recordings. The molecular work we have done captures fascinating sex-dependent differences in control mice as well as heterozygote mice for AGAP1. We can build on that to analyze how that translates into behavioral or developmental differences as we explore these genotypes in the future.

Using the heterozygote mice allows us to mimic clinical phenotypes better; however, heterozygote mice present many challenges in data sensitivity. The endosomal pathway is essential for proper neuronal function, and as such, it follows that this pathway has compensatory mechanisms. We did detect sex and genotype differences in endosomal protein levels and E/I proteins that are regulated by this pathway, which is fascinating. But we did not observe any changes in behavior. However, what if there were changes, but there were compensatory mechanisms that may have regulated the behavior in a way that we were unable to observe the changes. Our lab recently published a paper on the sex-dependent differences in the gut microbiomes of the shank3b +/- mice [46-49]. In this publication, we report that the male shank3b +/- mice have a more permissive, more diverse microbiome, and that the females do not have as diverse a microbiome as compared to the littermate controls. Could the alterations in microbiomes compensate for the protein alterations we observed in such a way that we would not observe any changes in behavior? There are several ways we could test for this. Future studies could include behavioral tests that include fecal bowel transplants and microbiome sequencing to determine if the microbiome is influencing behavior in a sex-dependent manner.

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