Brain region-specific disruption of Shank3 in mice reveals a dissociation for cortical and striatal circuits in autism-related behaviors

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Abstract

We previously reported a new line of Shank3 mutant mice which led to a complete loss of Shank3 by deleting exons 4—22 (Δe4—22) globally. Δe4—22 mice display robust ASD-like behaviors including impaired social interaction and communication, increased stereotypical behavior and excessive grooming, and a profound deficit in instrumental learning. However, the anatomical and neural circuitry underlying these behaviors are unknown. We generated mice with Shank3 selectively deleted in forebrain, striatum, and striatal D1 and D2 cells. These mice were used to interrogate the circuit/brain-region and cell-type specific role of Shank3 in the expression of autism-related behaviors. Whole-cell patch recording and biochemical analyses were used to study the synaptic function and molecular changes in specific brain regions. We found perseverative exploratory behaviors in mice with deletion of Shank3 in striatal inhibitory neurons. Conversely, self-grooming induced lesions were observed in mice with deletion of Shank3 in excitatory neurons of forebrain. However, social, communicative, and instrumental learning behaviors were largely unaffected in these mice, unlike what is seen in global Δe4—22 mice. We discovered unique patterns of change for the biochemical and electrophysiological findings in respective brain regions that reflect the complex nature of transcriptional regulation of Shank3. Reductions in Homer1b/c and membrane hyper-excitability were observed in striatal loss of Shank3. By comparison, Shank3 deletion in hippocampal neurons resulted in increased NMDAR-currents and GluN2B-containing NMDARs. These results together suggest that Shank3 may differentially regulate neural circuits that control behavior. Our study supports a dissociation of Shank3 functions in cortical and striatal neurons in ASD-related behaviors, and it illustrates the complexity of neural circuit mechanisms underlying these behaviors.

Introduction

Despite significant advances in identifying genetic defects in patients diagnosed with autism spectrum disorder (ASD), the anatomical basis and underlying neural circuit mechanisms that contribute to its core symptoms remain elusive1,2. These limitations represent a critical gap in our understanding of the disorder and hinder our ability to develop therapies targeting specific molecular or neural circuit abnormalities that underlie the condition. Human imaging studies of individuals affected by ASD have identified a pattern of morphological changes affecting many brain regions including the frontal cortex, hippocampus, amygdala, and striatum3,4. These clinical
studies have suggested that local hyper-connectivity and long-range hyper-connectivity in forebrain structures may underlie the pathogenesis of ASD5,6. Early changes in neural circuit development and plasticity can result in lifelong impairments in the neural systems that subserve the core features of ASD7.

In particular, there are a number of reports implicating corticostriatal circuits in ASD8–14. Support for their role in the expression of ASD-associated behaviors is derived, in part, from neuroimaging studies comparing neurotypical and ASD subjects. Aberrant striatal morphology and growth trajectories in ASD subjects have been identified by MRI4,15, with perturbations in functional connectivity between the prefrontal cortex and basal ganglia18–20. While several studies have found correlations between corticostriatal imaging phenotypes and repetitive behaviors15,21, limitations in technique, heterogeneity of patient populations, and inability to perform direct manipulations limit our ability to demonstrate causality between the anatomical and behavioral manifestations of the disorder.

As a complement to human studies, experiments utilizing rodents provide a more mechanistic way to evaluate the role of specific neural circuits in the expression of ASD-like behaviors. Neural projections between the amygdala and hippocampus, as well as those between the ventral tegmental area and nucleus accumbens have been identified, and stimulation of these projections alter sociability22,23. With respect to other core ASD domains, the basal ganglia are hypothesized to contribute to repetitive behaviors, which are thought to involve aberrant striatal-mediated learning24,25. Recent studies, using optogenetics to target the orbitofrontal cortex to ventral striatum circuit, have found altering activity between these brain regions can induce or alleviate repetitive self-grooming26,27. However, most neural circuit studies in rodents have not been conducted in genetically-engineered mouse models with sufficient construct or face validity for ASD.

The SHANK genes (SHANK1-3) encode critical scaffolding proteins for glutamatergic neurotransmission in the post-synaptic densities (PSD) of neurons. Autism-causing mutations have been identified in all three SHANK genes28. Nevertheless, most mutations are found in SHANK3, which accounts for 1–2% of all ASD cases. Moreover, patients with a deletion containing SHANK3 present with a high penetrance of ASD features28–30. SHANK3 displays a complex transcriptional regulation that is cell type and developmental stage specific in brain due to the combination of multiple intragenic promoters and extensive splicing of coding exons31. Interesting to note, for the small number of cases carrying single nucleotide variants (SNVs) in SHANK3, the genetically deleterious mutations such as frameshift and nonsense mutations are exclusively localized in exon 21 and the rest of SNVs are missense in other exons32. To examine the role of Shank3 in ASD-like behaviors, thirteen lines of Shank3 isomeric-specific mutant mice have been generated. These mice bear point mutations or deletions in various exons [Δe4–7, Δe4–9 (three lines), Δe9, Δe11, Δe13–16, e13–16\textsuperscript{flex}, Δe21, e21\textsuperscript{InsG3680} (two lines), and e21R1117X33–43]. Despite these mouse models, these isomeric-specific knockout lines have limited molecular construct validity as no patients with similar exonic deletions have been reported32 and only one ASD-pathogenic point mutation (InsG3680) has been identified within a single family44. We recently reported the first complete knockout of Shank3 (Δe4–22), which recapitulates the mutations seen in the majority of patients with SHANK3-causing ASD45. The global Δe4–22 mice display abnormal social behaviors, aberrant ultrasonic vocalizations (USVs), and increased repetitive responses that resemble the core behavioral features of the autism associated with SHANK3-related disorders. Hence, the Δe4–22 line of mutant mice provides a unique opportunity to dissect the anatomical and neural circuit mechanisms underlying their ASD-like behaviors.

Relatively few studies have directly compared phenotypes using brain-region-specific mutant mice for ASD models46,47. Here, we report the molecular, physiological, and behavioral consequences of Shank3 Δe4–22 deletions in specific corticostriatal regions using several different Cre drivers in transgenic mice. Our results reveal that Shank3 deficiency in the neocortex is critical for the expression of increased grooming behaviors, while the striatum is critical for the expression of perseverative exploratory behaviors.

Materials and methods

Generation of Shank3 mice with conditional deletion of exons 4–22

Shank3 Δe4–22 mice were generated using CMV-Cre to delete Shank3 in the germ-line45. Drd1-Shank3 and Drd2-Shank3 mice were generated by crossing the e4−22\textsuperscript{flex/flex} mice with dopamine (DA) D1 receptor (Drd1) Cre mouse (B6.Cg-Tg(Drd1a-Cre)EY262Gsat/Mmcd) and DA D2 receptor (Drd2) Cre mouse (B6.Cg-Tg(Drd2-Cre)ER44Gsat/Mmcd) [from The Gene Expression Nervous System Atlas (GENSAT) Project48]. Dlx5/6-Shank3 mice were generated by crossing e4−22\textsuperscript{flex/flex} mice with Distalless5a/6a (Dlx5/6) Cre mouse [Stock No. 008199; Jackson Laboratories, Bar Harbor, ME49]. NEX-Shank3 mice were generated by crossing e4−22\textsuperscript{flex/flex} mice with NeuroD6 (NEX) Cre mice50. For each experiment, conditional knockout animals (Cre-positive, e4−22\textsuperscript{flex/flox}) were compared to their own littermate control or “wild-type” animals (either Cre-negative e4−22\textsuperscript{flex/flox} or Cre+e4−22\textsuperscript{flex/flox}). No differences were observed between these two
genotypes from pilot data, so the genotypes were pooled into one control group for analysis. **Drd1a-tdTomato** mice were obtained from Dr. Nicole Calakos (Duke University, Durham, NC) and crossed to **Drd1-Shank3** and **Drd2-Shank3** mice for use in guiding the cell-type specific electrophysiological recordings. The natural **Disc1** mutation in 129/SvEv mice was segregated from the **Shank3** targeted mutation during the backcrossing. See supplement for additional information on genotyping protocols and animal husbandry.

**Behavioral testing**

Littermate WT and conditional **Shank3** KO mice (Supplemental Table 1) were tested in 6 cohorts of mixed
sex (except for adult vocalizations, which were recorded only from male mice), with testing beginning at 8–12 weeks of age. All experimenters were blinded to genotype of the mice throughout the studies and the scoring of their behaviors, identifying animals by a subject number until the entire battery of tests was completed and analyzed at which point the genotypes were revealed. Many of the methods described below have been reported previously by our group\textsuperscript{45}. See supplement for detail.

Whole-cell patch clamp recording from brain slices

Recordings of action potentials were performed from medium spiny neurons (MSNs) in the dorsolateral striatal slices prepared from \textit{Drd1-Shank3} and \textit{Drd2-Shank3} mice crossed with \textit{Drd1a-tdTomato} mice. After identifying direct pathway MSNs (D1) by the tdTomato signal, tdTomato-negative neurons were assumed to be indirect pathway MSNs (D2). Synaptic currents were recorded from hippocampal CA1 pyramidal neurons of \textit{NEX-Shank3} mice. See Supplement for detail.

Quantitative immunoblot analysis

Western blots were performed as previously reported by our group\textsuperscript{45}. See supplement for detail.

Statistical analyses

The data were analyzed with SPSS 21 (SPSS Inc., Chicago, IL) or Microsoft Excel and expressed as means ± SEM and analyzed by either two-tailed independent samples \textit{t}-tests, analysis of variance (ANOVA), and repeated-measures ANOVA, depending on the number of groups and conditions of the experiment (see Supplementary statistics dataset). Sample sizes were based upon previous experience with similarly designed experiments or from pilot experiments.

Results

Generation of conditional Shank3 knockout (KO) mice

Since it has been hypothesized that cortico-striatal circuits underlie ASD-like behaviors, we crossed the recently generated transgenic mouse with loxP sites flanking Shank3 exons 4–22 (\textit{e4–22}\textsuperscript{loxP/loxP}) to mice expressing Cre recombinase to disrupt the expression of Shank3 in cortical or striatal regions (Fig. 1a). We deleted Shank3 in forebrain excitatory neurons of the cortex and hippocampus by crossing \textit{e4–22}\textsuperscript{loxP/loxP} mice with \textit{NEX-Cre} mice\textsuperscript{39} to generate \textit{NEX-Shank3} mice, which begins to be expressed around embryonic day 11.5 (E11.5). To examine GABAergic neurons, and striatal MSNs in particular, we used \textit{Dlx5/6-Cre} mice\textsuperscript{49} to produce \textit{Dlx5/6-Shank3} mice, as it has been shown to be expressed in an enriched manner in GABA-ergic progenitors at similar timepoint (E12)\textsuperscript{54} which give rise to striatal MSNs and with relatively restricted robust expression in the striatum\textsuperscript{55}. Numerous groups\textsuperscript{55–58} have used it to generate striatal-targeted conditional knockout lines of mice with robust striatal targeting, but not complete specificity, and with minimal effects in Cre\textsuperscript{+} control animals (lacking the floxed gene of interest)\textsuperscript{59}. We also developed two additional lines of mice by crossing the \textit{e4–22}\textsuperscript{loxP/loxP} mice with \textit{Drd1-Cre} or \textit{Drd2-Cre} mice\textsuperscript{48}; thereby, selectively targeting the respective direct and indirect pathway MSNs of the basal ganglia. Parenthetically, expression of the
specific lines of Drd1-Cre (EY262) and Drd2-Cre (ER44) begins on day E16 (Drd1-Cre) and day E18 (Drd2-Cre), respectively, and are not 100% restricted to the striatum, similar to the endogenous expression of Drd1 and Drd2. Using primers designed to detect recombination between exons 4 and 22 of the Shank3 gene (Fig. 1a), we are able to detect the loss of Shank3 in the cortex and hippocampus of homozygousfloxed mice expressing NEX-Cre (NEX-Shank3) (Fig. 1b). Likewise, loss of Shank3 exons 4–22 could be identified in striatal DNA samples from homozygous floxed mice expressing Dlx5/6-Cre, Drd1-Cre, and Drd2-Cre (Dlx5/6-Shank3, Drd1-Shank3, Drd2-Shank3, respectively) (Fig. 1c–e). To quantify the extent to which Shank3 was deleted, we performed real-time PCR of genomic DNA and found that 25–50% of Shank3 exons 4–22 were deleted in the hippocampus and cortex, respectively, of NEX-Shank3 mice; 50% of Shank3 was deleted in Dlx5/6-Shank3 striatum; and 20–25% of Shank3 was deleted in Drd1-Shank3 and Drd2-Shank3 striata (Supplemental Figure S1a–d). We were not able to design primers technically for qPCR to quantify the recombination between exon 4–9 and exon 10–22 due to the long genomic distance. Using three primers for PCR in the same reaction, we were able to assess the ratio of recombination between exon 4–9 and exon 10–22 semi-quantitatively. As shown in the Fig. 1b–e, we detected a low percentage of exon 4–9 deletion in Cre targeted tissues. However, the exons 10–22 deletion was not detectable which suggest that the recombination did not occur or they may overlap with the exon 4–22 deletion. Shank3 is a transcriptionally complex gene with multiple promoters and extensive alternative splicing of coding exons. The exact number and repertoire of mRNA isoforms are predicted to be large and the expression of known isoforms is brain region and cell type as well as development specific. However, the transcript structure for most isoforms and expression patterns are largely uncharacterized due to the large size of mRNAs and lack of isoform specific antibodies. In a previous study, we have shown that we are able to examine five major mRNA isoforms (Shank3a-e) at the mRNA level using isoform-specific primer design. Using the same design, we performed quantitative reverse-transcription PCR (RT-PCR) on RNA samples harvested from dissected brain regions of the four lines of conditional knockout mice (Supplementary Figure S1e–h). In all four lines of conditional KO mice, the full length Shank3a was almost completely disrupted in the tissues as expected. However, the reduction of other queried isoforms varied among different lines. For instance, in NEX-Shank3 mice, Shank3b but not Shank3 c-e was significantly reduced in cortex (Supplementary Figure S1e). In Dlx5/6-Shank3 mice, Shank3b in striatum and Shank3d in striatum, cortex, and hippocampus were affected significantly (Supplementary Figure S1f). In Drd1-Shank3 mice, Shank3b, and Shank3d were reduced in striatum but Shank3a and Shank3d were also reduced (Supplementary Figure S1g). Lastly, in Drd2-Shank3 mice, Shank3b, c, d were significantly reduced and Shank3e showed a trend of reduction in striatum (Supplementary Figure S1h).

Reduction of Shank3 protein in the expected brain regions was also confirmed with crude PSD fractions isolated from the cortex, hippocampus, and striatum of all 4 conditional Shank3 knockout lines. Like our analyses of Shank3 mRNA, reduction in Shank3a protein was observed in the cortex and hippocampus of NEX-Shank3 mice (Fig. 1f) as well as in the striata of Dlx5/6-Shank3, Drd1-Shank3, and Drd2-Shank3 mice (Fig. 1g–i). The reduction of the large molecular weight band in the western blot is consistent with the reduced expression of Shank3a from the qRT-PCR. However, we were not able to assess the isoform specific reduction at protein level because it is unknown how many Shank3 protein isoforms exist in different brain regions. As shown in Fig. 1f–i, some residual protein isoforms remained, suggesting either the efficiency of Cre mediated recombination was incomplete or likely, that other cell types such as astrocytes or other neuronal populations were not targeted by the Cre recombinase in a given brain region where the expression of Shank3 isoforms has been described. However, attempts to examine the isoform or cell type specific expression of Shank3 in these lines of mutant mice were not successful due to lack of isoform specific and the inadequate quality for staining with Shank3 antibodies.

Shank3 conditional knockout mice engage in repetitive behaviors while social behaviors are intact

We next evaluated cohorts of the WT littermates and conditional KO mice from each line for expression of core features of ASD-related behaviors. Mice were tested in 6 cohorts of mixed sex littermates by blinded observers. The detail statistical analysis for all behavioral tests are summarized in the Supplementary statistics dataset. The behaviors were not analyzed by sex, as we have not seen sex-specific differences in the behavioral phenotypes of Δe4–22 mice (unpublished data) nor was this formally analyzed in our prior characterization of the global knockout. In a test for social affiliation, we observed that the conditional KO mice from each line, as well as the global Δe4–22 mice had no preference for either non-social stimulus (Supplemental Table 2). In testing, KO mice preferred interacting with the social over the non-social stimulus (Supplemental Table 2). In testing, KO mice preferred interacting with the social over the non-social stimulus (Supplemental Table 2).
test in the NEX-Shank3 and Dlx5/6-Shank3 mice using a simplified ethogram based on our previous finding that non-reciprocated social approach is significantly increased in global Δe4–22 mice. However, we instead found a significant increase in bi-directional interactions between Dlx5/6-Shank3 mice and the C3H intruders whereas the NEX-Shank3 did not differ from their WT controls (Supplemental Table 2), but saw no significant
We also examined ultrasonic vocalizations (USVs) in 6-Shank3, Drd1-Shank3 however, altered self-grooming was not observed in fi Shank3 mice (Fig.2c, d). As repetitive behaviors are evident in the global Δe4−22 mice, we monitored the duration of self-grooming in the home-cage of the different Shank3 lines. We found that NEX-Shank3 mice had a tendency for increased self-grooming but with significant variability (p = 0.086); however, altered self-grooming was not observed in Dlx5/6-Shank3, Drd1-Shank3, or Drd2-Shank3 mice that targeted the basal ganglia for Shank3 disruption (Fig. 2e).

Furthermore, skin lesions similar to the global Δe4−22 mice were observed in 4/15 NEX-Shank3 mice compared to 0/15 in WT controls; notably, targeted deletion of Shank3 in the striatum was insufficient to produce skin lesions in any of the three lines. This effect is consistent with an over-grooming phenotype similar to that in the global Δe4−22 mice (Fig. 2f) with some NEX-Shank3 engaging in very high levels of self-grooming similar to that of global Δe4−22 mice. However, the penetrance was reduced (~25% of NEX-Shank3 KOs developing skin lesions vs ~50% in global Δe4−22 KOs) resulting in greater variability in the expression of this behavior.

We next examined another form of repetitive behavior as monitored in the hole-board test. While Dlx5/6-Shank3 mice had significant reductions in the numbers of holes explored (Fig. 2g), perseverative or repetitive exploration of the same hole was augmented in Dlx5/6-Shank3 and Drd2-Shank3 mice (Fig. 2h). Together, these data suggest that loss of Shank3 in forebrain excitatory neurons contributes significantly to the expression of repetitive self-grooming, whereas loss of Shank3 in MSNs is responsible for the perseverative or repetitive exploration phenotypes seen in the global Δe4−22 mice.

Shank3 conditional knockout mice display distinctive comorbidities

We examined also multiple domains of learning which were reported to be abnormal in global Δe4−22 mice. To dissect the possible roles of the cortex-hippocampus and striatum to these responses, we focused on the NEX-Shank3 and Dlx5/6-Shank3 mice. Contextual fear has long been known to involve the hippocampus. Although freezing behaviors were augmented in the global Δe4−22 mice, both lines of the conditional KO mice demonstrated no genotype-dependent differences in freezing for contextual fear (Supplemental Figure S2a). Similarly, no genotype differences were observed for cued fear in any of the three genotypes (Supplemental Figure S2b). Given the profound deficits in instrumental learning seen in the global Δe4−22 mice and the known role for the striatum in operant conditioning, we utilized a lever-pressing task to determine whether deletion of Shank3 in forebrain excitatory or basal ganglia inhibitory neurons was responsible for this phenotype. While global Δe4−22 mice failed to acquire this task, unexpectedly learning responses in both Dlx5/6-Shank3 and NEX-Shank3 mice were similar to that of their WT littermates (Supplemental Figure S2c, d).

As patients with idiopathic and SHANK-related ASD and animal models of ASD often exhibit sensory abnormalities, including abnormalities of sensorimotor gating, we examined responses in a prepulse inhibition (PPI) paradigm. Based on the role of the ventral striatum in the regulation of PPI, we hypothesized that a reduction of Shank3 in the MSNs would alter PPI performance. Indeed, we found that PPI was augmented in global Δe4−22 and Dlx5/6-Shank3 mice relative to their WT controls, whereas PPI in NEX-Shank3 mice was similar to that of their WT littermates (Fig. 3a). However, startle activities were reduced in the global Δe4−22 and Dlx5/6-Shank3 mice (Fig. 3b) which complicates interpretation of the altered PPI response, although the
Fig. 3 Distinctions among the Shank3 conditional mice in anxiety-like behaviors and motor performance. a) Prepulse inhibition (PPI) where genotypes within each strain were analyzed separately. While all mice showed increased PPI with increasing prepulse intensity (RMANOVA, main effect of intensity, \( p \leq 0.001 \)) global \( \Delta e4-22 \) and Dlx5/6-Shank3 mutant mice showed enhanced PPI across various intensities of prepulse stimuli relative to their +/+ controls (main effect of genotype, \( p \leq 0.05 \)). No genotype differences were seen in NEX-Shank3 mice; \( n = 9–12/\text{genotype} \).

b) Startle activities in global \( \Delta e4-22 \) and Dlx5/6-Shank3 (t-tests, \( p \leq 0.02 \)) were reduced relative to their +/+ littermates, whereas startle amplitudes in NEX-Shank3 mice were similar to those of their +/+ littermates; \( n = 9–12/\text{genotype} \).

c, d) Elevated zero maze for anxiety-like behaviors. c) Similar to the global \( \Delta e4-22 \) mice, Dlx5/6-Shank3 mice spend more time in the open areas of the maze than their +/+ controls (t-tests, \( p \leq 0.05 \)); \( n = 9–18/\text{genotype} \). Responses in the NEX-Shank3, Drd1-Shank3, Drd2-Shank3 were similar to those of their +/+ controls. d) Dlx5/6-Shank3 (t-test, \( p = 0.006 \)) and Drd1-Shank3 (t-test, \( p = 0.050 \)) mice also make more transitions from the closed-to-open-to-closed areas, as is seen in global \( \Delta e4-22 \) mice (t-test, \( p = 0.038 \)); \( n = 9–18/\text{genotype} \). e) Open field. e) Global \( \Delta e4-22 \) mice traveled over a shorter distance in the open field (t-test, \( p = 0.056 \)), whereas locomotion in NEX-Shank3 mice was greater than that of their +/+ littermates (t-test, \( p = 0.004 \)). No significant differences in locomotion were seen in Dlx5/6-Shank3, Drd1-Shank3, or Drd2-Shank3 mice, \( n = 12–18/\text{genotype} \). f) Dlx5/6-Shank3 (t-test, \( p = 0.038 \)) Drd1-Shank3 (t-test, \( p = 0.050 \)), and global \( \Delta e4-22 \) mice (t-test, \( p = 0.010 \)) mice all demonstrated lower rearing behavior. By contrast, NEX-Shank3 (t-test, \( p = 0.025 \)) and Drd2-Shank3 mice (t-test, \( p = 0.015 \)) demonstrated increased rearing; \( n = 12–18/\text{genotype} \); \( \text{bb/1 h} = \text{beam breaks in 1 h} \). For all panels, *\( p < 0.05 \), compared to wild-type controls. All data are expressed as means ± SEM and were analyzed by independent samples two-tailed t-tests unless otherwise specified.
recapitulation in Dlx5/6-Shank3 mice of both findings observed in the Δe4–22 mice suggests that loss of Shank3 in striatal neurons may be responsible for the sensorimotor gating differences.

Given how strongly anxiety-like behavior, a frequent comorbidity in ASD patients including those with SHANK-related ASD, was affected in the global Δe4–22 mice\textsuperscript{45}, we hypothesized that this behavioral phenotype would be recapitulated in the NEX-Cre targeted mutants. Cre expression should disrupt Shank3 in the amygdala and ventral hippocampus—key neural substrates for anxiety-like behavior\textsuperscript{23,65,66}. We examined anxiety-like behavior in the elevated zero maze. Unlike their previously observed increase in anxiety-like behavior in the light-dark boxes\textsuperscript{45}, global Δe4-22 mice spent more time in the open areas of the maze and made more closed-to-open-to-closed arm transitions than their WT littermates (Fig. 3c). While the reason for this discrepancy is unclear, it was a robust phenomenon observed across multiple cohorts of mice in various unpublished studies. A similar phenotype was recapitulated in the Dlx5/6-Shank3 mice. An examination of transitions between the closed-to-open-to-closed areas of the maze revealed that the global Shank3 Δe4–22, Dlx5/6-Shank3, and Drd1-Shank3 mice engaged in more transitions than their WT littermates (Fig. 3d). Locomotor activity was examined also in the global Δe4–22 mice\textsuperscript{45}. Since the basal ganglia is known to play a key role in modulating motor activity\textsuperscript{25,67}, we hypothesized that locomotor activity would be affected by striatal deletion of Shank3. While global Δe4–22 mice displayed a tendency towards hypoactivity in this study and were significantly hypoactive in prior testing\textsuperscript{45}, the NEX-Shank3 mice were hyperactive (Fig. 3e).

Shank3 deletion in direct and indirect pathway MSNs exert cell-autonomous effects on neuronal excitability and reduces scaffolding to Homer1b/c

Since we found that various behavioral phenotypes seen in the global Δe4–22 mice were recapitulated by brain-region selective targeting of Shank3 deletion, we next queried whether any of the electrophysiological and biochemical phenotypes found in the global Δe4–22 mice could be dissociated using this conditional deletion approach\textsuperscript{45}. Since many of these cellular and synaptic phenotypes were most prominent in the striatum, we examined whether loss of Shank3 in direct or indirect pathway MSNs may account for these phenotypes. Using a transgenic reporter line of mice expressing tdTomato in Drd1-containing MSNs\textsuperscript{68}, we performed patch-clamp recordings on putative Drd1 (tdTomato+) and Drd2 (tdTomato−) neurons from Drd1-Shank3 and Drd2-Shank3 mice and their respective WT controls (Supplemental Figure S3a). We examined excitability in these cells, as MSNs from global Δe4–22 mice are hyperexcitable relative to WT controls\textsuperscript{45}. Single action potentials were evoked by a 10-ms current injection in 5-pA increments to determine the current threshold to initiate an action potential. D1 cells from Drd1-Shank3 mice had markedly decreased current thresholds for action potentials (Supplemental Figure S3b). We also analyzed the number of action potentials evoked over a wide range of current amplitudes. The numbers of action potentials evoked were significantly increased in D1 cells from Drd1-Shank3 mice than from D1 cells of WT littermates (Fig. 4a, b). However, control D2 cells from Drd1-Shank3 mice, in which Shank3 was not deleted, had thresholds and excitability profiles that were indistinguishable from D2 cells from WT controls (Supplemental Figure S3d). The converse was observed in Drd2-Shank3 mice, with D2 cells of the mutant animals being hyper-excitable relative to D2 cells of WT controls with increased action potential firing in response to current injection (Fig. 4c, d) although the threshold to action potential generation did not differ between genotypes (Supplemental Figure S3c). Control D1 cells from Drd2-Shank3 mice, in which Shank3 was not targeted, had responses similar to the D1 cells of their WT littermates (Supplemental Figure S3e). Other electrophysiological properties (Supplemental Table 4) such as input resistance, resting membrane potential, peak amplitude, and action potential kinetics were largely non-differentiated among genotypes. However, there was a significant increase in input resistance and significant reductions in the current threshold to initiate an action potential and a depolarized resting membrane potential in D1 neurons of Drd1-Shank3 mice relative to the D1 neurons of their WT controls. Hence, the electrophysiological results indicate that Shank3 is expressed in both D1 and D2 cells and its loss from either cell type is
Fig. 4 Loss of Shank3 in selected striatal neurons leads to cell autonomous alterations of synaptic function and PSD components. (a) Representative traces of evoked action potentials in D1 MSNs neurons from Drd1-Shank3 WT (+/+ or +/−) (black) and KO (−/−) (red) mice. The action potentials reflect responses to 200, 300, and 400 pA current injections, respectively. 

(b) Summarized data for the number of evoked action potentials (APs) at the indicated amplitudes of current injection in D1 MSNs from Drd1-Shank3 WT (+/+ or +/−) and KO (−/−) mice (2-way ANOVA, main effects of genotype and stimulation, $p < 0.001$, genotype x stimulation interaction, $p < 0.001$). 

c Example traces of evoked action potentials in D2 MSNs neurons from Drd2-Shank3 WT (+/+ or +/−) (gray) and KO (−/−) (pink) mice. 

d Summarized data for the numbers of evoked action potentials at the indicated amplitudes of current injection in D2 MSNs from Drd2-Shank3 WT (+/+ or +/−) and KO (−/−) mice (2-way ANOVA, main effects of genotype and stimulation, $p < 0.001$, genotype x stimulation interaction, $p < 0.001$). 

e–g Homer1b/c levels in the PSD from striatum where loss of Shank3 was targeted. (e) Dlx5/6-Shank3 mice show a reduction in Homer1b/c protein in striatal (ST) ($p = 0.002$), but not in cortical (CX) or hippocampal (HP) PSD samples; $n = 5$ mice/genotype. 

f Drd1-Shank3 mice have decreased Homer 1b/c in ST ($p = 0.018$), but not in the CX or HP samples; $n = 4$ mice/genotype. 

g Drd2-Shank3 mice have a loss of Homer1b/c in the ST ($p < 0.001$), but not in the CX or HP; $n = 4$ mice/genotype. For all westerns, independent samples two-tailed t-tests; representative images are shown and each western was replicated at least two times. For all panels, *$p < 0.05$, compared to wild-type controls. All data are expressed as means ± SEM.
sufficient to alter the excitability of these neurons autonomously.

We next examined whether changes in the PSD scaffolds of these cells could account for some of the observed effects. We focused first on Homer1b/c, given that it is consistently observed to be diminished across multiple mouse models of Shank3 deficiency34,35,40,41,43 and its protein level correlates with the degree of behavioral impairment in the global Δe4-22 mice45. Additionally, pharmacological manipulations of the metabotropic glutamate receptor mGluR5, which serves as a scaffold for Homer 1b/c and Shank3, can ameliorate some behavioral phenotypes in these mice45. Striatal PSD fractions from Dlx5/6-Shank3 (Fig. 4e), Drd1-Shank3 (Fig. 4f), and Drd2-
**Table 1 Phenotypes of Shank3 knockout mice**

| Experiment               | Δe4−22 | NEX-Shank3 | Dlx5/6-Shank3 | Drd1-Shank3 | Drd2-Shank3 |
|--------------------------|--------|------------|---------------|-------------|-------------|
| Sociability              | ↓ calls| ↓ calls    | ↓ calls       | ↓ calls     | ↓ calls     |
| USVs                     | ↓ length| ↓ length   | ↓ length      | ↓ length    | ↓ length    |
| Repetitive behaviors     | ↑ grooming | ↑ grooming | ←grooming      | ←grooming   | ←grooming   |
| Elevated zero maze       | ↑ exploration| ↑ exploration| ← exploration | ← exploration| ← exploration|
| Open Field               | ↓ distance| ↑ distance | ← distance    | ← distance  | ← distance  |
| Instrumental Learning    | ↓ lever pressing | ↑ lever pressing | ← lever pressing | ← lever pressing | ← lever pressing |
| Conditioned fear         | ↑ context| ← context  | ← context     | N/A         | N/A         |
| Pre-pulse inhibition     | ↑ PPI   | ← PPI      | ↑ PPI         | N/A         | N/A         |
| Rotarod                  | ↓ startle| ↓ startle  | ← startle     | ↓ startle   | N/A         |
| Excitability             | ↑ spiking| N/A        | N/A           | ↑ spiking   | ↑ spiking   |
| Homer1b/c                | ↓ in striatum | - in any region | ↓ in striatum | ↓ in striatum | ↓ in striatum |
| NMDAR currents           | N/A    | ↑ hippocampus | N/A           | N/A         | N/A         |
| GluN1/GluN2B             | - in any region | ↑ hippocampus | - in any region | N/A         | N/A         |

*Targeting Shank3 deletion by different Cre lines (NEX, Dlx5/6, Drd1, and Drd2) recapitulated different subsets of behavioral, electrophysiological, and biochemical features of the global Δe4−22 mice. ↑ indicates an increase, ↓ indicates a decrease, - indicates that the mutant is similar to the wild-type control. N/A indicates not applicable, as the experiment was not conducted.

**Shank3** (Fig. 4g) mice were found to have a significant reduction in Homer1b/c levels which were not observed in cortical or hippocampal samples from these three mouse lines.

**Shank3 deletion in forebrain excitatory neurons increases NMDAR synaptic function and subunit protein levels**

Given the unique subset of behavioral phenotypes present in NEX-Shank3 mice, we examined whether additional synaptic components may be dysregulated in these mice. As hippocampal circuitry is well-established and neuronal populations are more homogeneous in this structure than in cortical preparations, we recorded from hippocampal CA1 neurons of NEX-Shank3 mice. For these studies, we examined the functions of AMPARs and NMDARs which are scaffolded by Shank3 in the PSD and have been found to be altered in the hippocampi of some lines of isoform-specific Shank3 knockout mice34–36,39,41. First, we recorded NMDAR-mediated excitatory postsynaptic currents (EPSC<sub>p</sub>). Induction of NMDAR-EPSCs by a series of stimulus intensities was markedly enhanced in NEX-Shank3 mice (Supplemental Figures S4a, b). In contrast, AMPAR-EPSCs were unchanged in CA1 pyramidal neurons from the NEX-Shank3 mice compared to WT controls (Supplemental Fig. 4c, d). Additionally, the NMDAR- to AMPAR-EPSC ratio was significantly larger in CA1 pyramidal neurons from NEX-Shank3 mice than those from WT controls (Fig. 5a, b).

To examine the possible biochemical basis for our electrophysiological findings, we performed quantitative immunoblots of crude PSD proteins isolated from dissected brain regions of NEX-Shank3 mice and their WT littermates (Fig. 5c). Immunoblotting for the obligatory subunit of the NMDAR, GluN1 revealed a significant increase in NEX-Shank3 hippocampus with a trend for an enhancement in the cortex (Fig. 5d). We observed similar results when blotting for GluN2B (Fig. 5e); however, no significant changes were observed in GluN2A levels (Fig. 5f). In agreement with our electrophysiological data, immunoblotting for the obligatory AMPAR subunit, GluA1, revealed that levels in the cortex and hippocampus were similar between WT and NEX-Shank3 mice (Fig. 5g). Parenthetically, we failed to observe any significant changes in Homer1b/c protein in any of the NEX-Shank3 samples (Fig. 5h). This was expected given the mild to moderate changes in Homer1b/c levels observed in the neocortex of global Δe4−22 mice45.
**Discussion**

Our analyses of selective Shank3 deficiency in the forebrain and striatum have revealed several findings compared to the global Δe4–22 mice (Table 1)45. First, we demonstrate for the first time that deletion of Shank3 in excitatory neurons of the cortex and hippocampus, and in selective MSN striatal population results in abnormalities across different behavioral domains. Contrary to the prediction, targeted Shank3 deficiency in striatum by Dlx5/6-Cre, as well as, with the Drd1 or Drd2-specific Cre lines fails to produce the profound self-grooming phenotype observed in the global Δe4–22 mice. In contrast, self-injurious skin lesions are obtained by targeting forebrain-specific Shank3 deficiency with NEX-Cre. Second, we find that Dlx5/6-Shank3 mice engage in perseverative and repetitive behaviors in the hole-board. Third, we observe that alterations in some behaviors (e.g., motor activity) are differentially affected among the various lines of Shank3 conditional and global Δe4–22 mice. These data suggest that Shank3 may play disparate roles in specific cell types and these changes may regulate the neural circuits underlying ASD-like behaviors. Finally, Shank3 deficiency in corticostriatal regions fails to reproduce the impaired social interaction, abnormal ultrasonic vocalizations, and deficient instrumental learning observed in the global Shank3 Δe4–22 mice45. As a result, other brain regions appear to be more important in controlling these behaviors.

However, our findings should also be interpreted with caution due to several caveats or confounding factors primarily related to the transcriptional complexity of Shank3 as well as the specificity of Cre expression. The analyses of DNA in brain of conditional knockouts clearly demonstrated the occurrence of Cre-mediated recombination between loxP sites flanking exons 4–22 that are ~60 kb apart. We also detect a low percentage of recombination between exon 4–9 but not exons 10–22. The failure to detect exons 10–22 deletion indicates that the exon 4–22 deletion is reasonably sufficient in Cre-targeted cells. Quantitative analyses of Shank3a-e isoforms that are technically feasible were performed. The significant reduction of the Shank3a mRNA, the full length isoform in all lines of conditional knockout mice indicates the disruption of the major Shank3 isoform. The variable or no significant reduction in the expression of other Shank3 isoforms is not straightforward. The incomplete recombination between loxP sites and the persistent expression of Shank3 isoforms in cells not targeted by the individual Cre line are likely to contribute to the expression of the residual isoforms. However, the analysis of Shank3a-e isoforms and quantification of immunoblots could not fully elucidate the exact nature and predicted complexity of isoform-specific disruption of Shank3 mRNAs and protein isoforms because of a lack of the complete knowledge of Shank3 mRNA composition and protein isoforms and the technical difficulties in examining cell type and isoform specific expression. Further study is warranted to elucidate the full spectrum of Shank3 mRNA isoforms in different cell types and during development.

Excessive grooming in rodents is frequently used as an index for the stereotyped and compulsive behaviors in humans49. For instance, in a mouse model of obsessive-compulsive disorder (OCD), the Sapap3 knockout mice can over-groom to an extent where lesions around the face and neck appear70. Interestingly, re-expression of Sapap3 in the striatum of the homozygous mutants rescues the excessive grooming, suggesting that striatal dysfunction alone regulates this behavior. Further support for a striatal mechanism derives from experiments with Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Selective DREADD enhancement of activity in the D2-mediated indirect pathway, but not in the D1-mediated direct pathway, rescues the excessive grooming in isoform-specific Shank3B KO mice71. Other experiments, however, have indicated that this relationship may be more complex. An over-grooming phenotype can be evoked in EMX-Cre animals that are also co-expressed with the Cre inducible DIO-ChR2 vector through optogenetic activation of projections from the orbitofrontal cortex (OFC) to the striatum46. Additionally, optogenetic stimulation of OFC inputs to the striatum of Sapap3 mice can suppress the excessive grooming, which indicates that cortical input may remediate the striatal deficit in these mutants77. Our present findings provide a support for cortical control by demonstrating that forebrain-specific loss of Shank3 in NEX-Shank3 leads to presentation of excessive self-grooming lesions. By comparison, the Dlx5/6-specific, Drd1-specific, or Drd2-specific Shank3-KO did not recapitulate the over-grooming phenotype in our study. Collectively, the existing studies and present findings suggest a more complex mechanism involving both cortical and striatal circuitry in excessive grooming of Shank3 models. Future work will examine cortical/striatal interactions in greater detail in the Shank3 mice.

While targeting Shank3 deletion to the striatum does not produce over-grooming, Dlx5/6-Shank3 and Drd2-Shank3 mutant mice engage in perseverative behaviors in the hole-board test. This dissociation between types of repetitive behavior such as stereotyped self-grooming and perseverative exploration has been reported for other ASD animal models72. Martos and colleagues selectively ablated striatal cholinergic interneurons and reported that social behavior was perturbed and that the repetitive exploratory behaviors were augmented while self-directed responses such as grooming were not affected. In the present study, the dissociation between excessive
grooming and perseverative responses in Dlx5/6-Shank3 and Drd2-Shank3 mice indicate that different neural circuits underlie these behaviors and they emphasize the importance in carefully assessing behavioral endophenotypes in humans.

Some behavioral phenotypes in the global Δe4−22 mice were not observed in the conditional animals. For instance, USVs and sociability were not perturbed in the conditional mice, suggesting that excitatory cortical neurons or inhibitory striatal neurons are not critical or sufficient to modulate these behaviors. This point, however, does not indicate that Shank3 in these brain regions does not play some role in these responses because the USV study only examines one aspect of social communicative function and the sociability test does not evaluate the full range and complexity of social behavior in rodents. Other Shank3 mouse models also had relatively preserved sociability.24 Aside from communicative function and social behavior being abnormal in the global Δe4−22 mice, they were impaired in instrumental learning—a form of operant conditioning hypothesized to involve corticostriatal circuits and is thought to involve reward learning.24,25 We were surprised that forebrain or striatal specific Shank3 deletion did not perturb instrumental learning. There are several possibilities why we obtained this result. First, brain regions other than or in addition to principle cells of the cortex or inhibitory neurons in the striatum may control this instrumental behavior. Second, given the diversity of cell types in brain, there may be a cellular sub-type that is not targeted by our Cre lines and is essential for expression of these behaviors as seen in global Δe4−22 mice. Additionally, besides neurons, glia express also Shank3 and they were not targeted for Shank3 disruption in the four Cre lines used in our study.31 Third, incomplete Cre-mediated recombination in the conditional Shank3 knockouts may be sufficient to preserve their functioning. Fourth, since optogenetic cortical stimulation can override striatal dysfunction in Sapap3 mice,27 the loss of Shank3 in either cortex or striatum may be compensated by the expression of this gene in reciprocal brain areas; thereby, preserving neural circuit function. Finally, all Cre mouse lines in this study have this gene expressed in mid-gestation. Since Shank3 is expressed at an earlier time,31 there may be some developmental role for Shank3 that has yet to be identified. In this situation, Shank3 expression prior to excision by Cre recombinase may account for certain behavioral phenotypes reported in the global Shank3 Δe4−22 mice but absent in the conditional Shank3 animals. Despite all of these caveats at a molecular level, our overall conclusions are supported by functional studies at the cellular level by whole cell recordings in striatum and cortex. Indeed, the cellular phenotypes in Shank3 global knockout mice are well recapitulated in Shank3 conditional mice and these indicate that the deficiency of Shank3 in Cre targeted cells is sufficient.

The electrophysiological and biochemical characterizations of synaptic function and proteins in the conditional Shank3 mice have replicated the key observations in the global Shank3 Δe4−22 animals; however, they have revealed also some unexpected findings. The electrophysiological studies demonstrate that cell-type specific deletion of Shank3 in Drd1-containing or Drd2-containing MSNs is sufficient to recapitulate the hyper-excitability phenotype reported in the cell-type indiscriminate recordings from the global Δe4−22 mice.45 Responses from neighboring cells of the opposite cell-type (i.e., D2 cells in Drd1 mutants or D1 cells in the Drd2 mutants) are indistinguishable from the WT controls, suggesting that the effects of Shank3 deletion are cell autonomous despite likely reciprocal innervation.75 This hyper-excitability state may arise from a compensatory mechanism in cells lacking Shank3 which experience a reduction in synaptic transmission.45 Indeed, global loss of some Shank3 isoforms can result in early hyper-excitability which may perturb the development of corticostriatal circuits.76 Aside from the electrophysiological findings, our previous biochemical studies in the global Δe4−22 mice found Homer1b/c protein to be significantly reduced in striatal PSDs.45 This same result was recapitulated in the Dlx5/6-Shank3, Drd1-Shank3 and Drd2-Shank3 mutants, but not in the conditional mice where the cortical/hippocampal excitatory neurons were targeted. These findings indicate that Shank3 may form a scaffolding complex with Homer1b/c and metabotropic glutamate receptors in striatum with the exclusion of these functional interactions in other brain regions despite the abundance of these proteins in brain.

Hippocampal recordings from NEX-Shank3 mice revealed an unexpected increase in NMDAR-mediated currents without alterations in AMPAR-currents. Intriguingly, this enhancement in both function and NMDAR proteins is contrary to what has been reported in various lines of global isoform-specific Shank3 mice which show reduced NMDAR function and/or proteins.34,35,37,39,42,43 Our electrophysiological results in the NEX-Shank3 mice are consistent with the increase in NR1 and NR2B proteins in the hippocampus of these mutants; however, levels of these proteins are unchanged in the hippocampi of global Shank3 Δe4−22 mice. This disparate pattern in protein levels and function of NMDAR-associated components between the NEX-Shank3 and global Shank3 Δe4−22 mice suggests that in the former model Shank3 may modulate inhibitory inputs from some brain regions that affect hippocampal function. It should be emphasized that upregulation of NMDAR subunits has been observed also when Shank3 is selectively knocked down in developing hippocampal neurons.77 Hence, these collective results
indicate that selective alterations in Shank3 expression in
different neuronal subtypes can exert biochemical and
electrophysiological changes that may not be reflected in
the global loss of this protein. In the future, more specific
molecular and neural circuit studies may provide novel
insights into their phenomenon.

In summary, our study of the first Shank3 region-
specific conditional mice have yielded several new insights
into the function of this gene, as well as, illustrated the
complexity of dissecting neural circuit mechanisms of
behavior. From parallel analyses of multiple lines of
conditional mice, we demonstrate that certain behavioral
phenotypes in the global Shank3 Δe4–22 mice can be
attributed to brain-region or cell-type specificity. Intriguingly,
selective targeting of Shank3 in different brain
regions or cells leads to distinct alterations in the inter-
actions between Shank3 and other proteins in the PSD;
these interactions appear to have functional con-
sequences. However, interpretation of our findings may
be complicated by a lack of complete knowledge of the
complexity of Shank3 mRNAs and protein isoforms in
different cell types and during development. The pre-
sentation of abnormal behaviors was not always con-
gruent between the global and the conditional Shank3 Δe4
−22 mice. Some of these distinctions may be attributed to
the differences that Shank3 may play in the neural circuits
subserving certain behaviors. Together, our results
emphasize a need to examine in greater detail the separate
and collective roles that different brain regions exert in
the expression of Shank3-mediated behaviors. While our
study has not identified the brain regions or specific cell
types mediating all responses affected by Shank3 deletion,
it has provided new insights into the neural circuits
responsible for ASD-associated stereotyped and repetitive
behaviors and it has established a foundation for
mechanistic studies to understand how loss of Shank3
leads to synaptic and cellular dysfunctions associated with
these behavioral phenotypes. Further study to elucidate
the complete transcript structure and cell type specific
expression of Shank3 isoforms in brain during the
development is clearly warranted in future.

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electrophysiology. X.C. assisted with generating the mice. A.L.B., X.W., H.D.Y., N.
K., R.L.P., and R.M.R. did the statistical analyses. A.L.B., X.W., W.C.W., and Y.H.J.
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Conflict of interest
The authors declare that they have no conflict of interest.

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