

Review

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# Neuropathological insights from SHANK3 mutant animal models

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## Abstract

SHANK3 is a protein primarily found in the postsynaptic density (PSD) of excitatory synapses in the brain. Heterozygous mutations in the *shank3* gene have been linked to autism spectrum disorder (ASD) and intellectual disability. There are various animal models carrying mutant SHANK3 that have provided valuable insights into the pathogenesis of ASD. In this review, we will discuss these animal models, with a specific focus on the neuropathology observed in *shank3* mouse and monkey models. These models are particularly important as they share closer similarities to humans and are capable of more accurately recapitulating the neuropathological features observed in individuals with ASD. Mice with mutations in the *shank3* gene exhibit deficits in social behavior, communication, and repetitive behaviors, which are core features of ASD and support the link between SHANK3 and ASD. However, studies of monkey models with SHANK3 targeting by CRISPR/Cas9 have demonstrated that, unlike mice with completely knocked-out *shank3* genes, the monkey model with complete deletion of SHANK3 displays a reduction in the number of neuronal cells. This review discusses the species-specific neuropathology in SHANK3/*shank3* knockout mice and monkeys. The differences in neuropathology in SHANK3/*shank3* mutant mouse and monkey models suggest that non-human primate models are highly valuable for investigating the mechanism of neurodegeneration that may selectively occur in primate brains.

**Keywords:** CRISPR/Cas9, animal models, SHANK3, neurodegeneration



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## INTRODUCTION

The SH3 and multiple ankyrin repeat domains 3 (SHANK3) is a scaffold protein primarily expressed in the postsynaptic density of excitatory synapses in the brain. The *shank3* gene is located on mouse chromosome 15E3, rat chromosome 7q34, and human chromosome 22q13.3<sup>[1]</sup>. It consists of 22 exons that encode an N-terminal ankyrin repeat domain (ANK), Src homology domains (SH3), postsynaptic density-95/discs large/zone occludens-1 (PDZ), a proline-rich region including homer and cortactin binding sites (PRO), and a sterile alpha motif (SAM). Therefore, SHANK3 interacts with a number of synaptic proteins to delicately regulate synaptic function. For example, the ANK domain in SHANK3 binds directly to fodrin during spine and synapse formation and remodeling<sup>[2]</sup>. SH3 also interacts with CaMKII and is involved in Wnt signaling and dendritic spine remodeling<sup>[3]</sup>. Other domains, such as PDZ, PRO, and SAM, in SHANK3 are also involved in synaptic transmission and plasticity, the actin-based cytoskeleton, and synaptic targeting, suggesting that SHANK3 regulates synaptic function via its multiple domains<sup>[4-8]</sup>.

The expression of different isoforms in SHANK3 appears to be driven by various promoters in the *SHANK3* gene, which contains at least six intragenic promoters in humans and rodents and produces various types of SHANK3 transcripts and several promoter-specific isoforms (SHANK3a-f)<sup>[9-12]</sup>. These different isoforms have distinct structural domains and perform different biological functions. In the brain, these isoforms show significant temporal and regional differences. SHANK3a is expressed in the cortex, striatum, and hippocampus during the early developmental stages of synaptogenesis, whereas the SHANK3c/d isoform is predominantly presented in the cerebellum and SHANK3e is weakly expressed in all brain regions<sup>[13-15]</sup>. Interestingly, human SHANK3 mRNA is also highly expressed in the heart, with moderate expression levels in the brain and spleen<sup>[9,16,17]</sup>. Moreover, the expression of SHANK3 is characterized by selective isoform compensation<sup>[18]</sup>. Therefore, it remains to be elucidated how the isoform-specific expression of different SHANK3 isoforms is regulated.

In the mouse brain, SHANK3 protein is predominantly found in the postsynaptic density of excitatory synapses, where it has been widely reported to function in the formation of dendritic spines, synaptic transmission and plasticity, and cytoskeleton regulation<sup>[3,5-7,10,16-19]</sup>. In addition to the major role of *shank3* in regulating synaptic formation and function, the expression of SHANK3 in cells of the oligodendrocyte lineage plays a crucial role in the regulation of myelinating cell maturation<sup>[20]</sup>. Additionally, SHANK3 is expressed in multiple tissues, including skeletal muscle and intestinal epithelial cells, where it is involved in the regulation of barrier function<sup>[12,21-23]</sup>. Although SHANK3 is also found in the nucleus and cytoplasm<sup>[14,24]</sup>, the precise biological roles of SHANK3 in these subcellular compartments remain inadequately characterized. Future investigations of the expression patterns of various SHANK3 isoforms across diverse tissues and subcellular organelles, as well as their corresponding functional implications, will be pivotal in elucidating the function of SHANK3 and its involvement in ASD.

## SHANK3 MUTATIONS AND ASD

ASD is a neurodevelopmental condition that is clinically heterogeneous and highly heritable. Its main clinical features include impaired social interaction and repetitive behaviors. Heterozygous mutations in the *SHANK3* gene have been found to be closely associated with ASD, with truncating mutations in the *SHANK3* gene family accounting for approximately 1% of autism cases<sup>[25]</sup>. Phelan-McDermid Syndrome (PMDS) is the first reported neurodevelopmental disorder associated with heterozygous SHANK3 mutations. It is characterized by global developmental delay, absent or delayed speech, dysmorphic features, hypotonia, and ASD. PMDS is linked to SHANK3 mutations (22q13.3 deletions) that result in SHANK3 haploinsufficiency. While SHANK3 haploinsufficiency resulting from point mutations is sufficient to produce the extensive phenotypic characteristics linked to PMDS, genotype-phenotype correlation analysis has demonstrated that

the magnitude of the deletion is positively associated with the severity of clinical manifestations<sup>[17,26-28]</sup>. Additionally, individuals with smaller terminal deletions may exhibit more favorable developmental trajectories compared to those with larger deletions. The size of SHANK3 deletion fragments also correlates with disease severity.

Similarly, a cohort study conducted on a Brazilian population revealed a positive correlation between the size of SHANK3 deletion and the severity of renal abnormalities, lymphedema, and language impairment in PMDS patients<sup>[29]</sup>. Another cohort study from a Chinese population reported additional significant phenotypes, including heightened pain tolerance, impulsivity, repetitive behaviors, regression, and incessant crying<sup>[30]</sup>. Furthermore, imaging techniques have been extensively utilized to identify specific characteristics and structural brain abnormalities in ASD patients<sup>[31]</sup>. From a pathological perspective, young children diagnosed with ASD exhibit a notable decrease in neuronal and cytoplasmic volumes in the majority of regions examined compared to controls of the same age<sup>[32]</sup>. Conversely, a separate study revealed a 67% increase in the number of neurons in the entire prefrontal cortex (PFC) among male individuals with autism<sup>[33]</sup>. However, this datum is estimated to be the volume of reference for the regions of interest in order to derive total cell counts. It is important to exercise caution in interpreting these findings. The pathological and imaging reports of individuals with ASD commonly exhibit limitations such as small sample sizes and variations in the statistical methodologies employed. Therefore, it is crucial to establish a suitable animal model with SHANK3 mutations in order to effectively simulate ASD and investigate its pathogenesis.

## SMALL ANIMAL MODELS WITH SHANK3 MUTATIONS

Various small animal models carrying *shank/shank3* mutations, such as drosophila, zebrafish, mice, and rats, have been generated to extensively investigate the molecular phenotypes related to *shank3* mutations. Due to their high efficiency in genetic manipulations and rapid generation, drosophila and zebrafish have proven to be valuable tools for investigating the function of *shank3*. In one study, *shank* null drosophila models were found to be fully viable and fertile, showing no apparent morphological or developmental defects. However, these models did exhibit an abnormal structure of the central mushroom body calyx and reduced olfactory acuity<sup>[34]</sup>. Another *shank* null drosophila model demonstrated defects in synaptic bouton number and maturation<sup>[35]</sup>.

Similar synaptic defects related to *shank3* deficiency were observed in *shank3* null zebrafish models. The adult brains of *shank3b* deficient zebrafish showed significantly reduced levels of both postsynaptic homer1 and presynaptic synaptophysin. These reductions were accompanied by a decrease in locomotor activity, impaired social interaction, and significant repetitive swimming behaviors<sup>[36]</sup>. Another *shank3b* mutant zebrafish model also exhibited reductions in synaptic proteins and locomotor activity<sup>[37]</sup>. Furthermore, reduced sensory responsiveness in *shank3ab*<sup>-/-</sup> zebrafish was associated with decreased activity in sensory processing brain regions<sup>[38]</sup>. In zebrafish, CRISPR-Cas9-mediated mutations in the *shank3a* gene led to the downregulated expression of neuroligins, which are crucial for synaptogenesis. This downregulation could contribute to deficits in attention<sup>[39]</sup>.

It is important to acknowledge that zebrafish and drosophila are evolutionarily distant from humans; the expression of *shank3* in these small animals is distinct from that in mammals. For example, the zebrafish gene corresponding to human SHANK3 exists as two duplicates. A number of mouse models carrying *shank3* mutations have been established and extensively investigated. The mouse model with exon 21 deletion displays a reduced N-methyl-D-aspartate/alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (NMDA/AMPA) ratio in the hippocampal synapses, resulting in a significant rise in mGluR5 localization to synaptosomes, motor-coordination deficits, abnormal behaviors, and social interaction<sup>[40]</sup>.

However, the phenotypes of *shank3* mutant mice appear to be complex. Mice with *shank3* mutations in exon 9 did not display autistic behaviors, although they exhibited a reduction in the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in pyramidal neurons of the medial prefrontal cortex (mPFC), which contrasts with the observed increase in mIPSC frequency in the hippocampus<sup>[41]</sup>. The InsG3680 mutation in mice is associated with early-onset striatal synaptic transmission defects and impaired social interaction, as well as distinct alterations in synaptic transmission that were also seen in the prefrontal cortex of R1117X mutant mice<sup>[42]</sup>. Additionally, there is a notable alteration in the composition of PSD in adult R1117X and InsG3680 mutant mice. InsG3680 mutant mice exhibit a significant deficit in social interaction at an early disease stage compared with R1117X mutant mice<sup>[42]</sup>. The *shank3G* mouse model demonstrates deficiencies in motor learning and coordination, while exhibiting typical grooming behavior and an aversion to inanimate objects. However, social interaction deficits and anxiety-like behaviors are not observed in this model, although synaptic transmission and plasticity are impaired in these mouse models<sup>[43]</sup>. Mice with  $\Delta e4-22^{-/-}$  (deletions of exons 4-22) exhibited a deficiency of *shank3* that can hinder the metabotropic glutamate receptor 5 (mGluR5) - Homer scaffolding, leading to abnormalities in the cortico-striatal circuitry that are responsible for learning deficits and the manifestation of core behavioral characteristics associated with ASD<sup>[44]</sup>. The deficiency of *shank3* exon 9 in rats results in impaired long-term social memory and attention deficits. Consistently, synaptic plasticity deficits are observed in these rats, from the hippocampus to the prefrontal cortex<sup>[45]</sup>. In line with the critical role of *shank3* in synaptic function, *shank3*-deficient rats (exons 11-21) exhibited a decrease in spine density and alterations in synaptic proteins<sup>[46]</sup>. However, these mutant rats display normal social interaction behavior, despite impaired social memory, as well as learning and memory deficits in this rat model<sup>[46]</sup>.

Rodent models with *shank3* mutations provide a valuable tool for understanding the function of *shank3* and its role in ASD. For instance, these models have demonstrated that mutations or deletions of the *shank3* gene affect synaptic signal transmission and synaptic protein expression in various brain regions, such as the striatum, hippocampus, and prefrontal cortex. However, one notable finding is that unlike the severe clinical symptoms observed in humans with SHANK3 haploinsufficiency, *shank3<sup>+/-</sup>* mice show no or only very mild phenotypes. The abnormal behavioral phenotypes in mice are almost exclusively found in homozygous mutants<sup>[44,47,48]</sup>. Additionally, the inconsistent behavioral phenotypes of rodent models with *shank3* mutations suggest that mutations in different exons of the *shank3* gene and the nature of mutations may account for variations in behavioral phenotypes [Table 1]. Despite the closer phylogenetic proximity of rodents, such as mice and rats, to humans compared to fruit flies and zebrafish, significant disparities persist in terms of rodent brain development, neural circuitry, and anatomy compared to the human brain. As a result, several phenotypes associated with ASD display inconsistencies or contradictions across different mouse or rat models. Additionally, it is important to recognize the potential impact of diverse genetic backgrounds within the same species on the ASD-like phenotype triggered by *shank3* mutations. Therefore, there is a pressing need for more extensive research into the intricate pathogenic mechanisms that underlie the neurodevelopmental impairment associated with ASD, which may be unraveled by using non-human primate models that are closer to humans than small animals.

### Monkey models with SHANK3 mutations

Macaca monkeys possess high-order cognitive and social functions thanks to their well-developed neocortex. They are closer to humans in terms of their specialized brain function and structures than rodents<sup>[47,53,54]</sup>. The value of using primate models to study ASD has been supported by the generation of monkeys with altered expressions of MECP2 using lentivirus<sup>[55]</sup> and TALEN<sup>[56]</sup>-based methods. However, a SHANK3-edited monkey model was not reported until 2017. To date, two monkey models of SHANK3 mutants have been established using CRISPR/Cas9-mediated gene-editing technology in cynomolgus monkeys<sup>[51,52]</sup>. Despite the limited number of SHANK3 mutant monkeys generated, they provide important

**Table 1. SHANK3 mutant animal models**

Species/Background	Modifications approach	Targeted exons and isoforms	Molecular phenotype	Behavioral phenotype	Ref.
Drosophila/ <i>mef2-Gal4</i>	KO	- Minos transposable element ( <i>shank</i> <sup>D101</sup> homozygotes) - Loss of 97% coding region of the <i>shank</i> gene	- Glutamate receptors and active zones are not affected - A defect in postsynaptic development - Downregulation of Wnt signaling pathway - Defective NMJ bouton formation and maturation	- No reported	[35]
Drosophila/ <i>W<sup>1118</sup></i>	KO	- Exons4-12 ( <i>shank</i> <sup>BK</sup> ) - Exon10 ( <i>shank</i> <sup>T49</sup> ) - Exon5 ( <i>shank</i> <sup>138</sup> ) - Loss of all known SHANK protein isoforms	- Developmental defects of synapse in the calyx - Ultrastructural defects of synaptic boutons in the adult calyx - Normal NMJ development	- Reduced motor ability and defective olfactory acuity	[34]
Zebrafish/ <i>Tu</i>	KO	- Exon2 (ANK)/homozygous - Loss of <i>shank3b</i> - Exon9 (SH3, PDZ, SAM)/homozygous - Loss of <i>shank3a</i>	- Reduced protein expression of NeuN, homer1, and synaptophysin	- Reduced alertness or reduced danger awareness - Impaired social interactions and repetitive and stereotyped behaviors	[37]
Zebrafish/The Gulbenkian Institute of Science	KO	- Exon2 (ANK, SH3, PDZ, SAM)/homozygous - Loss of <i>shank3a</i>	- Downregulation of neuroligin gene expression - Upregulation of <i>BDNF</i> and <i>NeuroD</i> gene expression	- Social contagion effects and attention-recognition deficits	[39]
Zebrafish/The University of Miami zebrafish core facility	KO	- <i>shank3abΔN</i> <sup>-/-</sup> (ANK)/homozygous - Loss of <i>shank3a/b</i> - <i>shank3abΔC</i> <sup>-/-</sup> (near the PDZ)/homozygous - Loss of <i>shank3a/b</i>	- The absence of SHANK3 puncta is observed in the cerebellum and along the ventral neural tracts of the brainstem, while PSD-95 synaptic puncta remain unaffected	- Downstream brain regions fail to integrate and respond to dark transitions in larvae, both <i>shank3abΔN</i> <sup>-/-</sup> and <i>shank3abΔC</i> <sup>-/-</sup>	[38]
Zebrafish/ <i>Tu</i>	KO	- Exon2 (ANK, SH3, PDZ)/homozygous - Loss of <i>shank3b</i>	- Reduced homer1 and synaptophysin protein levels in the adult zebrafish brain	- Impaired locomotor activity - Abnormal repetitive movements and impaired social preference behaviors in the adult zebrafish	[36]
Mouse/Bruce4 <i>C57BL/6</i>	KO	- Exons4-9 (ANK)/heterozygous - Loss of <i>shank3a</i> and <i>shank3b</i>	- Reductions in glutamatergic synaptic transmission and plasticity - A reduced number of GluR1 immunoreactive puncta in the striatum radiatum	- Social interaction and social communication deficits	[49]
Mouse/ <i>C57BL/6J</i>	KO	- Exons4-9 (ANK)/homozygous - Loss of <i>shank3a</i> and <i>shank3b</i>	- Altered dendritic spine morphology - Impaired synaptic plasticity - Impaired activity-dependent GluA1 redistribution - Intact basal transmission function	- Abnormal social behavior - Aberrant motor behaviors and ultrasonic vocalizations - Repetitive behaviors - Deficient learning and memory	[10]
Mouse/ <i>C57</i> (Jackson)	KO	- Exons4-7 (ANK)/homozygous - Loss of <i>shank3a</i>	- Defects at striatal synapses and cortico-striatal circuits	- No lesions or anxiety-like behavior	[50]
Mouse/ <i>C57</i> (Jackson)	KO	- Exons13-16 (PDZ)/homozygous	- Reduced spine density, PSD length and thickness	- Anxiety-like behavior and excessive - Self-injurious grooming	[50]

		- Loss of <i>shank3a</i> and <i>shank3b</i>		- Dysfunctional social interaction behavior	
Mouse/C57BL/6	KO	- Exon 21 (PRO)/homozygous - Loss SHANK3 protein (> 150 Kd)	- Neuronal morphology appeared unaltered - No differences in spine density and shape - Impaired long-term potentiation	- Impaired spatial learning, poor motor coordination, avoidance towards inanimate objects, and excessive self-grooming in advanced adulthood - No alterations in ultrasonic vocalizations	[40]
Mouse/C57BL/6N	KO	- Exon 9 (ANK)/heterozygous - Loss of <i>shank3a</i>	- Reduced excitatory transmission and increased mIPSC frequency in hippocampus - Decreased mIPSC frequency but normal mEPSCs in mPFC	- Not autistic-like behavior - Increased rearing in a novel environment - Mildly impaired spatial memory	[41]
Mouse/C57BL/6J	KO	-Exon 21 (PRO)/homozygous -Loss of SHANK3 isoforms (> 150 Kd)	-No change in glutamate receptor or Homer1b/c expression in whole <i>shank3<sup>G6</sup></i> mice hippocampal lysates -Decreased phosphorylation of the GluN2B Tyr1472 site	-Impaired motor learning and coordination -An avoidance phenotype towards inanimate objects -Aberrant locomotor activity in response to novelty -Not express an anxiety-like phenotype -Minimal spatial learning differences -Not display social interaction deficits -Normal grooming	[43]
Mouse/C57BL6/S129sv	KO	- Exon 21 (PRO)/homozygous - Expressed SHANK3 (122 kDa) - R1117X mutation	- No change of mEPSC frequency and AMPA to NMDA current ratio - Reduced Homer protein, PSD93, SynGAP - Reduced NMDA receptor subunits - Not see a trend of upregulation of any of the synaptic proteins tested - Reduction of mEPSC amplitude - Reduction of the spine density layer 2/3 pyramidal neurons in the frontal association area	- Social interaction deficit, but it did not reach statistical significance	[42]
Mouse/C57BL6/S129s	KO	- Exon 21 (PRO)/homozygous - An almost complete loss of SHANK3 protein - InsG3680 mutation	- No change of mEPSC and AMPA to NMDA current ratio frequency - Increase of mEPSC amplitude - Reduced Homer protein, GluR1, SynGAP, SHANK2, and NMDA receptor subunits - No significant differences in either frequency or amplitude of mEPSC - Reduction of the spine density of layer 2/3 pyramidal neurons in the frontal association area	- An early-onset social interaction deficit	[42]
Mouse/C57BL/6J	KO	- Exons4-22 (ANK, PDZ, PRO)/homozygous - Loss of all known SHANK3 protein isoforms	- NAC firing deficit - Abnormalities in brain structure - Dysfunctional striatal synapses	- Core behavioral features of ASDs - Intact fear learning - Mildly perturbed hippocampal spatial memory - Severely impaired striatal learning - Abnormal ultrasonic vocalizations	[44]
Rat/Sprague Dawley	KO	- Exon 6 (ANK)/heterozygous - Loss of <i>shank3a</i>	- Induced LTP	- Impairs long-term social memory - Normal social interaction - Attention deficits	[45]
Rat/Charles River Laboratories	KO	- Exons11-21 (PDZ, SH3, PRO)/homozygous - Loss of all known SHANK3 protein isoforms	- Reduced spine density, PSD-95 - Reduced Homer and NR1 in homogenates from the striatum - Increased PSD-95 in the hippocampal PSD fraction of heterozygous rats	- Normal social interaction behavior - Impaired social memory and learning memory - Increased anxiety behavior and pain threshold - Self-grooming behavior and skin lesions	[46]

Monkey/Cynomolgus monkeys	KO	- Exons 6-12 (ANK, PDZ)/mosaic - All known SHANK3 protein isoforms reduced	- NR1 increased in the hippocampal homogenate fraction of heterozygous rats - Loss of neuronal cells - Increase of GFAP <sup>+</sup> astrocytes - Altered expressions of postsynaptic receptors and scaffold proteins	- Impaired social interaction and apparent stereotypical locomotion [51] - Delayed vocalization - No obvious structural abnormality - Lower glucose metabolism - Fluoxetine can alleviate the behavioral deficits
Monkey/Cynomolgus monkeys	KO	- Exon 21 (PRO)/FO: mosaic; F1: heterozygous - All known SHANK3 protein isoforms reduced	Not reported	- Reduced overall sleep efficiency and muscle strength [52] - Increased repetitive behaviors - Reduced exploration and social interaction - Fewer vocalizations - Decreased grey matter - Abnormal functional connectivity

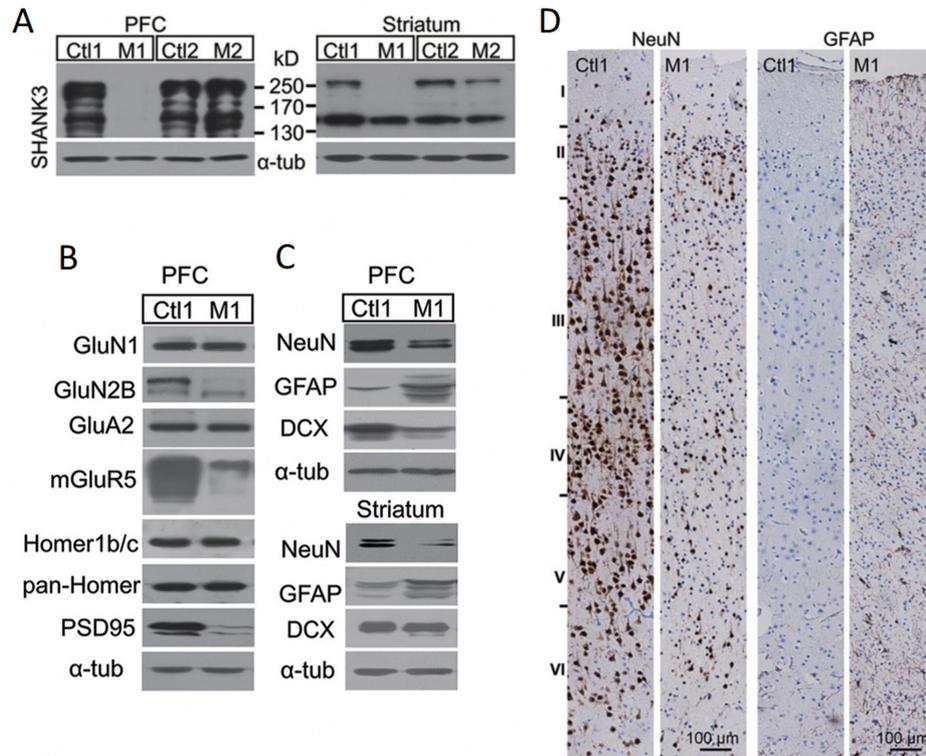
AMPA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANK: N-terminal ankyrin repeat domain; ASD: autism spectrum disorder; BDNF: brain-derived neurotrophic factor; KO: knock-out; LTP: long-term potentiation; mEPSC: miniature excitatory postsynaptic current; mIPSC: miniature inhibitory postsynaptic current; mPFC: medial prefrontal cortex; NAC: nucleus accumbens; NeuN: neuronal nuclei; NMDA: N-methyl-D-aspartate; NMJ: neuromuscular junction; NR1: NMDA receptor1; PDZ: postsynaptic density-95/discs large/zone occludens-1; PRO: a proline-rich region including homer and cortactin binding sites; PSD: postsynaptic density; SAM: sterile alpha motif; SH3: Src homology domains; SHANK3: SH3 and multiple ankyrin repeat domains 3.

insights into the function of SHANK3 and its role in ASD, which deserves a thorough discussion below.

### Targeted exons 6 and 12 of the monkey *SHANK3* gene

In 2017, Zhao *et al.* generated the first SHANK3-edited monkey<sup>[51]</sup>. Since a large deletion of *shank3* in the mouse gene did not yield obvious phenotypes and pathological changes, Zhao *et al.* aimed to establish a monkey model with a large SHANK3 gene deletion<sup>[51]</sup>. They targeted two sites (exons 6 and 12) of the monkey SHANK3 gene, which could completely disrupt all three longest SHANK3 isoforms in the fertilized eggs of cynomolgus monkeys. Although three SHANK3 mutant monkeys were obtained, two (M1, M2) died before or right after birth, and one (M3) survived. A higher-than-expected embryonic and perinatal lethality associated with SHANK3 targeting was observed, suggesting that SHANK3 is important for early development in primates. Analysis of SHANK3-targeted monkeys revealed that the M1 monkey brains showed almost complete deletion of SHANK3 protein, accompanied by a dramatic reduction in many postsynaptic receptors and scaffold proteins, such as GluN2B/mGluR5/PSD95. Meanwhile, the SHANK3-deficient monkey showed a significant loss of NeuN<sup>+</sup> neurons co-exhibited by an increase of GFAP<sup>+</sup> astrocytes [Figure 1]. These findings provide strong evidence for neuronal loss resulting from SHANK3 depletion, which has not been reported in any line of *shank3*-knockout mice. This demonstrates that SHANK3 plays a unique and critical role in early brain development in primate brains.

For the living SHANK3-targeted monkey (M3), a longitudinal and repeated investigation over a 2-year period had been performed<sup>[57]</sup>. This investigation revealed that the SHANK3-targeted monkey developed the core features of behavioral phenotypes of ASD, including impaired social interaction, apparent stereotypical locomotion, and delayed vocalization<sup>[57]</sup>. Although the exact meaning of delayed vocalization is not clear, the defect in vocalization may resemble



**Figure 1.** CRISPR/Cas9-mediated SHANK3 deletion results in neuronal loss in the monkey brain. (A) Western blot analysis of SHANK3 protein in the PFC and striatum of SHANK3-mutant monkeys. Ctl1 and Ctl2 are age-matched controls at gestational day 135 and full-term gestation, respectively; (B) Altered expressions of postsynaptic receptors and scaffold proteins in SHANK3M1 brain. Normal expressions of GluN1, GluA2, Homer1b/c, and pan-Homer but decreased expressions of GluN2B, mGluR5, and PSD95 were observed in the PFC of SHANK3M1; (C) Western blot analysis of NeuN, GFAP, and DCX in whole-cell lysates from PFC. SHANK3M1 showed decreased levels of NeuN and DCX but increased levels of GFAP in the PFC. Western blot analysis was repeated at least three times independently; (D) NeuN and GFAP staining (brown) in layers I-VI of PFC. NeuN staining showed a strong signal in the nucleus and a weak signal in the cytosol. Nuclei were stained with hematoxylin (blue). There were fewer NeuN+ neurons and more GFAP+ astrocytes in the PFC of SHANK3M1. Scale bar, 100  $\mu$ m<sup>[51]</sup>. DCX: Doublecortin; GFAP: glial fibrillary acidic protein; NeuN: neuronal nuclei; PFC: prefrontal cortex; PSD: postsynaptic density; SHANK3: SH3 and multiple ankyrin repeat domains 3.

delayed speech in many young patients with SHANK3 mutations. To investigate possible structural and functional changes, the authors conducted positron emission computed tomography (PET) and magnetic resonance imaging (MRI) in mutant and control monkeys. Morphological T1-weighted 3-dimensional MRI demonstrated that there were no obvious structural abnormalities in SHANK3-mutant monkeys. However, the <sup>18</sup>F-FDG-PET study showed that glucose metabolism in SHANK3-mutant brain tissues was significantly lower than in control brains. This phenotype is consistent with the lower glucose metabolic activity in the brains of some ASD patients<sup>[58,59]</sup>. To explore the therapeutic potential, they treated SHANK3-mutant monkeys with fluoxetine, a medication for major depression and obsessive-compulsive disorder. After treatment for 2 weeks, fluoxetine markedly alleviated repetitive behaviors, significantly increased active social interaction duration and frequency, and improved brain glucose metabolism in SHANK3-targeted monkeys<sup>[57]</sup>.

#### Targeted exons 21 of the monkey SHANK3 gene

Another SHANK3-targeted monkey model was generated to target exon 21 only<sup>[52]</sup> to create SHANK3 mutations similar to the InsG3680 mutation found in humans<sup>[60]</sup>. The authors not only conducted comprehensive behavioral experiments to analyze this monkey model but also obtained F1-generation

heterozygous SHANK3-mutant monkeys to ensure germline transmission of the SHANK3 mutations<sup>[52]</sup>. The behavioral assessment scores showed that overall sleep efficiency and muscle strength were reduced in SHANK3-mutant monkeys. However, the stereotyped or repetitive behaviors (such as licking fingers and cage bars) revealed a substantial increase compared to controls. Meanwhile, SHANK3-mutant monkeys displayed reduced levels of exploration, social interaction, and vocalization, which parallels some of the phenotypes found in children with ASD or PMDS<sup>[61,62]</sup>. Contrary to the previous SHANK3-targeted monkey model<sup>[57]</sup>, structural MRI analysis of the new SHANK3-mutant monkeys revealed that the volume of gray matter had decreased. In addition, reduced global connectivity and greater local connectivity were also observed in the new SHANK3 mutant monkeys.

Although there are small differences in behavioral phenotypes between the two SHANK3-targeted monkey models, most of the behavioral alterations in SHANK3-targeted monkeys were similar to the clinical characteristics of autism patients. However, unlike monkeys with a large SHANK3 gene deletion<sup>[51]</sup>, the monkeys with exon 21 targeting were not reported to have neuronal loss<sup>[52]</sup>. Thus, it is likely that complete loss of SHANK3 can more severely affect neuronal survival and development, whereas partial loss of SHANK3 or depletion of some isoforms of SHANK3 may lead to haploinsufficiency as seen in heterozygous SHANK3 mutations in humans.

Behavioral phenotypes of SHANK3 mutant monkey models highlight the complex regulation of SHANK3 expression and its multifaceted functions in large animals. Thus, large animals with SHANK3 mutations may provide unique pathogenesis insights that have not been identified in small animals. Indeed, dog models with SHANK3 mutations were also created and provided some interesting findings. The electrocorticograms (ECoG) revealed that SHANK3 mutant dogs responded to sound stimulation more intensely and were more sensitive to frequencies (1-4 kHz) that are sensitive to the human auditory system<sup>[63]</sup>. Stem cells from human exfoliated deciduous teeth transplantation improved social novelty preference, reduced social stress, and decreased serum IF- $\gamma$  levels in heterozygous SHANK3 mutant beagle dogs<sup>[64]</sup>. Considering that non-human primates are closer to humans than other animals, SHANK3-mutant monkeys would be a more faithful animal model for investigating the pathogenesis of human ASD.

## SPECIES-SPECIFIC NEURODEGENERATION

In heterozygous *shank3*<sup>+/-</sup> knockout mice, there are no or only very mild phenotypes, which is in contrast to the more severe clinical symptoms observed in patients with SHANK3 heterozygous mutations. Abnormal behavioral phenotypes in mice are almost exclusively found in homozygous mutants<sup>[44,47,48]</sup>. However, homozygous SHANK3 mutations have not been reported in humans, possibly because such mutations are embryonic lethal or can severely affect early brain development. In support of this idea, a large deletion of the monkey SHANK3 gene can cause remarkable neuronal loss<sup>[51]</sup>. The absence of such neuronal loss in complete *shank3* knockout mice clearly indicates that species-dependent differences are important for the development of neurodegeneration seen in humans.

Analogous to other neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases, genetically modified mouse models fail to recapitulate the overt and selective neurodegeneration observed in patient brains<sup>[65]</sup>. For example, homozygous mutations in PINK1, a kinase that is thought to be involved in mitophagy, can cause neurodegeneration in Parkinson's disease via the loss of function mechanism. However, the complete loss of Pink1 in knockout mice does not lead to neurodegeneration or obvious phenotypes<sup>[66-68]</sup>. In contrast, CRISPR/Cas9-mediated deletion of the *PINK1* gene in monkeys results in severe neurodegeneration<sup>[69]</sup>. Furthermore, this primate-specific neurodegeneration caused by PINK1 deficiency is due to reduced protein phosphorylation rather than mitochondrial homeostasis defects<sup>[65]</sup>.

Thus, non-human primate models may enable us to identify the pathogenesis that occurs in the human brain but is hardly replicated in small animals.

The identification of neuronal loss in SHANK3 mutant monkeys also highlights the important function of SHANK3 for neuronal survival during early brain development. It is highly likely that in primate brains, SHANK3 is required for neuronal differentiation and maturation, such that the complete loss of SHANK3 would affect the survival of neurons. However, further studies are needed to pinpoint the mechanisms of neuronal loss in SHANK3-deficient primates. On the other hand, partial deficiency in the expression or function of SHANK3 due to heterozygous mutations or deficiency in some isoforms is more likely to affect synaptic function and neuronal circuitry, leading to autistic phenotypes without neurodegeneration. Given that the expression of SHANK3 is driven by multiple promoters to yield various isoforms, the use of non-human primates would allow for investigating the relationship between neuronal loss and different isoforms. Such studies would provide new insight into the function of SHANK3 and a better understanding of how SHANK3 mutations alter behaviors to cause ASD.

It is important to note that in Zhao *et al.*'s report, only one SHANK3-targeted monkey exhibited almost complete deletion of the *SHANK3* gene and severe neuronal loss<sup>[51]</sup>. The authors did not find any evidence suggesting that the CRISPR/Cas9 targeting used in their study resulted in off-target events responsible for such severe neurodegeneration. While the phenotypes observed in this unique SHANK3 mutant monkey support the notion that complete loss of SHANK3 can lead to severe neuronal loss, further studies utilizing additional SHANK3 mutant monkeys are necessary to validate this concept. Additionally, the neuronal loss observed in the newborn SHANK3 mutant monkey could be attributed to neurodegeneration, impairment of neurogenesis or neuronal differentiation, which warrants further investigation. A recent study demonstrated that SHANK3<sup>+/-</sup> brain organoids are significantly smaller and contain fewer neurons compared to wild-type controls<sup>[70]</sup>. Thus, an alternative model for investigating the crucial role of SHANK3 in neuronal development could involve utilizing human brain organoids with CRISPR targeting SHANK3<sup>-/-</sup>.

## THE LIMITATIONS OF THIS ARTICLE

This article presents a comprehensive analysis of various animal models exhibiting shank3 mutations, including zebrafish, Drosophila, mice, rats, dogs, and monkeys, with a focus on molecular mechanisms, behavior, and neuropathology. However, it lacks in-depth exploration regarding specific biological distinctions, such as variations in the manifestation of stereotyped behaviors. The discussion is primarily exploratory, attributing these variances to the diversity within the autism spectrum, disparities in animal model targeting strategies, species distinctions, and other contributing factors. From a clinical perspective, there is a dearth of publicly available data concerning autistic individuals with SHANK3 mutations, thus limiting this article to discussions centered around existing animal models. The primary emphasis lies on the neuropathology caused by SHANK3 deletion, an occurrence observed exclusively in primates but constrained by sample size. This finding underscores the need for future validation, necessitating further research to unravel the neuropathological mechanism of autism.

## DISCUSSION

The utilization of large animal models in research is hindered by high costs, lengthy breeding cycles, and operational complexities, resulting in small sample sizes. Nonetheless, large animals, particularly non-human primates, offer distinct advantages in studying brain structure and nerve cell development, enabling researchers to mimic human neuropathies.

Due to the scarcity of neuropathological studies on autism patients and the heterogeneity of autism itself, animal models of autism face significant challenges. Shank3 is among the numerous genes associated with autism. A number of small animal models have demonstrated that SHANK3 is linked to autism-like behaviors and have elucidated its role in synapse formation. However, synaptic dysfunction alone may not adequately explain the complex pathogenic mechanism of SHANK3 mutations. The observation that the onset heterozygosity observed in patients differs from the partial homozygosity seen in small animal models underscores the significance of utilizing non-human primates to investigate the mechanisms of ASD.

It is important to consider the various promoters and forms of the shank3 gene and protein when selecting antibodies and interpreting results. Further research is necessary to understand how different forms of Shank3 contribute to autism and their roles in nerve cells and organs at different developmental stages, which will require validation through animal models. Despite their limited sample sizes, non-human primate models with the deletion of the Shank3 gene display neuronal loss not observed in small animal models. Non-human primates can spontaneously develop Alzheimer's disease (AD) and Parkinson's disease (PD), displaying unique neuronal loss patterns in their models. However, despite advancements, non-human primate models for ASD remain underutilized. Additional research is imperative to confirm and supplement existing data due to the scarcity of animal studies. Specifically, further investigation is needed to understand the mechanisms underlying neuronal loss in non-human primate models lacking Shank3. Addressing this inquiry will not only illuminate the novel functionalities of SHANK3, but also aid in advancing clinical investigations pertaining to individuals with Shank3 mutations.

## **DECLARATIONS**

### **Authors' contributions**

Wrote the first draft of the commentary: Zhang JW, He DJ

Edited and contributed to the final draft: Li XJ

### **Availability of data and materials**

Not applicable.

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### **Conflicts of interest**

All authors declared that there are no conflicts of interest.

### **Ethical approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

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