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SHANK1 and autism spectrum disorders

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Autism spectrum disorders (ASD) are highly heterogeneous pediatric developmental disorders with estimated heritability more than 70%. Although the genetic factors in ASD are mainly unknown, a large number of gene mutations have been found, especially in genes involved in neurogenesis. The Neurexin-Neuroligin-Shank (NRXN-NLGN-SHANK) pathway plays a key role in the formation, maturation and maintenance of synapses, consistent with the hypothesis of neurodevelopmental abnormality in ASD. Presynaptic NRXNs interact with postsynaptic NLGNs in excitatory glutamatergic synapses. SHANK proteins function as core components of the postsynaptic density (PSD) by interacting with multiple proteins. Recently, deletions and point mutations of the *SHANK1* gene have been detected in ASD individuals, indicating the involvement of *SHANK1* in ASD. This review focuses on the function of SHANK1 protein, *Shank*1 mouse models, and the molecular genetics of the *SHANK1* gene in human ASD.

autism spectrum disorders, SHANK1, synapse, genetics, mouse model

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Autism spectrum disorder (ASD) is a complex neurodevelopmental disease characterized by impaired social interaction and language development and repetitive and stereotyped behaviors and interests. ASD is more frequent in males than females, with an approximate ratio of 4∶1 [1]. Twin and family studies indicate that genetic factors play an important role in the etiology of autism with the heritability estimate more than 90% [2,3]. Though the causes of ASD are largely unknown, neurexin-neuroligin-shank (NRXN-NLGN-SHANK) pathway genes mutations have been implicated in ASD [4–20]. NLGN proteins, expressed highly in the brain, are postsynaptic adhesion molecules interacting with presynaptic NRXNs [9]. SHANK proteins function as core components of the postsynaptic density (PSD) by interacting with multiple proteins [21]. The impact of this pathway on synaptic function provides an important perspective for understanding the pathogenesis of ASD. The SHANK family has three members: SHANK1, SHANK2 and SHANK3. All SHANK family members are expressed in the brain and are present at PSD of excitatory synapses. *SHANK3* was the first and one of the best characterized genes implicated in ASD in *SHANK* family [19,20]. Recently, deletions and point mutations of the *SHANK1* gene have been detected in ASD individuals, indicating the involvement of *SHANK1* in ASD [22]. This review focuses on the function of SHANK1 protein, *Shank1* mouse models, and the molecular genetics of the *SHANK1* gene in human ASD.

1 The structure and function of SHANK1 protein

All three SHANK proteins have similar sets of domains: N-terminal multiple ankyrin repeats, a Src homology 3 (SH3) domain, a PSD-95/Discs large/ZO-1 (PDZ) domain, a long proline-rich region containing homer- and cortactin-binding sites, and a sterile alpha motif (SAM) domain.

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Through these multiple domains, SHANK family proteins interact with more than 30 synaptic proteins, consistent with the function of scaffolding proteins [23].

The major reported directly interacting proteins with SHANKs are GKAP (guanylate kinase-associated protein), GRIP (glutamate receptor-interacting protein), Homer and Cortactin. GKAP binds to the SHANK PDZ domain by C terminus sequence QTRL and interacts with NMDA (*N*-methyld-aspartate) receptor by PSD-95 protein [24,25]. GRIP protein interacts with SHANK by SH3 domain and also with membrane receptor AMPAR (AMPA-type glutamate receptor) [26,27]. There is a proline-serine-rich segment of more than 1,000 residues between PDZ domain and SAM domain, which mediates multiple sets of protein interactions by binding with SH3, EVH1 (enabled/vasodilator-stimulated phosphoprotein homology 1) and WW domains. Homer protein family includes one EVH1 domain, interacting with SHANKs by PPXXF sequence [28,29]. Homer mediates release of intracellular calcium in response to receptor stimulation by binding with mGluRs (metabotropic glutamate receptors) and IP3 (inositol 1,4,5-trisphosphate) receptors. Cortactin is an F-actin-binding protein, recognizing KPPVPPKP sequence of proline-rich region of SHANKs by its SH3 domain. Cortactin is rich in contact sites of cell-matrix, lamellipodia of cultured cells and growth cones of neurons, mediating the organization of the actin cytoskeleton in the cell cortex and in dendritic spines [30,31]. Thus, SHANK has the potential to interact indirectly with three major classes of postsynaptic glutamate receptor via their interacting proteins: NMDA receptors via the PSD-95/GKAP complex, mGluRs via Homer, and AMPA receptors via GRIP. The C-terminal SAM domain is a region critical for the postsynaptic localization of SHANK2 and SHANK3 while SHANK1 utilizes its PDZ domain for its synaptic localization [32–34].

In rats, SHANK2 and SHANK3 are expressed widely in many tissues such as the brain, kidney, heart, liver and spleen while SHANK1 mRNA and protein are expressed almost exclusively in the brain [35,36]. In cultured developing neurons, SHANK is highly expressed in growth cones and accumulates at synaptic junctions [25,31]. Overexpression of SHANK1 alters spine morphology, leading to the enlargement of spine heads [34]. On the other hand, *Shank1* knockout mice have smaller spines than wild type, suggesting a role for SHANK1 in affecting the size of spines [37]. Depolarization elicits a significant increase in levels of SHANK1 in the contiguous network immediately below PSD and only a modest increase in SHANK2 [38]. Moreover, while the increase in SHANK1 is reversed in 30 min, the increase in SHANK2 for the most part is maintained. These data indicate that SHANK1 is a dynamic element within the spine, involved in activity-induced, transient structural changes, while SHANK2 appears to be a more stable element of the postsynaptic complex [38].

Synaptic activity and excitatory stimuli elicit structural changes in spine morphology, a process called synaptic plasticity. These structural and molecular changes are believed to represent the basis for learning and memory. SHANK family members may play different roles in synaptogenesis and synapse maturation. Their levels at the PSD are tightly regulated via Zn^{2+} ions in an isoform-specific way [39]. SHANK2 and SHANK3 are sensitive to Zn^{2+} ions via their SAM domains, whereas SHANK1 is Zn^{2+} insensitive. Immature synapses exhibit a striking sensitivity to extracellular levels of Zn^{2+} ions that is not shared by mature synapses. This sensitivity is intimately linked to the differential expression and selective binding of Zn^{2+} ions to SHANK2 and SHANK3 but not SHANK1. SHANK family members are recruited consecutively to postsynaptic sites at different stages of development in primary hippocampal cultures. SHANK2 was the first to appear at PSDs, presented in all synapses while SHANK1 was detected in one third of all synapses at DIV (days *in vitro*) 7. During late development of synaptic contacts (DIV 21), 95% of all synapses were labeled with all three SHANK family members [38]. *Shank1* knockdown together with zinc depletion also reduced the number of "mushroom/stubby" synapses and leads to a shift towards smaller spine size [39].

Tissue-specific methylation is one of the mechanisms for regulation of gene expression. DNA methylation status of human *SHANK* genes was analyzed in lymphocytes, brain cortex, cerebellum and heart. Seven CpG islands of *SHANK1* and *SHANK2* genes and five of *SHANK3* were identified and analyzed for DNA methylation [40]. The majority of the CpG islands in *SHANK1* and *SHANK2* show extensive variability in methylation without apparent tissue-specificity. *SHANK3*, on the other hand, undergoes tissue-specific methylation at most of its CpG islands and highly methylated in tissues where its expression is low or absent, suggesting that *SHANK3* expression might be regulated by epigenetic mechanisms such as DNA methylation. Promoter-associated CpG islands of all SHANK members were always completely unmethylated.

2 Mouse models of *Shank1*

Shank1 mutant mice with a deletion of exons 14 and 15 were produced and characterized by Hung et al. [37] in 2008. These two exons encode almost the entire PDZ domain, a highly conserved region of *Shank1*. Since *Shank1* mutant mice in the B6 background had high mortality and *Shank1* mice in the 129Jae background strain had very low locomotion, two lines were crossed to obtain a mixed C57BL/6/129SvJae (B6/Jae) background. The molecular and behavioral phenotypes of *Shank1* mutant mice were studied. *Shank1*$^{-/-}$ mutant mice showed altered protein composition of the PSD. There was a significant reduction of GKAP (~30%) and Homer1b/c (~20%), two scaffold proteins that bind directly with SHANK, while no signifi-

cant difference in the levels of NMDA, AMPA, PSD-95, GRIP, β-PIX (PAK-interacting exchange factor-β) and cortactin. Consistent with the biochemical results, the density of GKAP puncta was significantly decreased in *Shank1*$^{-/-}$ mutant mouse brain. The immunostaining pattern of Homer was more diffuse though there was no significant change in density of Homer puncta in *Shank1*$^{-/-}$ knockout mouse brain. These data support the idea that SHANK1 is important for synaptic accumulation of GKAP and Homer. No gross abnormalities in the size or histological structure of the brain (including cortex, hippocampus, and cerebellum) were detected. In CA1 pyramidal neurons of hippocampus, significantly smaller spine size, reduced PSD thickness and a selective loss of the largest PSDs were observed in *Shank1*$^{-/-}$ knockout mice compared with wild type mice, suggesting that SHANK1 may be critical for the development and/or maintenance of the largest subset of PSDs and synapses in particular. *Shank1*$^{-/-}$ mutant mice showed decreased basal synaptic transmission as a result of a reduction in the number of functional synapses. The ratio of AMPAR and NMDAR EPSCs (excitatory postsynaptic currents) (AMPA/NMDA ratio) was not significantly different between wild type mice and *Shank1*$^{-/-}$ mutant mice, suggesting that the *Shank1* deficiency does not affect the proportion of synaptic AMPA and NMDA receptors.

The behavior of adult *Shank1*$^{-/-}$ mutant mice was investigated in a variety of assays [37]. *Shank1*$^{-/-}$ mutant mice were significantly less active in a novel open-field environment than the wild-type littermate control mice, as measured by horizontal activity, total distance traveled and movement time. *Shank1*$^{-/-}$ mutant mice showed increased anxiety-like behavior in open-field testing and the light/dark transition test. *Shank1*$^{-/-}$ homozygous mice were poor breeders, did not nurture their pups, and their litters generally died before weaning. *Shank1*$^{-/-}$ mutant mice have enhanced spatial learning but impaired long-term retention of this memory in eight-arm radial maze task in which animals must learn and remember the position of baited arms between trials while rapidly establishing memory of previously visited arms within a trial. In such a task, the *Shank1*$^{-/-}$ mutant mice showed a steeper learning curve and reached a better performance level with fewer reference memory errors and fewer working memory errors than the wildtype control mice. Together, these data indicate that *Shank1* deficient mice learn faster and more effectively during repetitive training in the eight-arm radial maze.

Autism has three core diagnostic symptoms including social communication deficits, language development delay, and stereotyped or repetitive behaviors and interests. Silverman et al. [41] developed multiple mouse behavioral assays to investigate autism-relevant phenotypes in *Shank1* mutant mice. Both females and males of all three genotypes (*Shank1*$^{-/-}$, *Shank1*$^{+/-}$, and *Shank1*$^{+/+}$) were tested in the study, while only *Shank1*$^{-/-}$ mutant males were studied in the first report of *Shank1*$^{-/-}$ knockout mice. Social behaviors were examined through three aspects: reciprocal social interactions in juvenile mice, adult sociability using automated three-chambered task in adult mice, and social interest in the olfactory habituation/dishabituation task. No genotype differences were observed for six parameters, i.e., nose-to-nose sniff, anogenital sniff, body sniff, push-crawl, push-past, and follow events, when characterizing active social interactions in juvenile *Shank1* mice. All of *Shank1* genotypes (–/–, +/–, and +/+) failed to demonstrate significant sociability, which could not be attributed to their background strain since the hybrid B6/Jae mice showed normal sociability. Three potential explanations for the lack of sociability in the *Shank1* line were proposed by the authors. First, altered behavior of the *Shank1*$^{+/-}$ mutant mother mice could affect performance on behavioral tasks via epigenetic modulation. Second, home cage interactions among littermate pups may affect later social performance. Third, genetic drift across generations could have introduced variations in the mixture of genes from the two original background strains across individual subject mice. Stereotyped and repetitive behaviors were measured by self-grooming in *Shank1* mutant mice. Though all *Shank1* genotypes mice exhibited high levels of self-grooming, no significant differences in self-grooming scores were detected across genotypes.

Anxiety-like behavior was assessed using the light/dark test and the elevated plus-maze task [41]. *Shank1*$^{-/-}$ mutant mice displayed significantly fewer transitions between the light and dark compartment as compared with *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ heterozygotes, consistent with previous findings. However, other parameters of the light/dark task and the elevated plus-maze task did not show difference across genotypes. The authors concluded that these results indicate a mild anxiety-like behavior attributable to the *Shank1* mutation.

The major phenotypes of *Shank1* mutant mice were motor impairments evaluated using three tasks, open field, rotarod, and wire hang [41]. *Shank1* null mice displayed less total distance and spent less time in the center of the arena as compared to *Shank1*$^{+/+}$ littermates in a novel open field. *Shank1*$^{-/-}$ null mutant mice fell from the accelerating rotarod faster and from the inverted wire mouse cage lid faster than *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ heterozygous littermate mice. Overall, *Shank1*$^{-/-}$ mutant mice showed reduced exploratory locomotion and reduced motor coordination, balance and neuromuscular strength.

Social communication of *Shank1* mutant mice was further evaluated using assays for ultrasonic vocalizations (USV) and scent marking in an open field, which are believed to be two major modes of mouse communication [42]. Deficits in several elements of social communication and early developmental milestones were detected in *Shank1* null mutant mice. Mouse pups emit USV when isolated

from their mother and littermates to elicit maternal search and retrieval behavior [43–46]. In adult mice, high USV levels are detected in males when courting and copulating with females. Several genetic mouse models of autism were reported to display reduced levels of pup USV, unusual calling patterns, or reduced levels of male USV production in response to females or female urine [47–51]. As pups, *Shank1*$^{-/-}$ mutant mice emitted fewer USV and spent less time calling than *Shank1*$^{+/+}$ littermate control mice when isolated from mother and littermates [42]. Calls emitted by *Shank1*$^{-/-}$ mutant mouse pups were shorter, higher in peak frequency, but less frequency modulated than the ones emitted by *Shank1*$^{+/+}$ control pups. These altered parameters of calls emitted by *Shank*$^{-/-}$ mutant pups may decrease their signal value and elicit less maternal care-giving responses. As adults, *Shank1*$^{+/+}$ wildtype males changed their calling pattern dependent on their previous exposure to a female, but *Shank1*$^{-/-}$ mutant males were unaffected by prior female experience though both *Shank1*$^{+/+}$ and *Shank1*$^{-/-}$ male mice emitted a similar amount of USV when exposed to female urine. The lack of experience-induced changes in USV production may represent an inability to modulate social behaviors in response to experiences with social cues. In addition to USV, adult male mice display scent marking behavior, depositing urinary pheromone traces in close proximity to the location of female urine. *Shank1*$^{-/-}$ mutant mice deposited fewer urine traces in proximity to the female urine spot than *Shank1*$^{+/+}$ littermate control mice. Reduced levels of locomotor behavior were observed in *Shank*$^{-/-}$ mutant mice, replicating the previous studies. The surface righting reflex was delayed in *Shank1*$^{-/-}$ mutant as compared to *Shank1*$^{+/+}$ mice. Appearance of some physical developmental milestones, i.e. pinnae detachment and incisor eruption were delayed in *Shank1*$^{-/-}$ mutant pups [42].

3 *SHANK1* mutations are associated with ASD

As compared with numerous reports of *SHANK2* and *SHANK3* mutations in ASD, there is so far only one report about the involvement of *SHANK1* in ASD [22]. Copy-number variations (CNVs) screening was performed in a cohort of 1,158 unrelated Canadian individuals (898 males and 260 females) and 456 unrelated European individuals (362 males and 94 females) and sequence-level mutations of *SHANK1* were tested in 509 unrelated ASD (384 males and 125 females) and 340 intellectual disability (ID, 191 males and 149 females) individuals by Sato et al. [22]. They initially identified a hemizygous microdeletion of 63.8 kb which eliminated exons 1–20 of *SHANK1* and the neighboring *CLEC11A* gene in a four-generation family. Four male carriers were diagnosed with Asperger disorder or broader autism phenotype (BAP) whereas two female carriers exhibited anxiety and shyness but would not be considered to have ASD or BAP. Another unrelated male case with the diagnosis of high-function autism was identified to have a *de novo* 63.4 kb hemizygous deletion which eliminated the last three exons of *SHANK1* and the entire centromeric synaptotagmin-3 (*SYT3*) gene. No equivalent deletion was observed in 15,122 control individuals. The frequency of deletions at the *SHANK1* locus is significantly higher in ASD cases than in controls. Remarkably, by whole-exome sequencing in two male individuals carrying the deletion of *SHANK1* in the multigenerational family, a nonsense mutation (Tyr313*) in the *PCDHGA11* gene, a member of the protocadherin gamma gene cluster thought to have an important role in establishing connections in the brain, was identified. The mutation was found to segregate precisely with the *SHANK1* deletion. It is possible that the Tyr313* mutation in *PCDHGA11* works in concert with the *SHANK1* deletion to modify (positively or negatively) the extent of the phenotype or that they are just randomly cosegregating.

The segregation of ASD in only male *SHANK1*-deletion carriers, but not female carriers, indicates gender-influenced autosomal penetrance differences at the *SHANK1* locus in ASD. *SHANK1* deletions are associated with ASD with higher functioning in males. The female carriers do not show evidence of ASD or BAP but have suffered from anxiety, which is considered to be a common comorbid condition but not a related phenotype to ASD. These results may help to explain the male gender bias in autism. Females need to carry more genetic liability than males in order to develop ASD. Consistent with these findings in humans, *Shank1* null mice exhibit increased anxiety-related behavior and deficits in several elements of social communication and developmental milestones.

By sequencing of all exons and splice sites of the *SHANK1* gene in 509 unrelated ASD and 340 ID individuals, 26 rare missense variants were identified in 23 ASD and 7 ID cases, which were not found in dbSNP (single nucleotide polymorphism) build 130 or in 285 control individuals [22]. Only two missense variants were predicted to be deleterious since they alter highly conserved residues within the PDZ domain and ANK (ankyrin) domain. Although they occurred in males with ASD, both variants were inherited with fathers with no symptoms of ASD.

Given that important roles of SHANK family in synaptic function and the detection of *SHANK2* and *SHANK3* mutations in ID and schizophrenia, it is reasonable to expect that *SHANK1* mutations may contribute to other psychiatric disorders. Lennertz et al. [52] genotyped 5 common SNPs in *SHANK1*, *SHANK2* and *SHANK3* and found one SNP rs3810280 in promoter region of *SHANK1* was associated with working memory in schizophrenia cases. This association was replicated in another cohort of 77 subjects with high risk of psychosis. Although the sample size was small, the association of SNPs of *SHANK1* with schizophrenia indicates the involvement of *SHANK1* in the pathogenesis

of neurodevelopmental disorders.

4 Perspectives

Although all SHANK family members have similar structure, the interaction proteins for each SHANK and related functions could be different. For example, SHANK2 and SHANK3 utilize their SAM domain for oligomerization and synaptic localization, while the PDZ domain of SHANK1 is critical for its postsynaptic localization. The different temporal and spatial distribution of SHANK1 indicates its non-redundant function with other two SHANK members. The protein partners of SHANK1 and their specific functions at excitatory synapses need to be illuminated.

The male carriers of *SHANK1* deletions were diagnosed with high-function autism. However, *Shank1*$^{-/-}$ mutant mice did not show robust social deficits. Mice lacking *Shank1* display smaller spine size, reduced PSD thickness and weaker basal synaptic transmission. On behavioral level, *Shank1*$^{-/-}$ mutant mice showed increased anxiety, enhanced spatial learning, motor impairments, and altered social communication (reduced levels of ultrasonic vocalizations and scent marking behavior). One interpretation could be that the absence of *Shank1* in mice may affect cognitive abilities including motor and spatial learning, instead of direct effect on sociability. Double knockout mice of *Shanks*, i.e. *Shank1* and *Shank2* double knockout mice, will be interesting model to investigate the expression pattern of synaptic proteins and behavioral phenotypes.

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