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# Enhancing inhibitory synaptic function reverses spatial memory deficits in *Shank2* mutant mice

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ABSTRACT

Autism spectrum disorders (ASDs) are a group of developmental disorders that cause variable and heterogeneous phenotypes across three behavioral domains such as atypical social behavior, disrupted communications, and highly restricted and repetitive behaviors. In addition to these core symptoms, other neurological abnormalities are associated with ASD, including intellectual disability (ID). However, the molecular etiology underlying these behavioral heterogeneities in ASD is unclear. Mutations in *SHANK2* genes are associated with ASD and ID. Interestingly, two lines of *Shank2* knockout mice (e6-7 KO and e7 KO) showed shared and distinct phenotypes. Here, we found that the expression levels of *Gabra2*, as well as of GABA receptor-mediated inhibitory neurotransmission, are reduced in *Shank2* e6-7, but not in e7 KO mice compared with their own wild type littermates. Furthermore, treatment of *Shank2* e6-7 KO mice with an allosteric modulator for the $GABA_A$ receptor reverses spatial memory deficits, indicating that reduced inhibitory neurotransmission may cause memory deficits in *Shank2* e6-7 KO mice.

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## 1. Introduction

Autism is a highly heritable neurodevelopmental disorder with behavioral deficits in three domains: social behavior, language development, and restricted interest and repetitive behaviors (Geschwind, 2009). Autism spectrum disorders (ASDs) are a group of developmental disabilities that can cause variable and heterogeneous phenotypes across these domains, which include autism, Asperger syndrome and Rett syndrome (Geschwind, 2009). In addition to the three core domains, ASD is frequently associated with other neurological abnormalities including hyperactivity, epilepsy, and sensory abnormalities (Abrahams and Geschwind, 2008; Koh et al., 2014). Importantly, 25–75% of ASD patients have learning disabilities (O'Brien and Pearson, 2004).

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Recent genomic studies demonstrated that the affected genes in ASD and their networks are primarily involved in synapse development, axon targeting, and neuron motility (Gilman et al., 2011; Voineagu et al., 2011). In addition, several other synaptic genes such as *NLGN4, NRXN1, SHANK2, SHANK3* and *CNTNAP2* had been associated with idiopathic ASDs (Alarcon et al., 2008; Berkel et al., 2010; Durand et al., 2007; Feng et al., 2006; Jamain et al., 2003). Among these, mutations in *SHANK* genes (*SHANK1, SHANK2, and SHANK3*), which encode postsynaptic scaffold proteins of excitatory synapses in the brain, are linked to ASD as well as schizophrenia (Choi et al., 2015; Schmeisser, 2015). *SHANK2* mutations are associated with ASD and intellectual disability and *Shank2* knockout (KO) mice showed deficits in social behaviors and learning, albeit with different cellular phenotypes (Berkel et al., 2010; Leblond et al., 2012; Schmeisser et al., 2012; Won et al., 2012). A *Shank2* mutant lacking exon7 of the *Shank2* gene (e7 KO) demonstrated ASD-like phenotypes, including social deficits, and this mutant showed decreased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) function (Schmeisser et al., 2012). In contrast, another *Shank2* mutant, lacking exon 6 and 7 of the *Shank2* gene (e6-7 KO), showed a decrease in *N*-methyl-D-aspartate receptor (NMDAR) function (Won et al., 2012). Accordingly, treatment of e6-7 KO mice with a partial agonist of NMDARs, D-cycloserine, or a positive allosteric modulator of metabotropic glutamate receptor 5 (mGluR5), 3-cyano-*N*-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), rescued NMDAR function and social deficits, suggesting that the decrease in NMDAR function caused social deficits in the mutant mice (Won et al., 2012). This discrepancy in the phenotypes observed between the different mutants of the same gene clearly demonstrates the heterogeneity of ASD and also provides an opportunity to study the molecular mechanism underlying the differences.

Alterations of GABAergic signaling and resulting imbalance of the excitatory/inhibitory (E/I) transmission in selective neuronal circuit are commonly found in many neurodevelopmental disorders including ASD (Braat and Kooy, 2015; Robertson et al., 2016). For example, several mouse models harboring ASD-associated mutations display altered ratio of E/I transmission (Cui et al., 2008; Gkogkas et al., 2013; Han et al., 2012; Houbaert et al., 2013; Lee et al., 2014, 2015; Tabuchi et al., 2007). Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult brain. Genetic studies in the Prader-Willi/Angelman syndrome harboring copy-number variations (CNVs) in chromosome 15q11–13, which contains many genes encoding for $GABA_A$ receptor subunits, have revealed the involvement of $GABA_A$ receptors in ASDs (Hogart et al., 2007; Scoles et al., 2011). In addition, the post-mortem tissue of ASD patients was shown to express fewer GABA receptor subunits (Fatemi et al., 2014; Oblak et al., 2011). $GABA_A$ receptors are ligand-gated receptors, permeable to chloride, and have a specific cellular and subcellular expression pattern according to their distinct receptor subtypes (Olsen and Sieghart, 2009). $GABA_A$ receptors containing an α1–3, βn, and γ2 subunit are mainly synaptic, whereas receptors containing α4–6 and δ subunits show mainly extrasynaptic localization (Belelli et al., 2009).

In this study, we first analyzed the genetic difference between two Shank2 mutants (e6-7 and e7 KO) by using the next generation sequencing technique and found that the mRNA level of *Gabra2* is reduced only in e6-7 KO, but not in e7 KO. Accordingly, GABA receptor-mediated synaptic transmission and inhibitory/excitatory (I/E) current ratios were reduced in e6-7 KO, which can be reversed by a $GABA_A$ receptor alpha 2 agonist, L838,417 treatment. Furthermore, a systemic administration of L838,417 reverses spatial memory deficits, but not the social deficit in adult *Shank2* e6-7 KO mice.

## 2. Material and methods

### 2.1. Mice

Eight to fifteen week-old male *Shank2* knock-out (KO) and wild type (WT) littermates were used for behavioral experiments. *Shank2* e6-7 KO and *Shank2* e7 KO had been backcrossed to C57Bl/6N and C57Bl/6J, respectively. Both lines on mixed background were used for the experiments: the e6-7 KO is on mixed background of 46% C57Bl/6J and 54% C57Bl/6N and the e7 KO is on mixed background of 75% C57Bl/6J and 25% C57Bl/6N based on SNP analysis (Mekada et al., 2009). Mice were kept on a 12 h light/dark cycle, and the behavioral experiment was performed during the light phase of the cycle. Food and water were provided ad libitum. Injured mice were excluded from all experiments. The Institutional Animal Care and Use Committee of Seoul National University approved the animal protocols and following experiments.

### 2.2. RNA-seq analysis

Hippocampi were isolated from 4-week-old male mice with either *Shank2* e6-7 KO or WT genotypes. RNA-seq libraries were prepared as previously described (Boo et al., 2015). Briefly, total RNA was extracted using TRIZOL. Poly(A) mRNA (5 μg) was isolated and fragmented using the Illumina Truseq RNA Sample Prep Kit with poly-T oligo-attached magnetic beads. Reverse transcription was performed using Superscript II reverse transcriptase (Life Technologies). The adaptor-ligated library was size-selected by band excision after agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen). The constructed libraries were sequenced on Illumina HiSeq 2500 (NICEM, Seoul National University) in the paired-end sequencing mode (2 × 101 bp reads). The sequencing raw reads were mapped onto the mouse genome reference mm10 using GSNAP (version 2013-11-27) (Wu and Nacu, 2010). Uniquely and properly mapped read pairs were used for further analysis. To identify differentially expressed genes (DEGs) between the WT and KO groups, the cuffdiff module in the Cufflinks package (version v2.1.1) was used (Trapnell et al., 2013) and differentially expressed genes were defined as those with changes of at least 1.5-fold between samples at a false discovery rate (FDR) of 10%. To assess expression levels of genes, the RPKM (reads per kilobase of exon per million mapped reads) measure was calculated (Mortazavi et al., 2008). PANTHER (Protein ANalysis THrough Evolutionary Relationships) Tools (http://www.pantherdb.org/) was used to divide the DEGs into functional protein classes and GO term enrichment analysis was performed similarly as previously described (Lee et al., 2011).

### 2.3. Quantitative real-time PCR (qRT-PCR)

RNA samples used for RNA-seq analysis were reverse-transcribed using SuperscriptIII. qPCR was carried out in triplicate using the ExTaqII SYBR Green Master Mix (Takara) in ABI7300 (Applied Biosystems). Cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Amplification of GAPDH was performed in parallel for each sample and used as an internal control. Primer Sequences are listed in Supplementary Table 6.

### 2.4. Western blot analysis

Mice were lightly anesthetized with isoflurane and then decapitated. The hippocampi were dissected out and then homogenized in the Frac buffer (30 mM Tris-Cl pH 7.4, 4 mM EDTA, 1 mM EGTA, Protease inhibitor cocktail). The homogenates were