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Shank1 regulates excitatory synaptic transmission in mouse hippocampal Parvalbumin-expressing inhibitory interneurons

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Abstract

The Shank genes (SHANK1, 2, 3) encode scaffold proteins highly enriched in postsynaptic densities where they regulate synaptic structure in spiny neurons. Mutations in human Shank genes are linked to autism spectrum disorder (ASD) and schizophrenia. Shank1 mutant mice exhibit intriguing cognitive phenotypes reminiscent of ASD individuals. However, the molecular mechanisms leading to the human pathophysiological phenotypes and mouse behaviors have not been elucidated.

We show in this study that Shank1 protein is highly localized in Parvalbumin-expressing (PV+) fast-spiking inhibitory interneurons in hippocampus. Importantly, lack of Shank1 in hippocampal CA1 PV+ neurons reduced excitatory synaptic inputs and inhibitory synaptic outputs to pyramidal neurons. Furthermore, we demonstrate that hippocampal CA1 pyramidal neurons in Shank1 mutant mice exhibit a shift in the *excitatory and inhibitory balance (E-I balance)*, a pathophysiological hallmark of ASD. The mutant mice also exhibit lower expression of gephyrin (a scaffold component of inhibitory synapses), supporting the dysregulation of *E-I balance* in hippocampus. These results suggest that Shank1 scaffold in PV+ interneurons regulates excitatory synaptic strength and participates in the maintenance of *E-I balance* in excitatory neurons.

INTRODUCTION

The Shank family proteins (Shank1, 2, 3, also known as ProSAP, Synamon, CortBP, Spank and SSTRIP) are enriched in postsynaptic densities (PSDs) and serve as scaffolds for a variety of postsynaptic molecules in excitatory neurons (Jiang & Ehlers, 2013). All Shank isoforms regulate the structure of dendritic spines, particularly spine heads, and are critical for the maturation of their structure in excitatory and medium spiny neurons (Sala *et al.*, 2001; Roussignol *et al.*, 2005; Haeckel *et al.*, 2008; Peca *et al.*, 2011; Durand *et al.*, 2012). Consistent with these findings, knockdown of any of the Shank isoforms leads to reduced spine size and/or density in neurons thereby perturbing excitatory synaptic transmission

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(Grabrucker *et al.*, 2011; Verpelli *et al.*, 2011; Berkel *et al.*, 2012). Furthermore, Shank knockout mouse lines exhibit abnormal synaptic structure and/or function in various brain regions (Hung *et al.*, 2008; Peca *et al.*, 2011; Wang *et al.*, 2011; Schmeisser *et al.*, 2012; Won *et al.*, 2012; Yang *et al.*, 2012). However, the pattern of expression of the SHANK genes and their functions in non-spiny neurons, such as inhibitory interneurons, are largely unknown.

Multiple lines of evidence indicate that the molecular organization of excitatory synapses in interneurons is different from that in excitatory neurons. In this regard, the subunit composition of AMPA receptors in interneurons is distinct from that in excitatory neurons (Jonas *et al.*, 1994). The receptor tyrosine-protein kinase ErbB4, the PSD-95 binding protein CITRON (a putative Rho effector), and Synapse-Associated Protein 97 are expressed more abundantly in interneurons than in pyramidal neurons (Zhang *et al.*, 1999; Mei & Xiong, 2008; Jaaro-Peled *et al.*, 2009; Fazzari *et al.*, 2010; Akgul & Wollmuth, 2013). Furthermore, there are fundamental differences in short- and long-term synaptic plasticity between inhibitory and excitatory neurons (McMahon & Kauer, 1997; Sun *et al.*, 2005; Lamsa *et al.*, 2007). Taken together, the excitatory postsynaptic molecular architecture of interneurons is different from that of excitatory neurons, and the types of postsynaptic neurons (projection versus inhibitory neurons) likely determine the physiological characteristics of excitatory synapses.

Parvalbumin-expressing (PV+) fast-spiking inhibitory interneurons send inhibitory axons to perisomatic areas of excitatory neurons and regulate neuronal synchronization (Freund & Katona, 2007). The dysregulation of PV+ neurons can cause a shift of the excitatory and inhibitory balance (*E-I balance*), which is considered one of the endophenotypes of several psychiatric disorders (Rubenstein & Merzenich, 2003; Rossignol, 2011). However, the molecular architecture of excitatory synapses in PV+ neurons, which serves an important role in regulating excitability of cortical and hippocampal circuits, is largely unknown.

In this study, we found that Shank1 is highly expressed in PV+ neurons, and its deficit in PV+ neurons causes a reduction of excitatory synaptic inputs and inhibitory outputs to CA1 pyramidal neurons. Furthermore, we observed that loss of Shank1 causes increased *E-I balance* by reducing inhibitory synaptic function and lowers expression of gephyrin in the hippocampal CA1 area. These results indicate that the Shank1 scaffold plays an important role in PV+ neuron-mediated synaptic circuits in hippocampus.

Materials and Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School. Shank1 mutant mice were generated previously and backcrossed with C57BL/6 and 129SvJae strains (gift from M. Sheng and R. Jaenisch, Massachusetts Institute of Technology, Cambridge, MA) (Hung *et al.*, 2008). The animals used in this study were in a 129SvJae/C57BL/6 hybrid genetic background. We generated PV-RFP mice by crossing PV-Cre mice (Carlén *et al.*, 2011) with a Cre-reporter mouse line carrying tdTomato (variant of red fluorescent protein, JAX:

007905) (Madisen *et al.*, 2010). To generate Shank1^{-/-}/PV-RFP mice, we first crossed Shank1 heterozygous (C57BL/6 background) mutants with PV-RFP mice for at least three generations to transmit the cre recombinase and RFP genes [Shank1BL6^{+/-}/PV-RFP]. Shank1^{-/-}/PV-RFP and wild-type mice were generated by crossing Shank1BL6^{+/-}/PV-RFP and Shank1^{+/-} (129SvJae) mutant mice. This mouse line expresses RFP in 80% of PV+ neurons with over 99% of the RFP-expressing cells exhibiting PV immunoreactivity in the hippocampus (confirmed in two PV-RFP brains, 748 out of 903 PV-positive neurons expressed RFP and 7 out of 748 RFP-positive neurons were negative to PV immunoreactivity).

Antibodies and Biochemistry

The following antibodies were used (dilution used for immunocytochemistry are indicated in parentheses): rabbit/mouse anti-calbindin, -parvalbumin and -calretinin (1:1000, SWANT); goat/mouse anti-parvalbumin (1:2000, SWANT); rabbit anti-GKAP (1:1000, gift from Dr. Morgan Sheng); rabbit anti-GABA_AR $\alpha 1$ (1:1000, EMD Millipore); mouse anti-GABA_AR $\beta 2/3$ and -Synaptophysin (1:1000, EMD Millipore); mouse anti-pan-Shank (1:3000, Neuromab); rabbit anti-Shank1a [western blotting: 1:1000 Shank1_1356 (gift from Dr. E. Kim, Korea Advanced Institute of Science and Technology, Korea), immunohistochemistry and immunocytochemistry: 1:10000, Abcam (ab66315) and Synaptic Systems (162013)]; mouse anti-PSD95 (1:2000, Neuromab); rabbit/mouse anti-Gephyrin (1:1000-3000, Synaptic Systems); mouse anti-Bassoon (1:3000, Synaptic Systems); mouse anti-VGAT and -VGluT1 (1:2000, Synaptic Systems); mouse anti-Homer (1:1000, Transduction Laboratories); secondary Alexa dye-conjugated anti-mouse (Alexa 488, 647), anti-rabbit (Alexa 488, 594, 647), anti-goat (Alexa 405, 488, 594), anti-guinea pig (Alexa 488, 647) antibodies (Invitrogen or Jackson ImmunoResearch Labs); HRP-conjugated anti-mouse and anti-rabbit antibodies (GE Healthcare, 1:2000). Fab fragment goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs) was applied prior to the application of primary mouse antibodies. The Triton-extracted P2 fraction was purified from the hippocampi of 5–7 week old mice, as described previously (Cho *et al.*, 1992; Futai *et al.*, 2013).

Immunohistochemistry

Mice (5–7 weeks old) were deeply anesthetized under isofluorane and transcardially perfused with heparin/saline and 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. The brains were removed and post-fixed in the same fixative for 24 hours at 4°C. The fixed brains were sectioned in the coronal plane at 10 and 30 μ m thicknesses on a microslicer (VT1200 S; Leica, Germany) to perform staining for gephyrin and Shank1, respectively. The sections were washed with PBS and then blocked with GDB buffer (30 mM phosphate buffer, pH 7.4, containing 0.2% gelatin, 0.5% Triton X-100, and 0.45 M NaCl) for 2 hours at room temperature. Sections were then incubated overnight at 4°C with primary antibodies against interneuronal markers, Shank1 or gephyrin. Following PBS washes, the sections were incubated at room temperature for 1.5 hours with secondary antibodies. Following a third round of PBS washes, the sections were mounted on slides with Vectashield mounting medium (Vector Laboratories, Inc.).

Primary hippocampal neuron culture, immunocytochemistry

Hippocampal primary cultures were prepared from the brains of individual mice at postnatal day 0–3 as described previously (Futai *et al.*, 2013). Cells were plated onto coverslips (Matsunami, Japan) coated with poly-D-lysine (80 µg/ml, BD) and laminin (2 µg/ml, BD) at a density of 200 cells/mm² in Neurobasal medium supplemented with 2% B27 supplement (Invitrogen). For immunocytochemistry against synaptic proteins in PV+ cells, hippocampal cultures were fixed at days *in vitro* 14 (DIV14) with 4% paraformaldehyde in PBS. The methanol fixation approach, a standard procedure to stain molecules in postsynaptic densities (Kim *et al.*, 2007), was not used in this study as this treatment dramatically reduced immunoreactivity against PV. Primary and secondary antibodies were applied in GDB buffer using the dilutions of antibodies described above. Primary neurons were incubated with primary and secondary antibodies for two and one hours at room temperature, respectively. The coverslips washed with PBS were mounted on slides with Vectashield (Vector Labs) mounting medium.

Neuronal Imaging

A spinning disk confocal microscope (Nikon TE-2000E2 and Leica TCS SP5 II; University of Massachusetts Medical School Imaging Core Facility) was used for imaging. The confocal images (512 × 512 or 1024 × 1024 pixels) of primary hippocampal cultures and slices were taken using 40X, 60X, 63X or 100X objective lenses. Each image was a Z-series projection of x-y images, and taken at 0.2 – 1 µm depth intervals. The size, intensity and density of immunopositive signals were evaluated by MetaMorph software (Molecular Devices). Shank1 signals in the dendritic segments of PV+ neurons and in the *stratum radiatum* region were obtained from the same images (Fig. 1A). Neurons that exhibited immunoreactivity against Shank1 antibody in the cell body and dendritic segments were classified as Shank1-decorated neurons. All measurements in Fig. 2 and 5 were carried out in a “blind” manner.

Laser Capture Microdissection (LCM) and Real-time PCR

Adult (5 – 7 week old, either sex) PV-RFP mice were euthanized and the brains were immediately frozen in dry ice-cooled 2-methylbutane (–60°C) and stored at –80°C. Coronal serial sections (10 µm) of the hippocampi were prepared using a cryostat (Leica, Germany) and mounted on pre-cleaned glass slides (Fisher Scientific). The sections were stored at –80°C until use. A Veritas Microdissection System Model 704 (Arcturus Bioscience) was used for LCM. Approximately 1000 – 2000 RFP-positive neurons (PV+ inhibitory interneurons) were obtained from the hippocampal CA1 region of each animal. The same number of RFP-negative neurons in CA1 stratum pyramidale was obtained as pyramidal neurons. Five to seven different mice were used for each test. Neurons were captured on CapSure MacroLCMcaps (Arcturus Bioscience) for mRNA isolation.

Total RNA was extracted from individual replicate samples using an RNAqueous-Micro Kit (Ambion). RNA samples extracted from hippocampal CA1 PV+ and pyramidal neurons were reverse-transcribed into cDNA using TaqMan Gene Expression Cells-to-CT Kit (Ambion).

Polymerase chain reactions (PCRs) were set up in 10- μ l reaction mixtures using TaqMan Gene Expression Assays (SHANK1: Mm01206737_m1, SHANK2: Mm01163731_m1, SHANK3: Mm00498775_m1, PV: Mm00443100_m1, Slc17a7 (VGluT1): Mm00812886_m1, GAPDH: Mm99999915_g1, Applied Biosystems). GAPDH transcript was used as an internal control to normalize gene expression levels. The expression of PV and Slc17a7, a marker of excitatory neurons, transcripts were measured against the samples that targeted PV+ and excitatory neurons to evaluate the quality of samples harvested by LCM. The expression of PV and Slc17a7 in two different cell-types are; PV: PV+ neurons, 0.18 ± 0.06 , excitatory neurons, 0.02 ± 0.01 ; Slc17a7: PV+ neurons, 0.19 ± 0.01 , excitatory neurons, 0.78 ± 0.06 , $N = 4$ mice. These results indicate that our LCM approach collected the specific cell types we expected. PCRs were performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). All reactions were performed in duplicate or triplicate. Relative amplicon quantification was calculated as the difference between Ct values of GAPDH and that of Shank1, Shank2 and Shank3.

Electrophysiology

Transverse hippocampal slices (400 μ m thickness) were prepared from 3- to 5-week-old mice (either sex) in ice-cold dissection buffer (in mM: 238 sucrose, 2.5 KCl, 1 CaCl₂, 5 MgCl₂, 26 NaHCO₃, 1 NaH₂PO₄, 11 glucose, gassed with 5% CO₂/95% O₂, pH 7.4) as described (Hung *et al.*, 2008; Ryu *et al.*, 2008). Slices were incubated in an interface or submersion incubation chamber containing extracellular artificial cerebrospinal fluid (aCSF; in mM: 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, 1 NaH₂PO₄, 11 glucose, gassed with 5% CO₂/95% O₂, pH 7.4) and allowed to recover for 30 min at 28°C and then maintained at room temperature (24–26°C) for at least 30 min. Slices were then transferred to a submerged recording chamber and continuously perfused with aCSF.

For whole-cell recordings, thick-walled borosilicate glass pipettes (Warner Instruments) were pulled to a resistance of 3–5 M Ω . For current-clamp recordings, pipettes were filled with internal solution containing the following (in mM): 115 potassium methanesulfonate, 20 CsCl, 10 HEPES, 2.5 MgCl₂, 4 adenosine triphosphate disodium salt, 0.4 guanosine triphosphate trisodium salt, 10 sodium phosphocreatine, and 0.6 EGTA, pH 7.25, with KOH. For voltage-clamp recordings, the potassium was replaced by cesium. To measure GABA_AR-mediated inhibitory postsynaptic current (IPSC) and AMPAR-mediated excitatory postsynaptic current (EPSC), NMDAR antagonist (D-APV, 0.05 mM; Ascent Scientific) dissolved in aCSF was present throughout the recording. A tungsten bipolar electrode (Frederick Haer Company, Bowdoin, ME) was placed in the *stratum radiatum* proximal to the *stratum pyramidale*, and the Schaffer collateral/commissural fibers and inhibitory inputs were stimulated at 0.1 Hz. GABA_AR-IPSC was first measured at $V_{\text{hold}} = 0$ mV. After obtaining forty to fifty consecutive stable IPSC responses, picrotoxin (0.10 mM; Sigma-Aldrich) was added to aCSF to eliminate the IPSC. Then, AMPAR-EPSCs were evoked at $V_{\text{hold}} = -60$ mV without changing the stimulus strength. Stimulus strength was set to produce an IPSC amplitude of ~ 1000 pA which leads to ~ 50 pA of AMPAR-EPSC. Measurements of GABA_AR-mediated miniature IPSCs (mIPSCs) were performed in the presence of D-APV, NBQX and tetrodotoxin (0.001 mM; Ascent Scientific). AMPAR-mediated miniature and spontaneous EPSCs (mEPSCs and sEPSCs) in PV+ neurons were

measured in the presence of picrotoxin and with or without tetrodotoxin, respectively. In Figure 3, all recorded PV+ neurons were filled with biocytin in order to identify PV+ basket cells. The measurement of firing activity was performed against PV+ neurons that established gigaohm seals ($> 2 \text{ G}\Omega$) under cell-attached voltage-clamp mode. Miniature and spontaneous synaptic events were analyzed using Mini Analysis software (Synaptosoft, Decatur, GA). Approximately three hundred events were sampled from each experiment; only events $>5 \text{ pA}$ were analyzed.

Dual whole-cell recordings were performed to monitor PV+ neuron-mediated unitary inhibitory synaptic transmission. RFP-positive neurons that were proximal to hippocampal CA1 stratum pyramidale, presumably basket and axo-axonic PV+ neurons, were chosen as presynaptic neurons. The neighboring CA1 pyramidal neurons (within $50 \mu\text{m}$ radius from PV+ neurons) were selected as postsynaptic neurons. Cesium-based internal solution was used for double whole-cell recordings of presynaptic interneurons. Pre- and postsynaptic neurons were voltage-clamped under -70 and 0 mV , respectively. Inhibitory synaptic transmission was evoked by applying one or two 70 mV depolarization pulses ($2\text{--}3 \text{ ms}$ duration, 50 ms interval) at 0.1 Hz . Consecutive paired stimuli ($25\text{--}50$ times) were applied to presynaptic neurons, and responses larger than 10 pA observed within 5 ms after the onset of depolarization pulses were considered as evoked unitary IPSCs. If any evoked response was observed during this period, the pair was considered synaptically connected.

All experiments and the analysis of data were performed in a blind manner. Recordings were performed using a MultiClamp 700B amplifier and Digidata 1440, and data were acquired and analyzed using Clampex 10 and Clampfit 10 (Molecular Devices, Union City, CA).

Statistical analysis

Results are reported as mean $\pm$ SEM. The statistical significance was evaluated by two-way ANOVA for multiple comparison, and by Student's *t*-test, paired *t*-test or Wilcoxon signed rank *t*-test with equal variance test for two-group comparison. Statistical significance was set at $p < 0.05$.

RESULTS

Shank1 is highly expressed in PV+ inhibitory interneurons

Shank family scaffolds are densely localized in spines of excitatory and medium-spiny neurons (Jiang & Ehlers, 2013). To investigate the expression of Shank in hippocampal non-spiny inhibitory interneurons, we performed double immunostaining against Shank1 and inhibitory neuronal markers. Importantly, we identified that Shank1 is highly expressed in adult hippocampal PV+ interneurons in wild-type but, as expected, not in Shank1^{-/-} mice (Fig. 1A). PV+ neurons in hippocampal CA1 area were highly reactive against Shank1 antibody (Fig. 1B). We compared the signal of Shank1 staining in the dendritic segment of PV+ neurons and the hippocampal CA1 stratum radiatum area where most pyramidal neurons form excitatory synapses. Both the size and the signal intensity of Shank1 puncta are significantly higher in PV+ neurons (averaged size of Shank1 puncta: dendritic segment of PV+ neurons, $0.55 \pm 0.05 \mu\text{m}^2$, hippocampal stratum radiatum area, 0.26 ± 0.02 , $p <$

0.0001, Wilcoxon signed rank *t*-test; averaged Shank1 signal intensity: dendritic segment of PV+ neurons, 2195.4 ± 108.8 arbitrary units; hippocampal stratum radiatum area, 2050.6 ± 100.5 ; $p < 0.001$, paired *t*-test. $N = 28$ PV neurons/3 mice). In addition, we observed strong VGluT1 signals, a marker of glutamatergic terminals, proximal to Shank1 puncta in PV+ neurons (Fig. 1C). These results suggest that Shank1 may play a key role at excitatory synapses in PV+ interneurons. These prominent Shank1 signals were not co-localized with other interneuronal markers, including Calb- and Calr-expressing inhibitory interneurons (Fig. 1D and E).

We also measured the expression of SHANK genes in PV+ neurons. PV+ neurons in PV-RFP mouse hippocampi (which specifically express red fluorescent protein in PV+ neurons) were dissected by LCM, and, using qPCR, we confirmed that all SHANK genes are expressed in both PV+ and pyramidal neurons (Fig. 1F).

Altered post synaptic density proteins in PV+ neurons lacking Shank1

Shank1 protein exclusively localizes at excitatory synapses in excitatory neurons and regulates spine structure (Sala *et al.*, 2001). However, the role of Shank1 in non-spiny neurons, such as PV+ inhibitory interneurons, has not been addressed. We next examined the expression of synaptic proteins in PV+ interneurons in dissociated hippocampal neurons cultured from wild-type or Shank1^{-/-} mice. As expected, Shank1 was easily detectable in wild-type interneurons but was not observed in PV+ interneurons from mutant mice (Fig. 2A and 2B).

Wild-type Shank1 is partially co-localized at dendritic clusters with the excitatory synapse marker PSD-95 (Fig. 2A). Similar to excitatory neurons where $45.2 \pm 10.8\%$ of Shank1 puncta co-localized with PSD-95 clusters ($n = 15$ neurons, image not shown), the Shank1 puncta in PV+ neurons also co-localized with PSD-95 ($32.1 \pm 9.8\%$; $n = 10$ neurons). It should be noted that the population of neurons with co-localized Shank1 and PSD-95 is much smaller than that in other reports because of the fixation method used in this study (see Materials and Methods) (Hung *et al.*, 2008). In Shank1^{-/-} PV+ neurons, the density of Shank puncta (labeled with pan-Shank antibody) along the dendrite was reduced to $\approx 50\%$ (Fig. 2B and 2C). The remaining Shank staining, presumably from Shank2 and Shank3 proteins, still localized at excitatory synapses. Furthermore, immunoreactivity against GKAP, PSD-95 and GluA1 were significantly reduced in Shank1^{-/-} PV+ neurons compared with wild-type neurons (Fig. 2C). There was no difference between cultured wild-type and Shank1^{-/-} PV+ interneurons in cluster density or staining pattern of Homer and Bassoon. These results indicate that Shank1 is important for the assembly of excitatory postsynaptic structure in PV+ neurons.

PV+ basket cells in Shank1^{-/-} mice exhibit reduced basal excitatory synaptic transmission

To study the function of Shank1 in PV+ neurons, we generated Shank1 wild-type and Shank1^{-/-} mice that express RFP in PV+ neurons, and measured basal excitatory synaptic transmission from RFP-positive neurons. We chose PV+ basket neurons, one of the major PV+ cell-types in CA1 *stratum pyramidale*, and performed whole-cell recordings to measure AMPAR-mediated spontaneous and miniature EPSCs (sEPSCs and mEPSCs) (Fig. 3A – F).

The amplitudes and frequencies of both sEPSCs and mEPSCs in PV+ Shank1^{-/-} basket cells were lower than those of wild-type mice (Fig. 3B and 3E). In addition, sEPSCs in PV+ Shank1^{-/-} basket cells exhibited slower rise times compared with that of wild-type mice (Fig. 3C and 3F). The AMPA receptor-mediated paired-pulse facilitation (PPF), a type of short-term synaptic plasticity that measures the change of presynaptic release probability, was similar in wild-type and Shank1^{-/-} slices [PPF induced by 50 ms of inter-stimulus interval: wild-type, 1.57 ± 0.19 (n = 8); Shank1^{-/-}, 1.60 ± 0.13 (n = 7); $p > 0.8$, Student *t*-test], implying that the change in sEPSC frequency in Shank1^{-/-} PV+ neurons is due to the reduction of the number of functional excitatory synapses. These results are consistent with our immunocytochemical studies indicating a reduction in the expression of the excitatory postsynaptic markers GKAP, PSD-95 and GluA1 in Shank1^{-/-} PV+ neurons (Fig. 2B and 2C). GluA1 is one of the major AMPAR subunits in PV+ neurons and regulates excitatory synaptic transmission in hippocampal PV+ neurons (Fuchs *et al.*, 2007) while PSD-95 is critical for AMPAR trafficking at synapses (Elias & Nicoll, 2007). Therefore, it is possible that the reduced GluA1 and PSD-95 signals in Shank1^{-/-} PV+ neurons caused the changes in amplitude and kinetics of sEPSCs. This result highlights that Shank1 in PV+ neurons has a different role in regulating excitatory synapses compared with that in pyramidal neurons, since CA1 pyramidal neurons exhibited only reduced miniature EPSC frequency (Hung, *et al.*, 2008).

Shank1^{-/-} mice display normal membrane excitability in PV+ neurons

It has been reported that Shank1 and Shank3 directly bind to Ca_v1.3, L-type Ca²⁺ channel subunits (Zhang *et al.*, 2005). In addition, Shank1 regulates Ca²⁺-sensitive Big K⁺-channel activity (Sala *et al.*, 2005) that shapes the width of action potentials by regulating after-hyperpolarization of action potentials at presynaptic and postsynaptic sites (Hu *et al.*, 2001; Sailer *et al.*, 2006; Matthews *et al.*, 2008). Thus, Shank1 may regulate not only excitatory synaptic transmission but also intrinsic membrane excitability in PV+ neurons.

One of the most prominent membrane properties of PV+ neurons is their firing capability, which underlies their characterization as “fast-spiking” neurons. Therefore, we compared the firing patterns of CA1 PV+ neurons in wild-type and Shank1^{-/-} mice. We performed whole-cell current clamp recordings from RFP-positive PV+ neurons and injected a series of current pulses into the neurons. All recorded RFP-positive neurons displayed fast-spiking properties. Neither the firing frequencies (Fig. 3G) nor the basic membrane properties of FS-interneurons in Shank1^{-/-} mice were different from those of wild-type PV+ neurons [resting membrane potentials: wild-type, -61.0 ± 1.1 (n = 8); Shank1^{-/-}, -59.9 ± 0.7 mV (n = 7); series resistance: wild-type, 20.6 ± 1.3 ; Shank1^{-/-}, 21.1 ± 1.6 MΩ; input resistance: wild-type, 122.6 ± 21.1 ; Shank1^{-/-}, 109.7 ± 9.7 MΩ; $p > 0.3$, Student *t*-test]. These results indicate that Shank1 does not play a major role in the membrane excitability of PV+ neurons.

Shank1^{-/-} mice show reduced PV+ neuron-mediated inhibitory synaptic transmission

The reduced excitatory synaptic transmission in Shank1^{-/-} PV+ neurons may decrease basal activity in these cells. We therefore performed cell-attached patch-clamp recording and measured PV+ neuronal excitability in hippocampal CA1 area (Fig. 4A). Interestingly, PV+

neurons in Shank1^{-/-} slices exhibited lower firing rates compared with those of wild-type slices. This result suggests that the reduced excitatory synaptic transmission in PV+ neurons (Fig. 3A–F) caused a decrease in their firing rates in Shank1^{-/-} slices.

Does the reduced firing rates of PV+ neurons in Shank1^{-/-} mice change the inhibitory outputs onto postsynaptic pyramidal neurons? It has been reported that glutamatergic inputs to GABAergic neurons modulate their inhibitory outputs (Chang *et al.*, 2014). Chronic reduction of PV+ neuronal activity may decrease their inhibitory outputs onto CA1 pyramidal neurons. To address this question, we performed simultaneous pre- and postsynaptic dual whole-cell voltage-clamp recordings from RFP-positive presynaptic CA1 PV+ neurons and postsynaptic pyramidal neurons located within 50 μ m of PV+ neurons in wild-type and Shank1^{-/-} mice (Fig. 4C and 4D). PV+ neurons were identified by RFP fluorescence (Fig. 4C, *left*). Presynaptic GABA release was evoked by applying single or double depolarization commands (Fig. 4C, **middle and right**). The amplitude of PV+ neuron-mediated inhibitory synaptic transmission and connective frequency were reduced in Shank1^{-/-} mice compared with that of wild-type mice (Fig. 4D, **left and middle**). Shank1^{-/-} PV+ neurons exhibited essentially similar levels of paired-pulse ratio as wild-type (Fig. 4D, *right*), suggesting that the reduced inhibitory synaptic transmission and connective frequency are not attributable to the difference of presynaptic release probability. These results suggest that reduced excitatory synaptic transmission in PV+ neurons weakens the inhibitory outputs of PV+ neurons to pyramidal neurons, although it is possible that the null knockout of Shank1 can indirectly change synaptic function in hippocampus.

Shank1^{-/-} mice show increased excitatory and inhibitory balance

What is the consequence of reduced PV+ neuron-mediated inhibitory synaptic transmission in the hippocampal CA1 circuit? The *E-I balance* plays a critical role in cognitive behavior (Zikopoulos & Barbas, 2013). Abnormal *E-I balance*, as caused by the dysfunction or loss of inhibitory interneurons, has been suggested as a pathophysiological hallmark in ASD, schizophrenia and animal models of these disorders (Rubenstein & Merzenich, 2003; Gatto & Broadie, 2010; Markram & Markram, 2010; LeBlanc & Fagiolini, 2011; Yizhar *et al.*, 2011). To address whether the abnormal PV+ neuron-mediated inhibitory transmission in Shank1^{-/-} mice leads to a *E-I imbalance*, we measured the *E-I balance* in hippocampal CA1 pyramidal neurons using an approach previously described (Futai *et al.*, 2013). We divided the amplitude of AMPAR-mediated excitatory postsynaptic currents (AMPA-EPSCs) by GABA_A-mediated inhibitory postsynaptic currents (GABA_A-IPSCs) in the same cells and refer to this as the *E-I ratio*.

Interestingly, the *E-I ratio* of Shank1^{-/-} mice was significantly higher than that of wild-type animals (Fig. 5A). It has been reported that excitatory synaptic strength is moderately reduced in Shank1^{-/-} hippocampal CA1 pyramidal neurons (Hung *et al.*, 2008); therefore, the degree of IPSC reduction in Shank1^{-/-} mice must be more severe than the decrease of excitatory synaptic transmission to account for the *E-I imbalance* that we detected. Shank1^{-/-} showed essentially the same paired-pulse depression (PPD) induced by the double stimulation of synaptic inputs with 50 ms of inter-pulse interval as wild-type (wild-type: 0.64 ± 0.03 , $n = 20$ from 6 mice; Shank1^{-/-}: 0.67 ± 0.02 , $n = 24/6$; $p = 0.36$, Student *t*-

test). Paired-pulse depression is one form of short-term synaptic plasticity that reflects the change of presynaptic release probability. Therefore, this result indicates that Shank1 knockout does not change the inhibitory presynaptic release probability.

We next examined the amplitudes and frequencies of the GABA_AR-mediated miniature IPSCs (Fig. 5B). The frequencies, but not the amplitudes, of the mIPSCs from Shank1^{-/-} mice were significantly reduced, suggesting that the increased *E-I ratio* is caused in large part by a reduction in the number of functional inhibitory synapses.

Gephyrin expression is down regulated in Shank1^{-/-} mice

To further investigate the mechanisms underlying the shift of *E-I balance*, we compared the expression of synaptic proteins in membrane fractions of Shank1^{-/-} and wild-type mice. Interestingly, expression of gephyrin, a scaffold molecule of inhibitory synapses, was significantly reduced in Shank1^{-/-} membrane fractions (Fig. 5C), consistent with the impairment of inhibitory transmission in Shank1^{-/-} mice. We next examined expression of gephyrin histochemically in the hippocampal CA1 area. Consistent with our western blot data, the size of gephyrin immunoreactivity in CA1 *stratum radiatum* (SR) and *pyramidale* (SP) in Shank1^{-/-} mice are reduced compared with Shank1 wild-type mice (averaged size of gephyrin puncta: wild-type, $0.09 \pm 0.01 \mu\text{m}^2$; Shank1^{-/-}, $0.05 \pm 0.002 \mu\text{m}^2$, $p < 0.01$; averaged puncta densities: wild-type, 13.73 ± 0.15 puncta / $100 \mu\text{m}^2$; Shank1^{-/-}, 13.18 ± 0.39 , $p = 0.49$, Student *t-test*. N = 4 brains from each genotype). (Fig. 5D). The density of gephyrin puncta in Shank1^{-/-} was comparable to that of wild-type mice, which is not consistent with our mIPSC results that showed that Shank1 KO mice exhibit normal mIPSC amplitudes (Fig. 5B). In this regard, it is interesting to note that gephyrin knockout moderately reduces mIPSC amplitude compared with wild-type, and further, that knockdown of gephyrin reduces the number of GABA_AR clusters (Levi *et al.*, 2004; Jacob *et al.*, 2005). Thus, subtle reduction of gephyrin may have more impact on the number of active synapses rather than the number of GABA_AR per synapse.

DISCUSSION

Molecular architecture of excitatory synapses in inhibitory neurons

Shank proteins serve as scaffolds for a variety of postsynaptic molecules, including GKAP, Homer, L-type Ca²⁺ channels and actin regulatory molecules, such as cortactin, Sharpin, α -fodrin, Abp1 and β -Pix (Jiang & Ehlers, 2013). Shank proteins are targeted to postsynaptic sites in spiny neurons and regulate spine size, which determines the number of AMPA receptors on the synapses (Naisbitt *et al.*, 1999; Matsuzaki *et al.*, 2001). Ectopic expression of Shanks enlarges spines in excitatory spiny neurons and induces spinogenesis in non-spiny neurons (Sala *et al.*, 2001; Roussignol *et al.*, 2005). However, previous studies have not addressed the physiological and structural roles of Shanks in non-spiny inhibitory neurons.

In the present study, we demonstrated that Shank1 is highly expressed in PV+ neurons. Furthermore, we confirmed the expression of major synaptic molecules, such as PSD-95, GKAP and Homer, in PV+ neurons, indicating that the composition of the postsynaptic architecture in inhibitory neurons is similar to that of excitatory neurons. However, the

consequences of Shank1 knockout in PV+ basket cells are more severe than that in pyramidal neurons. Shank1 deficit reduced spine size and the frequency of mEPSCs in pyramidal neurons, while the expression of other postsynaptic molecules is comparable to that in wild-type neurons (Hung *et al.*, 2008). In contrast, the absence of Shank1 protein in PV+ interneurons caused reduced expression of PSD-95, GKAP and GluA1, and led to abnormal frequency, amplitude and kinetics of sEPSCs and mEPSCs (Fig. 2 and 3). While the possibility that conventional Shank1 knockout can dysregulate PV+ neuronal function through non-cell autonomous fashion cannot be excluded, our results suggest that Shank1 plays an important role in the assembly of synaptic molecules at postsynaptic membranes in PV+ neurons. It is possible that the differential impact of Shank1 knockout between excitatory and PV+ neurons is a consequence of the distinct structures of the excitatory synapses in these two classes of neurons. The spine structure in excitatory neurons may act as a barrier of diffusion of postsynaptic proteins, thus minimizing the knockout effect of Shank1. In contrast, excitatory synapses in inhibitory interneurons, which mostly form directly on the dendritic shaft, may rely on the scaffold proteins to maintain synaptic structure (Douglas & Martin, 1998).

Roles of Shank scaffolds in *E-I balance*

We have demonstrated that Shank1 is highly expressed in PV+ neurons and regulates excitatory synaptic transmission in PV+ basket cells. Furthermore, Shank1^{-/-} mice display increased *E-I balance* in hippocampal CA1 pyramidal neurons due to the reduction of inhibitory synaptic transmission and reduced gephyrin expression. Since knockout or knockdown of gephyrin reduces GABA_AR-mediated currents by disrupting receptor function without changing total expression of GABA_ARs (Kneussel *et al.*, 1999; Levi *et al.*, 2004; Jacob *et al.*, 2005), these results complement each other. However, it is unclear how a Shank1 deficit causes abnormal inhibitory synaptic transmission as Shank1 is exclusively localized to excitatory postsynaptic sites. We found that Shank1^{-/-} PV+ neurons display reduced excitatory synaptic transmission and diminished firing rates. Neuronal activity is critical for synaptogenesis in the brain (Lu *et al.*, 2009) and excitatory inputs modulate the properties of synaptic outputs in GABAergic neurons (Chang *et al.*, 2014). It is possible that the weakened PV+ neuronal activity reduces inhibitory synaptic output on pyramidal neurons, and this may lead to a subsequent decrease in gephyrin expression. Generating a PV+ neuron-specific Shank1 knockout would allow us to directly address the roles of Shank1 protein in PV+ neurons on *E-I balance*. An alternative possibility that explains the reduction of inhibition in Shank1^{-/-} mice is the contribution of neuronal homeostasis. It has been reported that excitatory synaptic transmission in pyramidal neurons is slightly reduced in Shank1^{-/-} mice (Hung *et al.*, 2008). Therefore, reduced excitatory synaptic transmission in pyramidal neurons may cause the homeostatic reduction of inhibition, including a reduced number of active inhibitory synapses and expression of gephyrin, in a cell autonomous manner.

We evaluated *E-I ratio* in pyramidal neurons by taking the ratio of evoked EPSC and IPSC responses that are derived from non-specific synaptic inputs from different types of neurons, and observed increased *E-I ratio* in Shank1^{-/-} pyramidal neurons (Fig. 5A). In addition, we detected reduced inhibition in Shank1^{-/-} pyramidal neurons by recording mIPSCs that

measures the activity of all GABAergic synapses in pyramidal neurons (Fig. 5B). Therefore, reduced inhibitory synaptic transmission may not be solely due to abnormal inhibition through PV+ neurons. Other interneuronal types that exhibit lower clustering of Shank1 compared with PV+ neurons may also be dysregulated by Shank1 knockout.

Detailed behavioral tests have shown that Shank1^{-/-} mice exhibit better performance in radial maze task, impaired memory retention, impaired contextual fear memory, abnormal ultrasonic vocalization and scent marking behavior (Hung *et al.*, 2008; Silverman *et al.*, 2011; Wöhr *et al.*, 2011). In contrast to these intriguing phenotypes, the physiological characteristics previously observed in this mouse line were only subtly perturbed: moderately reduced basal excitatory synaptic transmission in CA1 pyramidal neurons, normal NMDAR-dependent long-term potentiation (LTP) and long term depression, and intact protein synthesis-dependent LTP.

Our data suggest that abnormal *E-I balance* can be a pathophysiological hallmark of Shank1^{-/-} mice exhibiting abnormal behavioral phenotypes reminiscent of neuropsychiatric disorders. Importantly, Shank3 transgenic mice also exhibit dysregulation of *E-I balance*, reduced gephyrin expression and normal Hebbian type of synaptic plasticity (Han *et al.*, 2013). The similarities of physiological phenotypes between Shank1^{-/-} and Shank3 transgenic mice highlight the potential significance of Shank mediated-*E-I balance* in neuropsychiatric disorders.

SHANK2 and SHANK3 are also characterized as disease-associated genes (Guilmatre *et al.*, 2013; Jiang & Ehlers, 2013) and their transcripts are expressed in hippocampal CA1 PV+ neurons (Fig. 1E). It is of paramount interest to elucidate the roles of Shank2 and Shank3 proteins in PV+ interneurons in hippocampus and cortex.

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Abbreviations

ASD	autism spectrum disorder
aCSF	artificial cerebrospinal fluid
E-I balance	excitatory and inhibitory balance
EPSC	excitatory postsynaptic current
IPSC	inhibitory postsynaptic current
LCM	laser capture microdissection
LTP	long-term potentiation
PV	Parvalbumin

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Figure 1. Shank1 is highly expressed in Parvalbumin-expressing interneurons

(A, B) Confocal images of double-labeled immunofluorescence staining against parvalbumin (PV, red) and Shank1 (green) from hippocampal CA1 pyramidal cell layer of wild-type ($+/+$) (left) and $Shank1^{-/-}$ ($-/-$) (right) mice. Note that the immunoreactivity of Shank1 in $Shank1^{-/-}$ tissue is minimal, confirming the specificity of the antibody. Enlargements of the indicated rectangular fields are shown at bottom. (B) Relative proportion of hippocampal CA1 PV+ neurons as a fraction of neurons decorated by Shank1 signals (left), and that of Shank1-decorated neurons as a fraction of PV+ neurons (right).

Numbers of neurons obtained from 4 wild-type mice were: Shank1-decorated PV+ neurons, 137; PV+ and Shank1 non-decorated neurons, 14; PV negative and Shank1 positive decorated neurons, 22. Note that the majority of PV+ neurons were immunopositive for Shank1. (C) Confocal images of triple-labeled immunofluorescence staining against PV (red), Shank1 (green) and VGluT1 (red) from proximal region of hippocampal CA1 stratum pyramidale (SP) of a wild-type mouse. Note that the dense VGluT1 signals (arrow heads) were localized close to Shank1 puncta in PV+ neurons. (D, E) Confocal images of double-labeled immunofluorescence staining against calbindin (Calb, D, red), calretinin (Calr, E, red) and Shank1 (green) from hippocampal CA1 stratum pyramidale (SP) and radiatum (SR) regions of wild-type (+/+) mice. (F) Relative expression of SHANK genes (SHANK1, SHANK2 and SHANK3) in PV+ and pyramidal (Py) neurons in adult mouse hippocampus. The expression level was normalized to that of GAPDH. Approximately 1000 PV+ and pyramidal neurons in hippocampal CA1 pyramidal cell layer per mouse were cut from one PV-RFP mouse (N = 6–8 mice). Scale bars: 10 μ m.

Figure 2. Altered expression of postsynaptic proteins in Shank1-deficient PV+ neurons

Triple- or double-labeled immunofluorescence staining for PV and synaptic proteins in hippocampal primary cultures (14 DIV) prepared from wild-type (+/+) or Shank1^{-/-} (-/-) mice. (A) PV+ interneurons in wild-type and Shank1^{-/-} cultures were triple-stained for PV, Shank1 and PSD-95. (B) Double-immunostaining for pan-Shank, PSD-95, GKAP, Homer, GluA1, or Bassoon, and PV. (C) Quantification of puncta density per 10 μm dendrite length (left) and puncta area (right) for the indicated proteins. Shank1^{-/-} dendrites show a significant reduction in pan-Shank, PSD-95, GKAP and GluA1 (n = 10 cells from 3 mice, 3 independent cultures) signals. The error bars show standard error. Scale bars, 10 μm. *, p < 0.05; **, p < 0.01; ***, p < 0.001, *student t-test*.

Figure 3. Shank1 regulates excitatory synaptic transmission in Parvalbumin-expressing basket cells

(A, D) *Top*, Consecutive sample sEPSC (A) and mEPSC (D) traces of PV+ basket cells in wild-type (black traces) and Shank1^{-/-} (grey traces) mice. *Bottom*, Averaged and normalized sample sEPSC (A) and mEPSC (D) traces in PV+ basket cells. (B, C, E and F) Summary of the frequency and amplitude (B and E), and kinetics (C and F) of sEPSCs and mEPSCs in wild-type (+/+) and Shank1^{-/-} (-/-) animals. The averaged EPSC frequency and amplitude for each cell were superimposed as circles while the bar graphs indicate the means $\pm$ SEM. Number of cells: sEPSC: wild-type, 16 cells from 7 mice; Shank1^{-/-}, 19 / 11; mEPSC: wild-type, 10 cells from 5 mice; Shank1^{-/-}, 10 / 4. (G) *Left*, Sample traces from CA1 fast-spiking PV+ basket cells in wild-type and Shank1^{-/-} hippocampal slices showing spikes elicited by current injections of 300 and 900 pA for 400 ms. *Right*, Summary graph of the frequency of action potentials in wild-type and Shank1^{-/-} animals. Input-output relationship [number of spikes elicited versus amount of current injection (400 ms duration)] was plotted for wild-type and Shank1^{-/-} animals. Neurons were held at the indicated resting membrane potentials. Number of cells: wild-type, 10 cells from 7 mice; Shank1^{-/-}, 11 / 6.

Figure 4. Shank1 deficit causes reduced basal firing rate in PV+ neurons and PV neuron-mediated inhibitory synaptic output onto CA1 pyramidal neurons
 (A) Sample traces of PV+ neurons in wild-type (black traces) and *Shank1*^{-/-} (gray traces) mice. (B) Summary graph of the firing rates measured by cell-attached recordings of PV+ neurons in wild-type and *Shank1*^{-/-} animals. Number of cells: wild-type, 7 cells from 7 mice; *Shank1*^{-/-}, 6 / 5. (C–D) Effect of Shank1 deficiency on unitary inhibitory synaptic transmission between hippocampal CA1 PV+ and pyramidal neurons. (C) Left, superimposed fluorescent and Nomarski images. Averaged sample unitary IPSC (uIPSC) traces by one (middle) and double (right) presynaptically applied depolarization commands. (D) Summary of uIPSC amplitude (left), connectivity (middle) and paired-pulse ratio (right).

Number in each bar represents the number of synaptically connected cell pairs (left and right), total number of cell pairs tested (middle).

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Figure 5. Shank1 deficit causes reduced *E-I* ratio by reducing inhibitory synaptic function and gephyrin expression

(A) Shank1^{-/-} mice display reduced *E-I* ratio compared with wild-type mice. *Left*, Sample traces (average of 10 consecutive responses) mediated by GABA_AR (upward) and AMPAR (downward) from wild-type (+/+) or Shank1^{-/-} (-/-) hippocampal slices. Stimulus artifacts were truncated. Calibration, 200 and 50 pA, 20 msec. *Right*, Summary graph of *E-I* ratio of wild-type (total n = 20 cells from 6 mice) and Shank1^{-/-} mice (n = 24 / 6, student *t*-test) (see text for definition). (B) Shank1^{-/-} mice reduce inhibitory synaptic transmission. *Left*, Sample traces of mIPSC events (top) and the average traces of mIPSC traces (bottom) obtained from wild-type (+/+) or Shank1^{-/-} (-/-) hippocampal slices. The averaged traces scaled to match the amplitude and aligned at the onset of response are shown at the bottom right (norm). Note that the time course of events is the same. Calibration, 40 pA, 500 msec (top), 5 pA, 20 msec (bottom). *Right*, Summary graphs of the frequency (left) and amplitude (right) of mIPSCs in wild-type (+/+) and Shank1^{-/-} (-/-) animals. Number of cells: wild-

type, total 9 cells from 3 mice; Shank1^{-/-}, 9 / 3. (C, D, E) Shank1^{-/-} mice exhibit reduced expression of gephyrin. (C) *Left*, Immunoblot analysis of membrane (P2) fractions from individual wild-type (+/+) and Shank1^{-/-} (-/-) mice for the indicated proteins. *Right*, Quantitation of various proteins in membrane fractions. Syn: Synaptophysin. (D) Confocal images of gephyrin puncta in hippocampal CA1 stratum pyramidale (SP) and radiatum (SR) areas from wild-type and Shank1^{-/-} mice. *Left*, double-labeled staining for gephyrin and DAPI. *Right*, the gephyrin images were deconvoluted and quantified after thresholding of fluorescence intensity (grey images).