GSK3β inhibition restores cortical gamma oscillation and cognitive behavior in a mouse model of NMDA receptor hypofunction relevant to schizophrenia

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Cortical gamma oscillations are believed to be involved in mental processes which are disturbed in schizophrenia. For example, the magnitudes of sensory-evoked oscillations, as measured by auditory steady-state responses (ASSRs) at 40 Hz, are robustly diminished, whereas the baseline gamma power is enhanced in schizophrenia. Such dual gamma oscillation abnormalities are also present in a mouse model of N-methyl-D-aspartate receptor hypofunction (Ppp1r2cre/Grin1 knockout mice). However, it is unclear whether the abnormal gamma oscillations are associated with dysfunction in schizophrenia. We found that glycogen synthase kinase-3 (GSK3) is overactivated in corticolimbic parvalbumin-positive GABAergic interneurons in Grin1 mutant mice. Here we addressed whether GSK3β inhibition reverses both abnormal gamma oscillations and behavioral deficits with high correlation by pharmacological and genetic approach. We demonstrated that the paralog selective-GSK3β inhibitor, but not GSK3α inhibitor, normalizes the diminished ASSRs, excessive baseline gamma power, and deficits in spatial working memory and prepulse inhibition (PPI) of acoustic startle in Grin1 mutant mice. Cell-type specific GSK3β knockdown, but not GSK3A knockdown, also reversed abnormal gamma oscillations and behavioral deficits. Moreover, GSK3β knockdown, but not GSK3A knockdown, reverses the mutants' in vivo spike synchrony deficits. Finally, ex vivo patch-clamp recording from pairs of neighboring cortical pyramidal neurons showed a reduction of synchronous spontaneous inhibitory-postsynaptic-current events in mutants, which was reversed by GSK3β inhibition genetically and pharmacologically. Together, GSK3β inhibition in corticolimbic interneurons ameliorates the deficits in spatial working memory and PPI, presumably by restoration of synchronous GABA release, synchronous spike firing, and evoked-gamma power increase with lowered baseline power.

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INTRODUCTION

The disturbance of neural oscillations in the gamma frequency band (30–100 Hz) is considered to be a core pathophysiologic feature of schizophrenia, particularly in the generation of cognitive dysfunction [1, 2]. For example, clinical electroencephalogram has shown reduction of auditory steady-state responses (ASSRs) at 40 Hz in patients with schizophrenia, which are electrophysiologic responses entrained to the frequency and phase of a periodic auditory stimulus generated by auditory cortex activity [3, 4]. The impairment of visual-evoked gamma oscillation in schizophrenia was also observed during Gestalt stimuli [5]. In addition to impairment in evoked gamma oscillations, spontaneous or baseline gamma-band cortical activity during the resting state is also reported in schizophrenia [6–8]. However, it is unclear whether such abnormal gamma oscillations are causally linked to cognitive impairments in schizophrenia.

Preclinically, administration of uncompetitive N-methyl-D-aspartate receptor (NMDAR) antagonists, such as ketamine and MK-801, in rodents dose-dependently reduces sensory-evoked gamma responses [9, 10] and increases spontaneous or baseline gamma oscillations [11–13]. Furthermore, positive associations have been reported between abnormalities in EEG gamma power and deficits in prepulse inhibition (PPI) of acoustic startle induced by the NMDAR antagonists, suggesting that gamma power abnormalities may be responsible for sensory processing disturbance [14]. Schizophrenia-related behaviors, including PPI and Y-maze alternation (to assess spatial working memory), are also defective in a genetically-engineered mouse model of NMDAR hypofunction [15]. In this model, indispensable NMDAR subunit Grin1 is missing from ~50% of cortical and hippocampal GABAergic neurons, a majority of which are parvalbumin (PV)-positive fast-spiking interneurons, in early postnatal development (Ppp1r2cre/Grin1 knockout (KO) mice) [16]. Notably, 40-Hz click train-evoked ASSR deficits and spontaneous local field potential broad-band power increase in the pre-stimulus period are both observed in this mutants [17]. Genetic Grin1 deletion selectively from PV neurons in awake mice also resulted in increased baseline gamma power [18, 19] and impaired optogenetically evoked-gamma oscillations [19], suggesting that Grin1-deleted PV neurons play a role in abnormal gamma oscillations. However, it remains to be
addressed whether abnormal gamma oscillations are strongly associated with cognitive dysfunction in the genetic NMDAR hypofunction model.

Glycogen synthase kinase-3 (GSK3) is a Ser/Thr protein kinase that is ubiquitously expressed in all mammalian tissues and subcellular organelles, including the brain with high expression. Accumulating evidence suggests that GSK3 regulatory pathways are altered and GSK3 activity increases in the schizophrenia [20, 21]. GSK3 comprises two structurally and functionally related serine/threonine kinases encoded by two distinct genes, GSK3A and GSK3B. Both kinases are inherently active under resting conditions, and are primarily regulated by phosphorylation at two levels: (i) inhibitory phosphorylation of serine residues S21/S9 in GSK3α/β and (ii) tyrosine phosphorylation at Y279/Y216 in GSK3α/β, which augments their activity and relieves substrate-priming by other kinases [22]. Notably, nonselective GSK3 inhibitors have been shown to alleviate the behavioral impairments induced by ketamine treatment [23].

Here we report that proper GSK3β activity in corticolimbic GABAergic neurons is crucial for the emergence of abnormal gamma oscillations and cognitive behavior in aforementioned Grin1 mouse model [24]. Although there was no difference in GSK3α expression levels ([16]) and GSK3-phospho (Y279/Y216) in PV-negative interneurons or non-PV neurons. The corrected total cell fluorescence (CTCF) was calculated as previously described [26]. Results are presented as normalized fluorescence, which is the ratio

MATERIALS AND METHODS
All experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham and Southern Research. For detailed experiential procedures, see Supplementary Information.

Animals
We employed Ppp1r2-cre/floxed-Grin1 KO mice (or simply Grin1 mutant mice), in which genetic deletion of obligatory Grin1 subunit is introduced in ~50% cortical and hippocampal GABAergic interneurons from the postnatal second week [16]. To reduce the kinase activity of GSK3A or GSK3B in Grin1-deleted GABAergic neurons, either floxed-GSK3A or floxed-GSK3B mouse strain was bred to Grin1 mutant mice to generate GABAergic neuron-specific GSK3 heterozygous KO mice (namely, GSK3A- or GSK3B-knockdown mice). The generation of floxed-GSK3A mouse strain is described in Supplementary Fig. S1. The floxed-GSK3B strain was obtained from Dr. J. Woodgett [25].

Immunohistochemistry
Immunohistochemistry was performed as previously described [16]. Briefly, sections containing auditory cortex were double-immunostained with rabbit anti-GSK3α (1:200, G08-63R-25, Signal-Chem, USA), rabbit anti-GSK3β (1:1000, ab32391, Abcam, USA), or rabbit GSK3β phospho-Y216 (1:150, ab75745 Abcam, USA), and mouse anti-PV (1:5000, 235, Swant, Switzerland). Images of the auditory cortex were captured using confocal microscope (Nikon A1). NIH ImageJ software was used to measure the integrated density of GSK3α, GSK3β and GSK3-phospho (Y279/Y216) in PV-positive interneurons or non-PV neurons. The corrected total cell fluorescence (CTCF) was calculated as previously described [26].

Fig. 1 Up-regulation of glycogen synthase kinase 3 (GSK3) in cortical Grin1-deleted PV-positive neurons. Immunoreactivity levels in the somata of cortical PV neurons against GSK3α, GSK3β, or phosphorylated-Y216-GSK3β (Y279-GSK3α), normalized by the average levels of each immunoreactivity (IR) of the non-PV (pyramidal) neurons, and are shown by the level of floxed-Grin1 control non-PV neurons as 100%. Although there was no difference in GSK3α (a) and GSK3β (b) expression levels (b), the levels of phosphorylated GSK3 at Tyrosine residues (c) were increased in the PV neurons of Grin1 mutant mice (p = 1.01E–04, t test), suggesting over-activation of GSK3 in the NMDAR-deleted PV neurons. The number of brain sections obtained from three mice (8–10 week-old, both sexes) per genotype/immunostaining is shown in bar graph. Panels D and E show the images of GSK3 phospho-Tyr immunoreactivity (IR) (Left) and PV-IR (right) of floxed-Grin1 control mice (d) and mutant mice (e). Note that GSK3β phospho-Y216 antibody cross-reacts with phospho-Y279 in GSK3α. Arrowheads show cells for double-positive for phospho-Tyr-GSK3 and PV. **p < 0.01.
of CTCF value of the protein to the CTCF values of corresponding proteins in non-PV neurons of floxed-control mice.

GSK inhibitors
A non-selective GSK3 inhibitor, SB216763 (2.5 mg/kg, i.p.; Tocris, Ellisiville, MO) was dissolved in 10% DMSO and 10% Tween80/PBS. Another non-selective GSK3 inhibitor, TDZD-8 (2.5 mg/kg, i.p.; Abcam, Cambridge, MA) was dissolved in 1.25% DMSO and 5% Tween80/PBS. These two non-selective GSK3 inhibitors are brain-permeable as previously described [27, 28]. The paralog-selective GSK3β inhibitor BRD3731 (30 mg/kg, i.p.) and paralog-selective GSK3α inhibitor BRD0705 (30 mg/kg, i.p.) were synthesized by
Fig. 2 Non-selective GSK3 inhibitor alleviated in vivo stimulus-evoked gamma oscillation deficits and cognitive dysfunction in Grin1 mutant mice. a Examples of the averaged ASSR LFP trace (middle, z-score) and its evoked power spectrogram (bottom) in response to 40-Hz click trains (upper; 80 dB intensity, 500 ms duration) from a floxed-control mouse (left) and a Grin1 mutant mouse (right). Time 0 is tone onset. The red dashed area (35–44 Hz frequency and during 200 ms before cessation of the click train) was for the following ASSR analyses. b Evoked-power amplitudes at 35–44 Hz during 200 ms before cessation of click-train stimuli were obtained by time-frequency decomposition of averaged LFP power across 50 trains of stimuli following subtraction of spontaneous power amplitude (z-score, 200-ms segment) at the mid-time point of the ISI (20 s duration) from LFP power amplitude (z-score). Evoked power amplitudes were evaluated before and after i.p. injection of TDZD-8 (non-selective GSK3 inhibitor) in control (15 channels) and mutant mice (15 channels) per animal (top) and per channel (bottom) [before TDZD-8 treatment, controls vs mutants, F(1,28) = 217.2, drug × genotype, p < 0.001 (per channel), F(1,8) = 3.33, p < 0.001 (per animal), two-way Repeated Measures ANOVA with Tukey-Kramer post hoc test]. TDZD-8 normalized ASSR power at 40 Hz in Grin1 mutant mice (p < 0.001 (per channel), p = 0.049 (per animal), two-way Repeated Measures ANOVA with Tukey-Kramer post hoc test), but not in the control mice (p = 1.00 (per channel), p = 0.95 (per animal)). No difference between TDZD-8 treated mutant and control mice per channel (p = 0.09, fully rescued), c Phase locking to 40-Hz click-train stimuli before and after TDZD-8 treatment also partly reversed the phase locking deficits to 40 Hz click-train stimuli in Grin1 mutant mice per channel (bottom) [before vs after treatment, F(1,28) = 18.8, drug × genotype, p < 0.001 (per channel), F(1,8) = 30.3, p < 0.001 (per animal), two-way repeated measures ANOVA with Tukey-Kramer post hoc test], but not in control mice (p = 0.97 (per channel), p = 0.63 (per animal)). No difference between TDZD-8 treated mutant and control mice per animal (p = 0.11, fully rescued). d Elevated baseline power at 35–44 Hz in the pre-stimulus period was normalized by TDZD-8 in Grin1 mutant mice saline vs TDZD-8, drug × genotype, F(1,28) = 33.7, p < 0.001 (per channel), F(1,8) = 13.1, p < 0.01 (per animal), two-way Repeated Measures ANOVA with Tukey-Kramer post hoc test. No difference between TDZD-8 treated mutant and control mice per channel (p = 0.56) and per animal (p = 0.75, fully rescued). e TDZD-8 did not affect the N1 amplitudes elicited by the click-train stimuli in Grin1 mutant mice although the N1 amplitudes were lower in the mutant mice compared to floxed-controls (controls vs mutants, p < 0.001 per channel, p < 0.01 per animal, two-way Repeated Measures ANOVA with Tukey-Kramer post hoc test). f In Y-maze spontaneous alternation task, the alternation index of the mutant mice was near the chance level (50% dotted line), suggesting spatial working memory deficit (control mice vs original Grin1 mutants, F(1,32) = 8.35, p < 0.005, one-way ANOVA with Tukey-Kramer post hoc test). The same index in a different cohort of mutants was returned back to the level (over 65%) of control mice one hr later (dotted line), suggesting spatial working memory deactivation restored in vivo by TDZD-8. g Statistical analyses were conducted using JASP (version 0.12.1) (Univ Amsterdam open-source data analysis software). Student’s t test, paired t test and factorial analysis of variance (ANOVA) were employed wherever appropriate. When main effects or interaction effects were significant, Tukey-Kramer post hoc analysis was conducted to determine which groups differ significantly from other groups. Data are presented as mean ± s.e.m. Significance was considered at p < 0.05.

RESULTS
Over-activation of GSK3 in cortical Grin1-deleted PV-positive neurons
To explore whether GSK3 is up-regulated in the Grin1 mutant mice, we measured fluorescence intensity of auditory cortical neurons produced by fluorescent-labeled antibodies against GSK3α, GSK3β, and phospho-GSK3 (at Y279 for GSK3α and at Y216 for GSK3β), an auto-activated form of GSK3 [31, 32], respectively (Fig. 1). Although sex differences were observed. More details are provided in Supplementary Methods.

Ex vivo multiple patch clamp recording
To measure spontaneous inhibitory postsynaptic currents (sIPSCs) from pairs of neighboring (<50 µm apart) neurons ex vivo, multiple patch-clamp recordings were performed in auditory cortex layer 2/3 in the presence of CNQX (20 µM) and AP5 (50 µM). Synchronous events were detected by custom written Microsoft Excel (Statcel 4th ed.) macro by defining the sIPSC events (> 5pA) coinciding within ±10 ms of signal window. More details are provided in Supplementary Methods.

Mouse behavioral tests
Y-maze spontaneous alternation was conducted to assess spatial working memory, as previously described [16]. PPI of acoustic startle was also performed to measure a pre-attentive aspect of cognitive function [30]. Male and female data were mixed because no clear sex difference was observed.

Statistical analyses
Statistical analyses were conducted using JASP (version 0.12.1) (Univ Amsterdam open-source data analysis software). Student’s t test, paired t test and factorial analysis of variance (ANOVA) were employed wherever appropriate. When main effects or interaction effects were significant, Tukey-Kramer post hoc analysis was conducted to determine which groups differ significantly from other groups. Data are presented as mean ± s.e.m. Significance was considered at p < 0.05.

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the expression of GSK3α (Fig. 1a) and GSK3β (Fig. 1b), both, are higher in the cortical layer 2/3 PV neurons compared to the nearby non-PV neurons (largely pyramidal neurons), no difference in the protein levels was detected between the genotypes in either PV neurons or non-PV neurons. However, tyrosine phosphorylation at Y279/Y216 in GSK3α/β was significantly intensified in the PV neurons of mutant mice (increased by 40%), compared to the PV neurons of floxed control mice (Fig. 1c), suggesting that NMDAR deletion in PV neurons leads to over-activation of GSK3 in the PV neurons.

Non-selective GSK3 inhibitor reversed stimulus-evoked gamma oscillation deficits, spontaneous gamma oscillation and cognitive dysfunction in Grin1 mutant mice

To assess the impact of predictive GSK3 over-activity on the in vivo stimulus-evoked LFP gamma oscillations, we used a non-selective...
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**Fig. 3** Paralog-selective GSK3β inhibitor (BRD3731), but not GSK3α inhibitor (BRD0705), alleviated gamma oscillation deficits and cognitive dysfunction in Grin1 mutant mice. **a** Representative examples of the averaged 40 Hz ASSR (middle, z-score) and spectrogram (bottom) in response to 40 Hz click trains (upper; 80 dB intensity, 500 ms duration) from a mutant mouse with saline injection (left) and a mutant mouse with BRD3731 injection (right). Time 0 is tone onset. The red dashed area (35–44 Hz frequency and during 200 ms before cessation of the click train) was for the following ASSR analyses. **b** Stimulus-evoked 40 Hz ASSR before and after i.p. injection of either BRD3731 or BRD0705 in floxed-control (15 channels) and mutant mice (15 channels) before and after BRD3731, but not BRD0705, alleviated the baseline gamma power deficits at 40 Hz in Grin1 mutant mice by per-animal and per-channel analyses (15 channels before and after BRD3731, F(2,84) = 1.72, drug × genotype, p = 0.01 (per channel), F(2,24) = 3.01, p < 0.01 (per animal); 15 channels before and after BRD0705, p = 0.998 (per channel), p = 1.00 (per animal), two-way ANOVA with Tukey-Kramer post hoc test). No difference between BRD3731-treated mutant and control mice per channel (p = 0.75) and per animal (p = 0.23, fully rescued). Neither BRD3731 nor BRD0705 affected the evoked-gamma oscillation in floxed-control mice (before and after BRD3731, p = 0.93; before and after BRD0705, p = 0.57, two-way ANOVA with Tukey-Kramer post hoc test).  

As previously described [16], 40-Hz click trains evoked the N1 potential (defined as the amplitude of the first prominent negative peak) followed by the robust increase in the power magnitudes, in particular at low gamma frequency range, in the floxed-Grin1 control mice (Fig. 2a). Genotypic comparison revealed a profound reduction of the evoked ASSR power amplitudes at 40 Hz in the Grin1 mutant mice (Fig. 2a, b). One hr after administration of TDZD-8, the evoked powers were restored in the mutant mice whereas no drug effect was observed in controls (Fig. 2b). The analysis of total ASSR power using the same LFPs reach the same conclusions (Supplementary Fig. S3A and Supplementary Result and Discussion). In addition, we found that the degree of phase locking in the ASSR to 40-Hz click trains was lower in the mutants, which was also reflected in the mutant mice, whereas it was unaffected in the floxed-control mice (Fig. 2c). TDZD-8 also normalized abnormally high baseline gamma power amplitudes during the last 10 s prior to the onset of the first click-train (referred to a pre-stimulus period), to the baseline level of control mice (Fig. 2d). On the other hand, the N1 amplitudes evoked by click trains were smaller in the mutant mice, which were unaffected by TDZD-8 (Fig. 2e). These results suggest that acute GSK3 inhibition alleviates the evoked-power deficits and reversed the baseline gamma power in the pre-stimulus period of the mutant mice.  

We also assessed whether TDZD-8 restored the normal behavior in the Grin1 mutant mice. TDZD-8 restored the Y-maze spontaneous alternation, which was diminished in the mutant mice as previously described [16] (Fig. 2f). Pretreatment with TDZD-8 also alleviated the deficits in PPI of startle in the mutant mice (Fig. 2g). These results suggest that acute GSK3 inhibition ameliorates cognitive dysfunction of the Grin1 mutant mice.  

Paralog-selective GSK3β inhibitor, but not GSK3α inhibitor, normalized the deficits in gamma oscillation and cognitive dysfunction of Grin1 mutant mice  

To determine the over-activation of which isoform, GSK3α or GSK3β, elicits the gamma oscillation deficits, we used a paralog-selective GSK3β inhibitor BRD3731 and a GSK3α inhibitor BRD0705. Administration of BRD3731, but not BRD0705, reversed the gamma oscillation deficits in the mutant mice (Fig. 3a, b). On the other hand, neither BRD3731 nor BRD0705 affected the evoked-gamma oscillation in the floxed-control mice. The total ASSR power analysis reach the same conclusion (Supplementary Fig. S3B). In addition, administration of BRD3731, but not BRD0705, also augmented phase locking factor at 40 Hz in the mutant mice (Fig. 3c). Pretreatment with BRD3731, but not BRD0705, suppressed abnormally high mutants’ baseline gamma and beta power to the normal level in the pre-stimulus period (Fig. 3d and Supplementary Fig. S5). On the other hand, neither BRD3731 nor BRD0705 affected the N1 amplitudes of the mutant mice, nor any parameters in the floxed-control mice (Supplementary Fig. S4A). Finally, BRD3731, but not BRD0705, rescued the spatial working memory of Grin1 mutant mice in Y-maze alternation task (Fig. 3e). Pretreatment with BRD3731, but not BRD0705, also ameliorated the mutants’ PPI deficits (Fig. 3f). The similar results were seen in the other drug treatments (Supplementary Fig. S5A). These results suggest that GSK3β inhibition, but not GSK3α inhibition, is crucial for restoration of gamma oscillations and cognitive function.  

GABAergic neuron-selective GSK3β knockdown reverses stimulus-evoked gamma oscillation deficits, excessive baseline gamma oscillations and cognitive dysfunction  

To address whether GABAergic neuron-specific GSK3β inhibition is sufficient for restoration of gamma oscillations and cognitive behavior, we bred either a floxed-GSK3β or a floxed-GSK3β mouse strain to the Ppnr1zcre/Grin1 KO mice to produce the corticobasal GABAergic neuron-selective heterozygous deletion of GSK3A or GSK3β gene, respectively, on Grin1 mutant genetic background (GABAergic neuron-selective knockdown). Genetic GSK3β knockdown, but GSK3A knockdown, fully alleviated the defective gamma power amplitudes (Fig. 4a and Supplementary Fig. S3C) and phase locking factors at 40 Hz of the Grin1 mutant mice (Fig. 4b). GSK3β knockdown, but not GSK3A knockdown, also fully normalized baseline gamma oscillation during the pre-stimulus period (Fig. 4c). However, neither genetic GSK3A nor GSK3B knockdown altered the N1 amplitudes (Supplementary Fig. S3D). We also examined whether knockdown of GSK3-isoform restores the cognitive behavior. Genetic GSK3β knockdown, but not GSK3A knockdown,
improved the Y-maze spontaneous alternation (Fig. 4e) and PPI of the Grin1 mutant mice (Fig. 4f). No difference was detected in the startle amplitudes between the genotypes (Supplementary Fig. S5B). These results suggest that GSK3β inhibition in corticolimbic GABAergic neurons is sufficient for restoration of gamma oscillations and cognitive function.

GABAergic neuron-selective GSK3β knockdown alleviated in vivo spike synchrony and ex vivo synchronous sIPSC deficits

To assess whether GSK3 inhibitors impact on spike synchrony in vivo, we measured multi-unit activity from somatosensory cortex in the Grin1 mutant mice before and after drug treatment. Individual spikes were obtained from nearby pyramidal neurons.
through the tetrodes’ cell-cluster analysis during simple linear track exploration (Fig. 5a). As previously reported [16], the Grin1 mutant mice showed much lower correlation coefficients of spikes from pairs of pyramidal neurons compared to the control mouse (Fig. 5c). Pretreatment with the non-selective GSK3 inhibitor SB216763 or TDZD-8 both increased in vivo spike synchrony as measured by cross-correlation magnitude, in the Grin1 mutant mice (Fig. 5b). To determine which isoform of GSK3 results in vivo spike synchrony defects, multi-unit recording from somatosensory cortex was also performed in the Grin1 mutants (Fig. 5e). Immunoreactivity in the PV neurons (presumably Grin1-deleted neurons, in the auditory cortex of the Grin1 mutant mice, (2) normalization of the diminished evoked-gamma oscillations, and behavioral deficits in the Grin1 mutant mice by administration of non-selective GSK3 inhibitors, (3) reversal of the evoked-gamma oscillations, baseline gamma power, spatial working memory and PPI by paralog-selective GSK3β inhibitor (BRD7371), and (4) restoration of evoked-gamma oscillations, baseline gamma power, and cognitive behaviors by corticolimbic GABAergic neuron-selective GSK3β genetic knockdown. These findings suggest that GSK3β inhibition in cortico-limbic GABAergic neurons rescues cognitive behavior in the NMDAR hypofunction mouse model, in strong association to the restoration of normal synchronous oscillatory brain activity at gamma frequency both in the stimulus and pre-stimulus period. Consistently, in vivo deficits in spike synchrony from pairs of cortical layer 2/3 pyramidal neuron was reversed by GSK3β inhibition and by GABAergic neuron-specific knockdown of GSK3β, but not GSK3α. Ex vivo reduction of synchronous sIPSC event number in pairs of pyramidal neurons were also restored by GSK3β inhibition and by GABAergic neuron-selective GSK3β genetic knockdown, suggesting that evoked-gamma oscillation deficit in the Grin1 mutant mice is attributed to the synchronous GABA release impairment and subsequent pyramidal cells’ spike synchrony deficits.

Two distinct gamma power abnormalities originating from Grin1-deleted PV neurons

Abnormal cortical gamma oscillatory activity appears to underlie impairments in higher cognitive functions associated with schizophrenia. One finding from the landmark observation by Kwon et al. [3], several groups have confirmed the reduction of the evoked power and synchronization to 40-Hz click-train stimuli in patients with schizophrenia [33–35]. On the other hand, spontaneous gamma power has also been reported to increase during the pre-stimulus baseline period in patients experiencing positive auditory symptoms [6] or during the resting state [7, 8]. However, it has been debated whether the mechanisms of producing such two gamma abnormalities are shared or distinct. We previously demonstrated that Grin1 deletion in cortical GABAergic neurons elicits such dual gamma abnormalities in the same animal [17]. We postulated that the evoked gamma power deficits could be due to the impaired feed-forward inhibition of thalamo-cortical circuits in response to acoustic stimuli by the defective cortical PV neurons, which may attenuate the phase-locked firing of pyramidal neurons at gamma frequency range [17]. On the other hand, the reason for the elevated baseline gamma power may be attributable to the aberrant or “noisy” spike firing of pyramidal neurons, due to the cortical disinhibition by defective PV neurons. The present study corroborates this prediction and further suggests a shared underlying mechanism between the two, because both abnormalities are consistently reversed together upon various GSK3β activity manipulations. The responsible cell-type is likely to be PV neurons, because PV neuron-specific Grin1 deletion in mice results in
optogenetically-evoked gamma oscillation deficits and increased baseline power [18, 19]. In the Grin1 mutant mice of this study, a majority (75–84%) of cortical PV neurons is Grin1-deleted, whereas Cre recombination frequency is much lower in other PV-negative GABAergic neurons [16].

Curiously, the spontaneous gamma power (200-ms segment) at the mid-time point of ISIs was normal (in z-score) and unaffected by the GSK3β inhibition. Since auditory layer 2/3 PV neurons are consistently suppressed in active wakefulness by acoustic stimuli [36], functional insufficiency of Grin1-deleted PV neurons may be masked.
This decrease was reversed by application of BRD3731 (23.01 ± 2.4%, *p < 0.001, paired *t* test). Cross-correlation coefficient of mutants was higher than when the same spike trains data set was randomly shuffled, suggesting that low cross-correlation coefficient of mutants is not due to a random temporal overlap of spike trains from pyramidal cell pairs. Each dot indicates the individual data per animal or per channel. The number of channel is shown in parentheses next to the number of animals.

Representative traces showing spontaneous IPSC (siPSC) activity evoked, simultaneously recorded from a pair of layer 2/3 pyramidal cells from auditory cortex of the *Grin1* mutant mice before and after BRD3731 application, synchronized events are indicated by asterisks.

Ex vivo whole-cell patch-clamp recording from pairs of neighboring pyramidal cells in cortical layer 2/3 showed reduced synchronous siPSC events in *Grin1* mutants compared to controls (23.97 ± 2.9% for flexed-controls (n = 11 pairs) vs 11.97 ± 1.3% for mutants (n = 10 pairs), **p < 0.001). This decrease was reversed by application of BRD3731 (23.01 ± 2.4%, n = 8 pairs) to *Grin1* mutants and in GSK3B knockdown on the mutant background (19.43 ± 1.65%, n = 11 pairs). **p < 0.01 and *p < 0.05.

Summary plots of inter-event intervals of sIPSCs under the given experimental conditions (286.3 ± 39.6 ms for flexed-control; vs 429.0 ± 89.0 ms for *Grin1* mutant, p = 0.19; mutant vs mutant with BRD3731 application: 304.8 ± 79.2 ms, p = 1.00; mutant vs GSK3B knockdown on mutant background, 273.4 ± 35.1 ms, p = 1.00). Student’s *t* test.

Figure 5: Selective GSK3β knockdown in GABAergic neurons increases in vivo action potential synchrony and ex vivo synchronous siPSCs from pairs of cortical pyramidal cells in *Grin1* mutant mice. A, Representative raw trace of multi-unit recording from somatosensory cortex from flexed-control mouse (bottom). After spike sorting, two pyramidal cell activities are shown (top), which was used for cross-correlation analysis. Red arrows indicate the synchronous unit activities of the paired pyramidal cells. B, Plots of cross-correlation magnitudes before and after treatment with GSK3 inhibitor. The deficits of in vivo spike synchrony in *Grin1* mutants disappeared 1 h after *i.p.* treatment with GSK3 inhibitor (TDZD-8 (6.4 μM) and SB216763 (16 μM) by both per-animal (top) and per-channel (bottom) analyses (SB216763: 15 pairs, *p < 0.05., paired *t* test, TDZD-8: 21 pairs, *p = 0.0005, paired *t* test). C, The degree of cross-correlation of in vivo spike firing of pairs of pyramidal cells in somatosensory cortex was much lower in *Grin1* mutant mice (61 pairs) compared to flexed-control mice (67 pairs). In vivo spike synchrony was restored when GSK3β was heterozygously knocked-out (knockdown) in *Grin1*-deleted GABAergic neurons (31 neuron-pairs from GSK3B knockdown mice vs 61 pairs from the original *Grin1* mutants, F(5,290) = 43.4, p < 0.001, one-way ANOVA with Tukey-Kramer post hoc test); but not when GSK3A was knocked down (10 pairs from GSK3A knockdown mice vs 61 pairs from original *Grin1* mutants, p = 0.99, one-way ANOVA with Tukey-Kramer post hoc test). Cross-correlation coefficient of mutants was higher than when the same spike trains data set was randomly shuffled, suggesting that low cross-correlation coefficient of mutants is not due to a random temporal overlap of spike trains from pyramidal cell pairs. Each dot indicates the individual data per animal or per channel. The number of channel is shown in parentheses next to the number of animals.

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Limitations

We repeatedly showed that systemic or GABAergic neuron-selective GSK3β inhibition restores the in vivo spike synchrony, gamma oscillations and cognitive behaviors altogether, suggesting that spike synchrony, gamma oscillation and cognitive function are tightly associated. However, it should be noted that, while LFP gamma oscillations were assessed in the auditory cortex and in vivo spike synchrony in somatosensory cortex, the behavioral tests assessed may be dependent on other brain regions, such as the prefrontal cortex and/or hippocampus [49, 50]. Thus, a direct link has not been established between evoked-gamma oscillations and cognitive function. Consistent demonstration of reversal responses by GSK3 inhibition among the manipulations may suggest that restoration of gamma oscillations and spike synchrony elicited by GSK3 inhibition at PV-positive interneurons may constitute a shared mechanism across the cortical areas.

Secondly, this study involved a relatively small sample size (five mice for each genotype) owing to the difficulty in recording the stimulus-evoked LFPs from mouse primary auditory cortex [17]. However, the results under per-animal analysis design were fully replicated by under per-channel analysis design, suggesting the robustness of the findings.

In summary, we demonstrated a strong association between the integrity of cortical gamma oscillations and cognitive function, by manipulating GSK3β activity. The precise cellular mechanism regarding how GSK3β kinase inhibition ameliorates the synchronous GABA release, in vivo spike synchrony and LFP gamma oscillations has remained unsolved. However, impaired synchronous inhibition of pyramidal neurons by *Grin1*-deleted PV neurons appears to be a key mechanism underlying schizophrenia-like phenotypes. Further study is warranted towards potential development of therapeutic interventions for schizophrenia.

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AUTHOR CONTRIBUTIONS

KN and KN designed research. KN performed in vivo electrophysiology and behavior tests and analyzed the data. MS performed ex vivo electrophysiology, analyzed the data. JHJ supervised the electrophysiological data analysis. KS performed immunohistochemistry and analyzed the data. BRD compounds were synthesized by RH CR, and BH contributed to transgenic work. KN and KN wrote the paper.

ADDITIONAL INFORMATION

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