**Schizophrenia Bulletin** vol. 48 no. 4 pp. 804–813, 2022
https://doi.org/10.1093/schbul/sbac040
Advance Access publication May 6, 2022

# Deletion of Schizophrenia Susceptibility Gene Ulk4 Leads to Abnormal Cognitive Behaviors via Akt-GSK-3 Signaling Pathway in Mice

**Ling Hu[1,2,6], Bing-Yao Zhou[2,6], Cui-Ping Yang[3], Da-Yun Lu[4], Yun-Chao Tao[2], Lin Chen[3], Lei Zhang[3,*], Jun-Hui Su[3], Ying Huang[1], Ning-Ning Song[1,2], Jia-Yin Chen[1,2], Li Zhao[1], Yi Chen[3], Chun-Hui He[3], Yu-Bing Wang[3], Bing Lang[5], and Yu-Qiang Ding[*,1–3,*]**

[1]Department of Laboratory Animal Science, Fudan University, Shanghai, China; [2]State Key Laboratory of Medical Neurobiology and MOE Frontiers Center for Brain Science, Institutes of Brain Science, Fudan University, Shanghai, China; [3]Key Laboratory of Arrhythmias, Ministry of Education, East Hospital, and Department of Anatomy and Neurobiology, Tongji University School of Medicine, Shanghai, China; [4]CAS Key Laboratory of Receptor Research, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China; [5]Department of Psychiatry, National Clinical Research Centre for Mental Health, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China

*To whom correspondence should be addressed; Shanghai 200032, China; tel: +86 021 5423 7169, e-mail: dingyuqiang@vip.163.com

[6]These authors contributed equally to this work.

***Objectives:*** **Despite of strenuous research in the past decades, the etiology of schizophrenia (SCZ) still remains incredibly controversial. Previous genetic analysis has uncovered a close association of Unc-51 like kinase 4 (ULK4), a family member of Unc-51-like serine/threonine kinase, with SCZ. However, animal behavior data which may connect Ulk4 deficiency with psychiatric disorders, particularly SCZ are still missing.** ***Methods:*** **We generated Emx1-Cre:Ulk4$^{flox/flox}$ conditional knockout (CKO) mice, in which Ulk4 was deleted in the excitatory neurons of cerebral cortex and hippocampus.** ***Results:*** **The cerebral cellular architecture was maintained but the spine density of pyramidal neurons was reduced in Ulk4 CKO mice. CKO mice showed deficits in the spatial and working memories and sensorimotor gating. Levels of p-Akt and p-GSK-3α/β were markedly reduced in the CKO mice indicating an elevation of GSK-3 signaling. Mechanistically, Ulk4 may regulate the GSK-3 signaling via putative protein complex comprising of two phosphatases, protein phosphatase 2A (PP2A) and 1α (PP1α). Indeed, the reduction of p-Akt and p-GSK-3α/β was rescued by administration of inhibitor acting on PP2A and PP1α in CKO mice.** ***Conclusions:*** **Our data identified potential downstream signaling pathway of Ulk4, which plays important roles in the cognitive functions and when defective, may promote SCZ-like pathogenesis and behavioral phenotypes in mice.**

*Key words:* Unc-51-like kinase 4/schizophrenia/sensori motor gating/Akt/GSK-3/cerebral cortex

## Introduction

SCZ is a debilitating mental disorder which displays a variety of symptoms including delusion, auditory and visual hallucination, disorganized speech and impaired working memory.[1,2] Despite extensive research, the underlying molecular and neural basis remains elusive. A genome-wide association study has identified 108 genetic loci significantly associated with SCZ.[3,4] Unfortunately, only a handful of candidate genes have achieved clear mechanical insights on its contribution to SCZ.[5]

Recurrent deletion of *Unc-51-like kinase 4* (*ULK4*) has been proposed as a genetic variant of major mental disorders including SCZ.[6] Deletion of ULK4 was also reported in 7/5,891 patients suffering developmental disorders such as learning difficulty and language problems in the study of Brain and Body Genetic Resource Exchange (BBGRE) cohort.[6] In addition, ULK4 intragenic microdeletion together with BRWD3 (Bromodomain And WD Repeat Domain Containing 3) partial microduplication was discovered in siblings with cognitive impairment, autistic features, and obesity.[7] Apart from these findings from the variants, the common variants of ULK4 also predispose people at risk to major mental disorders.[6] The data from four family cohorts comprising autistic samples from Han Chinese and European ancestry revealed strong *cis*-association between functional ULK4 SNPs and mRNA expression in postmortem autistic brains and antipsychotic treatment response in SCZ patients.[8]

How ULK4 works properly in vivo remains controversial. ULK4 belongs to the family of *unc-51-like serine/threonine kinase (ULK1-4)* and *unc-51* was originally found to participate in the endocytosis and axon growth in *Caenorhabditis elegants*.[9] Deletion of Ulk4 in mice leads to reduced neural stem cell pool,[10] hypomyelination of corpus callosum[11] and aberrant corticogenesis.[12] Although Ulk4$^{-/-}$ mice are viable, they develop severe congenital hydrocephalus which interferes with the properly functional characterization of Ulk4 in vivo.[13]

In this study, we generated Ulk4 CKO mice in which Ulk4 was selectively deleted in the cerebral cortex and hippocampus. The CKO mice exhibited impaired spatial and working memories and sensorimotor gating with elevated activity of Akt-GSK-3 signaling. We provided evidence that Ulk4 might form a protein interactome via binding two phosphatases, PP1α and PP2A. Critically, administration of okadaic acid (OA), an inhibitor of PP2A and PP1α, restored the abnormal Akt-GSK-3 signaling in CKO mice. Our results provide mechanistic insights for understanding the in vivo function of Ulk4 and when defective, can cause deleterious effects to the brain and precipitate SCZ-like behaviors in mice.

## Methods

All experimental procedures were reviewed and approved by the Animal Committee of Department of Laboratory Science, Fudan University, China.

### *Animals*

The Ulk4$^{tm1a(KOMP)Wtsi}$ sperm harboring knockout-first construct was purchased from the Knockout Mouse Project (KOMP) Repository (www.KOMP.org) at University of California, Davis. The mouse strains used in this study were generated and maintained on C57BL/6J background.

### *Western Blot, Golgi Staining and Dendritic Spine Counting*

Western blot, Golgi staining and spine counting was carried out as described previously[14] (See supplementary material for detail).

### *Immunohistochemistry and In Situ Hybridization*

Immunohistochemistry staining and in situ hybridization were carried out as described previously[15,16] (See supplementary material for detail).

### *Proteome Analysis*

To identify potential interactors of ULK4, SH-SY5Y cells over-expressing pLex-Flag-ULK4 by transient transfection were cultured in the presence of 15 μmol/L MG132 for 10 h before harvest. Cell lysates were collected to perform immunoprecipitation (IP) experiment with anti-Flag (Sigma, A2220) and proteome analysis.[17]

### *Behavioral Tests*

The Morris water maze (MWM),[18] delayed matching-to-place T maze,[19] open field[20] and prepulse inhibition (PPI)[21] were conducted as previously described (See supplementary material for details).

### *Drug Administration*

SB-216763 (Tocris) was dissolved in DMSO and diluted with saline containing 5% Tween-80 followed by intraperitoneal injection at a dosage of 10 mg/kg. The PPI test was performed at 30 min after SB-216763 injection. OA (Tocris) was prepared in 50% DMSO in saline and administered intracerebroventricularly in a volume of 1 μL at a rate of 1 μL/min as previously described.[22]

### *Statistical Analysis*

Statistical analyses were performed using GraphPad Prism 8.0 software. Student's *t*-tests and ANOVA were employed where appropriate. Bonferroni's post hoc test was mentioned in figure legends. Data were presented as mean ± SEM. Significance was considered at $P < .05$.

## Results

### *Generation of Ulk4 CKO Mice*

We first determined the expression profile of Ulk4 in the forebrain using in situ hybridization. In line with previous findings,[23] Ulk4 mRNA was distributed throughout the cerebral cortex and hippocampus (figure 1A). To delete Ulk4 specifically in the excitatory neurons of these two regions, we crossed Ulk4$^{flox/flox}$ mice[12] with Emx1-cre[24] to obtain Emx1-Cre: Ulk4$^{flox/flox}$ mice (Ulk4 CKO mice). The deletion of Ulk4 was also confirmed at transcriptional level using a pair of primers targeting the regions flanking exon 8 of Ulk4 (Ulk4-F/R) (figure 1B). As expected, two bands (752bp and 677bp) were detected in the cerebral cortex and hippocampus of Ulk4 CKO mice, but not in the cerebellum which does not express Cre recombinase.[24] In contrast, only the 752 bp band was amplified in control mice, showing that the exon8 (75 bp) was successfully deleted (figure 1C). This was further confirmed by qRT-PCR as the Ulk4 transcripts were reduced by approximately 90% in the cerebral cortex (figure 1D) and hippocampus of CKO mice. As a comparison, we also analyzed the mRNA expression of other Unc-51-like kinase family members (Ulk1-3 and STK36) and did not find any difference between the CKO and control mice (figure 1E). The CKO mice were viable and displayed comparable body weight with littermate controls at adult stage (figure 1F).

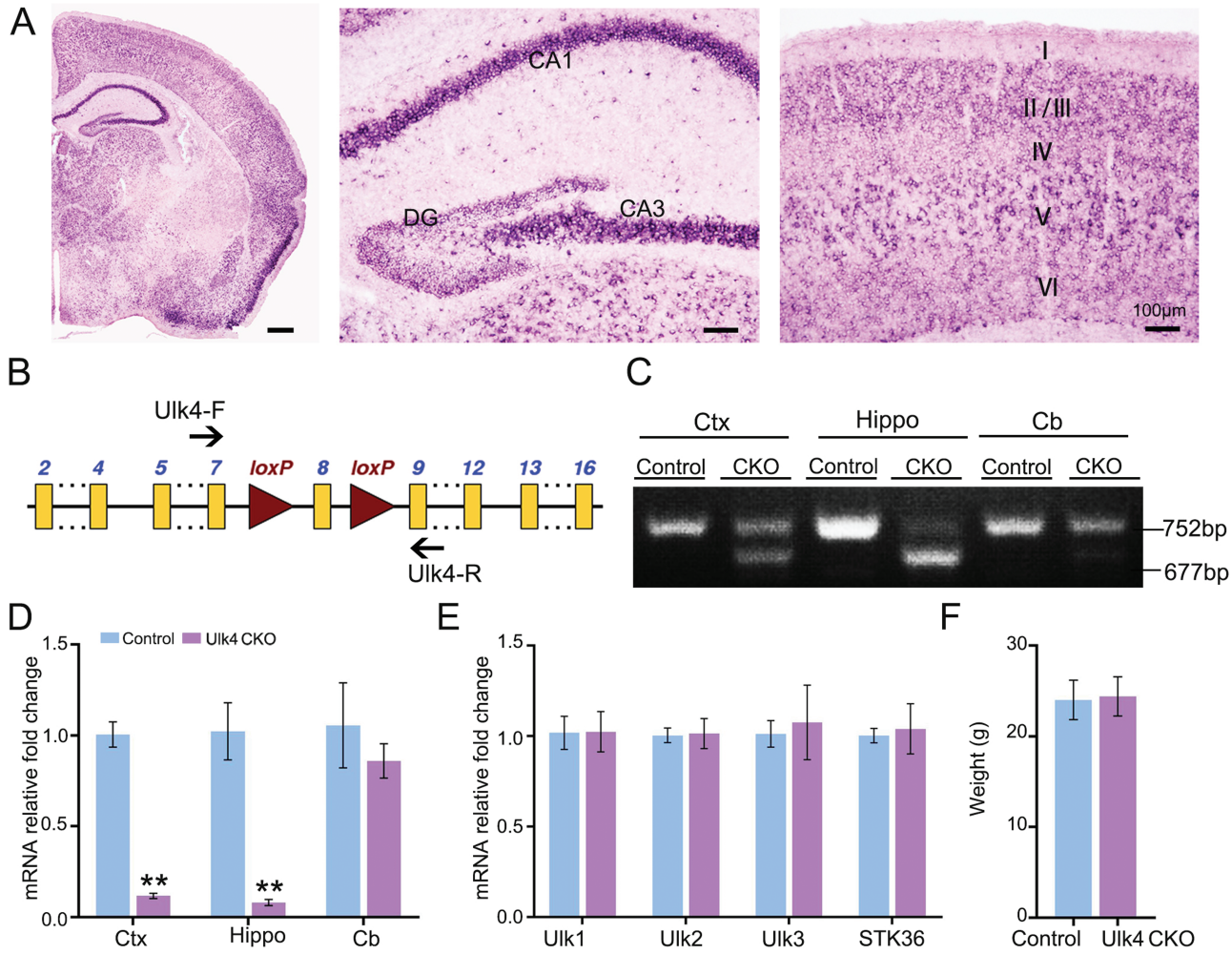


**Fig. 1.** Generation and validation of Ulk4 CKO mice. (A) The expression of Ulk4 throughout the cerebral cortex (layers II/VI) and hippocampus. Scale bar = 400 μm in the left panel and 100 μm in the middle and right panels. (B) Diagram showing the genetic makeup of Ulk4 flox mice. (C) Using the cDNA as template and primer pair indicated in B, two bands (752 bp and 677 bp) were detected in Ulk4 CKO mice in the cerebral cortex and hippocampus where the cre recombinase was expressed. In the cerebellum, only the 752 bp band was amplified in both control and Ulk4 CKO mice. (D) qRT-PCR also showed that Ulk4 transcript was significantly reduced in the cerebral cortex and hippocampus but not in cerebellum of Ulk4 CKO mice. (E) The expression profile of other Unc-51-like family members (Ulk1-3 and STK36) were not changed in CKO mice. mRNA levels in CKO mice are normalized to that of control mice. $N = 4$ for each group; ** $P < .01$; one-way ANOVA with Bonferroni post hoc test. (F) No significant difference in the body weight was observed between adult CKO and control mice (4–6 month old). $N = 10$ for each group. Student's $t$ test. Ctx, cortex; Hippo, hippocampus; Cb, cerebellum; I-VI, cortical layers I-VI; CA1 and CA3, CA1-CA3 regions of hippocampus; DG, dentate gyrus.

### *Ulk4 CKO Mice Displayed Impaired Spatial and Working Memories Together with Deficient Sensorimotor Gating*

We then carried out a battery of behavioral tests to determine possible behavioral abnormalities in Ulk4 CKO mice. In the open field test, no significant difference was found in the travel distance or average velocity between control and Ulk4 CKO mice (supplementary figures S1A and S1B). Similar results were obtained in dark-light choice test (supplementary figures S1C and S1D) and in the elevated plus maze (supplementary figures S1E and S1F) as well as in three-chamber social interaction test (supplementary figures S1G and S1H). Collectively, spontaneous locomotor activity, anxiety level and social behaviors are not obviously altered in CKO mice.

To examine whether the cognitive function is defective in Ulk4 CKO mice, we performed the Morris water maze (MWM) and delayed matching-to-place T maze tests. During the learning period of the MWM test, the Ulk4 CKO mice exhibited a longer latency in finding the hidden platform than the control mice on days 4–7, suggesting a deficit in spatial learning (figure 2A). During the test day, Ulk4 CKO mice showed a much longer latency to find the platform compared with the control mice (figures 2C and 2E), and a significantly decreased number of target zone crossings (figures 2B and 2E). Noteworthy, these changes were not due to the deficits in the motor function as the swimming velocity was comparable between control and CKO mice (figure 2D). Delayed matching-to-place T maze is a well-defined behavior paradigm assessing

working memory in mice.[19] The CKO mice displayed a reduced number of correct arm entries and concomitantly increased latency to find sweet jelly when the interval between a sample run and a choice run last for 3 min (figures 2F and 2G). These results indicated that Ulk4 deletion leads to profound impairment of spatial learning and memory retrieval, and deficits in working memory.

We next set out to explore the ability of sensorimotor gating by PPI test. The CKO mice displayed a significant reduction of PPI at the 65, 73, and 82 dB pre-pulse levels compared with control mice (figure 2H). Noteworthy, the startle response did not differ between control and CKO mice, suggesting no hearing deficit (figure 2I). This result strongly indicated that the sensorimotor gating is disturbed in CKO mice.

### *Ulk4 CKO Mice had Unchanged Layer Architecture but Reduced Spine Density of Cortical Pyramidal Neurons*

Previous studies have shown that Ulk4 null knockout mice display thinner cerebral cortex and enlarged lateral ventricles.[12] Thus, we first quantified the thickness of cortical individual layers with Nissl staining together with other layer-specific markers (Cux2 and CDP for layers II-IV, Rorβ for layer IV, Plxnd1 for layer V and TLE4 for layer VI). The Ulk4 CKO mice had comparable thickness of layers I–VI in the cortex (figures 3A–3F) along with the normal size of the lateral ventricle (supplementary figures S1H and S1I). Similarly, they also presented normal architecture of hippocampus as evidenced by Nissl staining and region-specific genes including Math2, KA1, Man1α, and EphA6 (supplementary figures S2A–S2E). In addition, it has been reported that adult hippocampal neurogenesis is also implicated in the pathogenesis of SCZ.[25,26] We thus performed immunostaining of DCX, MCM2, and Ki67, which are expressed in the neuroblasts and proliferating NSCs,[27] and did not find any difference between control and CKO mice (supplementary figures S2F–S2H).

Reduced spine density of cortical pyramidal neurons has been frequently reported in the postmortem brains with SCZ and is believed to be a key hallmark of SCZ.[28–31] We then performed Golgi staining and evaluated the morphology of pyramidal neurons in the somatosensory cortex. Although dendrite tree was not obviously changed, the spine density of cortical neurons was significantly decreased in Ulk4 CKO mice (figures 3G and 3H).

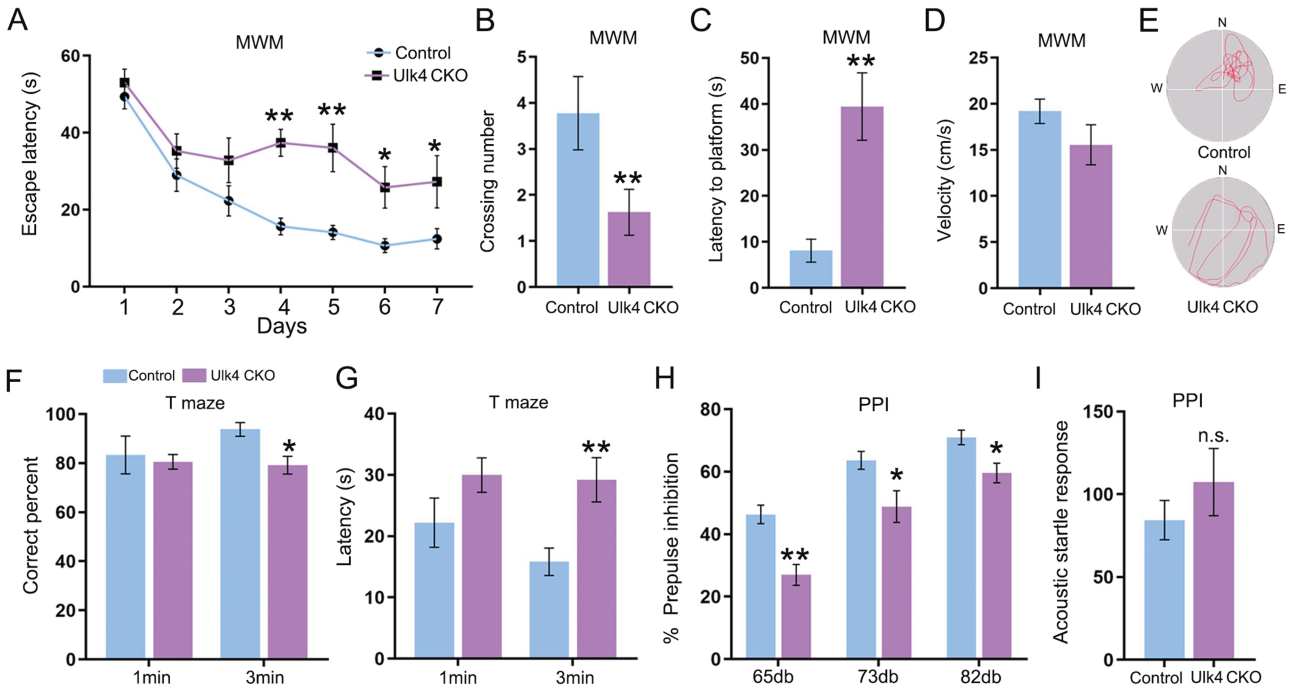


**Fig. 2.** Ulk4 CKO mice exhibited impaired spatial and working memories and defective sensorimotor gating. (A–C) In learning phase of Morris water maze (MWM) test, the Ulk4 CKO mice showed significantly longer latency to find platform than the control mice (A, two-way repeated ANOVA) and the Ulk4 CKO mice presented less platform crossing number during the memory trial (B) and longer latency to find the platform in test phase (C). $N = 10$ for control and $N = 8$ for CKO mice, ** $P < .01$; * $P < .05$. (D) The Ulk4 CKO mice showed a similar swimming velocity with control mice. $N = 10$ for control and $N = 8$ for CKO mice, Student's $t$ test. (E) Representative traveling trajectory of the control (top) and CKO mice (bottom) in the MWM test. (F, G) The Ulk4 CKO mice showed a reduced number of correct arm entries (F) and an increased latency to find sweet jelly when the interval last for 3 min (G). No difference was found when interval last for 1 min. $N = 6$ for control and $N = 9$ for CKO mice. Two-way repeated ANOVA, * $P < .05$; ** $P < .01$. (H, I) The Ulk4 CKO mice had a significant PPI deficit compared with control mice at three different pre-pulse intensities (H) but the startle response was comparable (I). Two-way repeated ANOVA for PPI test and Student's $t$ test for startle response comparison. *$P < .05$, ** $P < .01$. $N = 14$ for control and $N = 13$ for Ulk4 CKO.

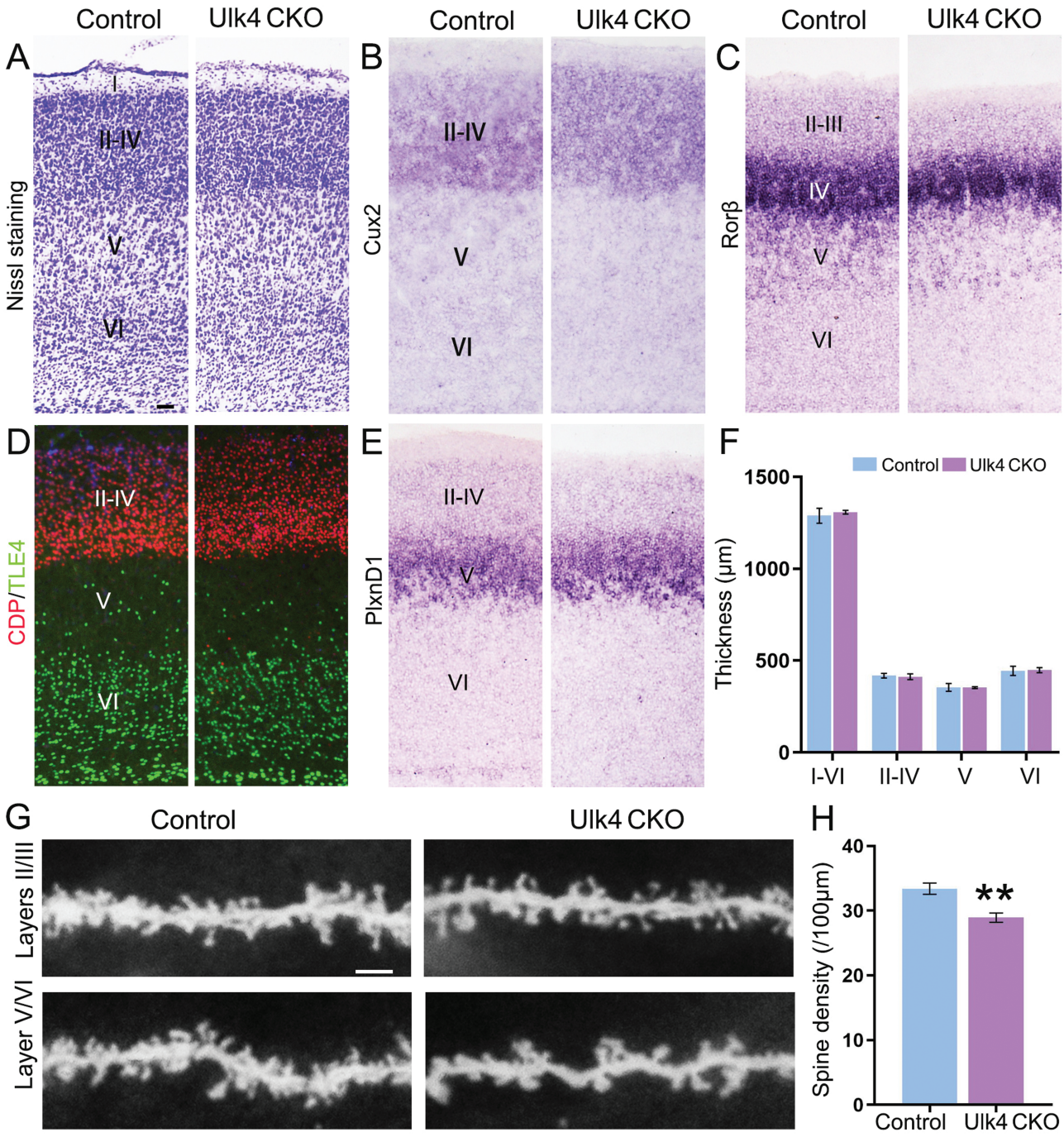


**Fig. 3.** Unchanged cortical layers but reduced spine density of pyramidal neurons in CKO mice. (A–E) Compared to the control mice, the CKO mice presented normal cortical lamination as shown by Nissl staining (A) and layer-specific markers, including Cux2 for layer II-IV (B), Rorβ for layer IV (C), CDP for layer II–IV (D), TLE4 for layer V–VI (D), PlxnD1 for layer V (E) in P7 mice. Scale bar = 50 μm. (F) Quantification of the thickness of the individual layers shown by Nissl staining. Student's *t* test. $N = 3$ for each group. (G) Representative images of dendritic spines from the neurons in the layers II/III and layer V/VI in the two groups. Scale bar = 10 μm. (H) Decreased spine density was found in Ulk4 CKO mice in comparison with control mice. Student's *t* test, ** $P < .01$, $N = 34$–40 in each group.

### *Elevated GSK-3 Activity was Responsible for the Disturbed PPI Response of Ulk4 CKO Mice*

The aberrant PPI response and reduced spine density of cortical pyramidal neurons in the CKO mice prompted us to investigate the underpinning molecular mechanisms regulated by Ulk4. As abnormal Akt-GSK-3 pathway in the brain is one of the key molecular pathways implicated in the pathogenesis of SCZ,[32–34] we carried out western blot to investigate whether this pathway is changed in the brain of Ulk4 CKO mice. The total levels of Akt, GSK-3α,

or GSK-3β were not different between the two groups, whereas the levels of p-Akt (Ser473), p-GSK-3α (Ser9), and p-GSK-3β (Ser21) were significantly downregulated in CKO mice, showing an increased activity of Akt-GSK-3 pathway in the absence of Ulk4 (figures 4A and 4B).

We also cultured 293T cells which were transfected with control and pLex-Flag-ULK4 (Flag-ULK4), respectively, to investigate whether overexpression of human ULK4 (full-length) influences the activity of Akt-GSK-3 pathway. Consistently, the levels of p-Akt (Ser473), p-GSK-3α (Ser9), and p-GSK-3β (Ser21) were increased (figures 4C and 4D). Thus, we speculated that Ulk4 may co-ordinate brain functions via regulating the activity of Akt-GSK-3 pathway.

To validate the involvement of GSK-3 in the abnormal PPI of CKO mice, we re-run the PPI test after the acute systemic administration of SB-216763, a selective inhibitor of GSK-3.[35] Our results showed that SB-216763 significantly improved the performance of PPI in Ulk4 CKO mice, which showed a comparable PPI reaction at 82 dB compared with control mice without affecting the acoustic startle response (figures 4E and 4F). These results indicated that elevation of GSK-3 activity in the brain might be a key factor contributing to the deficient sensorimotor gating in Ulk4 CKO mice.

### *Ulk4 may Regulate Akt-GSK-3 Pathway via PP2A and PP1α*

To determine the molecular mechanism which underlies the modulatory roles of Ulk4 to GSK-3 activity, we cultured SH-SY5Y cells transfected with pLex-Flag-ULK4 plasmid and the harvested proteins were subject to immunoprecipitation and proteome analysis to screen potential ULK4 interactors (figure 5A). Compared with control group, ULK4 overexpression caused a significant

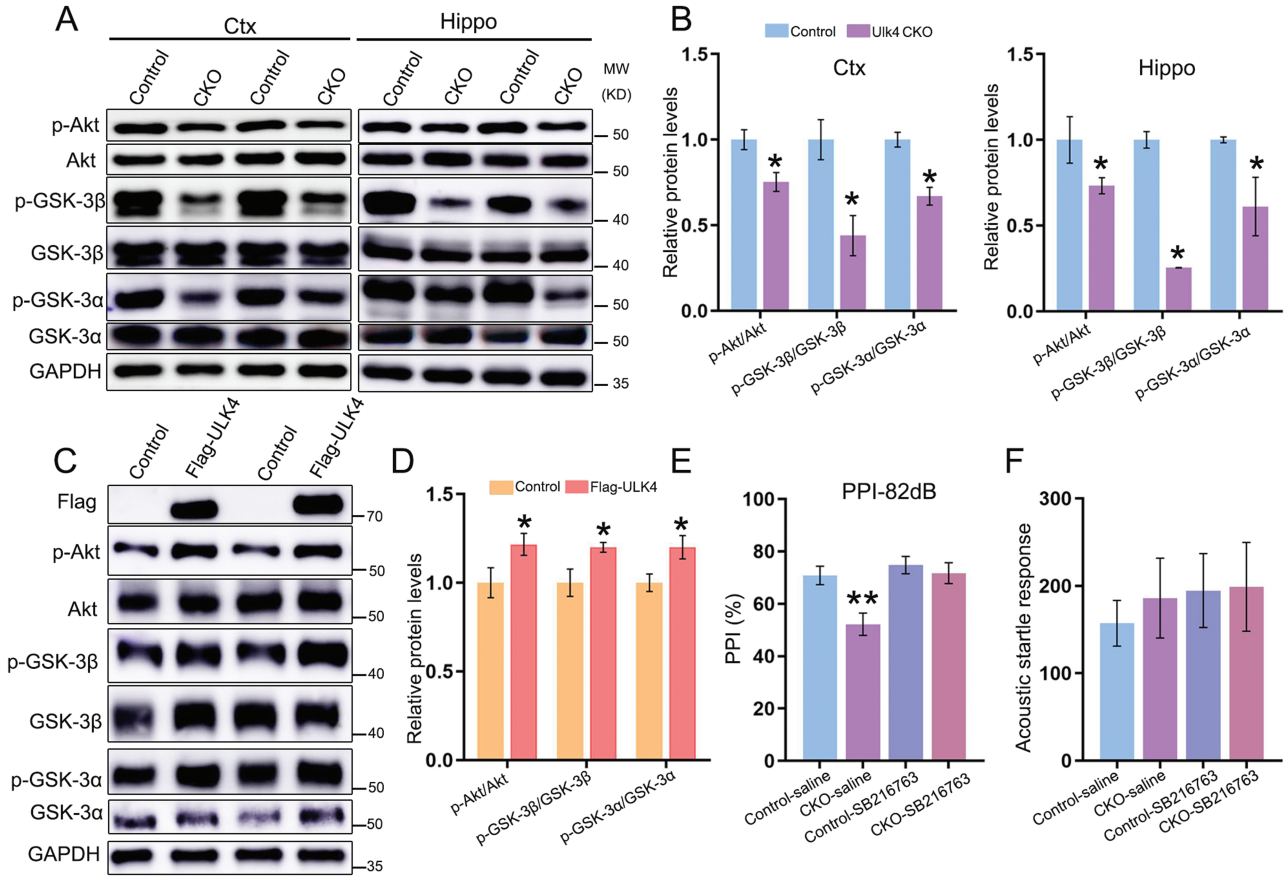


**Fig. 4.** Ulk4 CKO mice presented significantly down-regulated expression of p-Akt and p-GSK-3 in the cerebral cortex and hippocampus. (A) Representative western blots for the proteins of interest in the cerebral cortex and hippocampus. (B) Quantitative data of the relative protein expression levels showed significantly decreased p-Akt, p-GSK-3α, and p-GSK-3β in the cerebral cortex (left) and hippocampus (right) of Ulk4 CKO mice. Student's *t* test, $*P < .05$, $N = 5$ for each group. Ctx, cerebral cortex; Hippo, hippocampus. (C) Representative western blots for the proteins of interest in the 293T cells transfected with control plasmids and Flag-ULK4-overexpressing plasmid. (D) Quantitative data of the relative protein expression levels showed significantly increased p-Akt, p-GSK-3α and p-GSK-3β in ULK4-overexpressing group (Flag-ULK4) compared with control group. Student's *t* test, $*P < .05$, $N = 5$ for each group. (E) SB-216763 restored the impaired PPI in CKO mice at pre-pulse intensity of 82dB. $** P < .01$, One-way ANOVA with Bonferroni post hoc test. (F) The startle responses were not altered among the four groups. $N = 6$ in each group. One-way ANOVA with Bonferroni post hoc test.

enrichment of serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform (PPP2R2A, 55 kDa), serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform (PPP2CA) and serine/threonine-protein phosphatase PP1α catalytic subunit (PPP1CA) (figure 5A), which were further confirmed by western blot (figure 5B). On the other hand, the expressing levels of these proteins (termed as PP2A B subunit, PP2A C subunit and PP1α, respectively) and their phosphorylated forms were not altered in Ulk4 CKO mice (figure 5C). It is well known that PP2A and PP1α are protein phosphatases and negatively regulate Akt-GSK-3 signaling pathway.[22] Therefore, it is likely that the PP2A/PP1α-involved regulatory machinery is defective in the absence of Ulk4, which leads to the elevated Akt-GSK-3 activity in the CKO brain.

To obtain in vivo evidence to support our hypothesis, we injected OA, one of the potent protein-phosphatase inhibitors acting on both PP2A and PP1α, into the lateral ventricles (1 μL, 100 μM) of Ulk4 CKO mice. Western blot results showed that the reduction of p-Akt, p-GSK-3α, and p-GSK-3β in the brain of Ulk4 CKO mice was restored to a comparable level with control mice at 30 min after OA injection (figures 5D and 5E). Thus, it is likely that Ulk4 forms a complex with PP2A/PP1α which negatively regulates the activity of Akt-GSK-3 signal. Thus, it is likely that the deletion of Ulk4 blocks this regulation and leads to aberrant behavioral phenotypes such as impaired sensorimotor gating ability (figure 5F).

## Discussion

SCZ is a common but poorly understood chronic brain disease and genetic predisposition is substantial (60%–80%). We previously reported that recurrent deletion of ULK4 is a risk factor for major mental disorders including SCZ.[6] In this study, we demonstrated that the deletion of Ulk4 in the cerebral cortex and hippocampus leads to impairments of working and spatial memories, and deficient sensorimotor gating. However, Ulk4 CKO

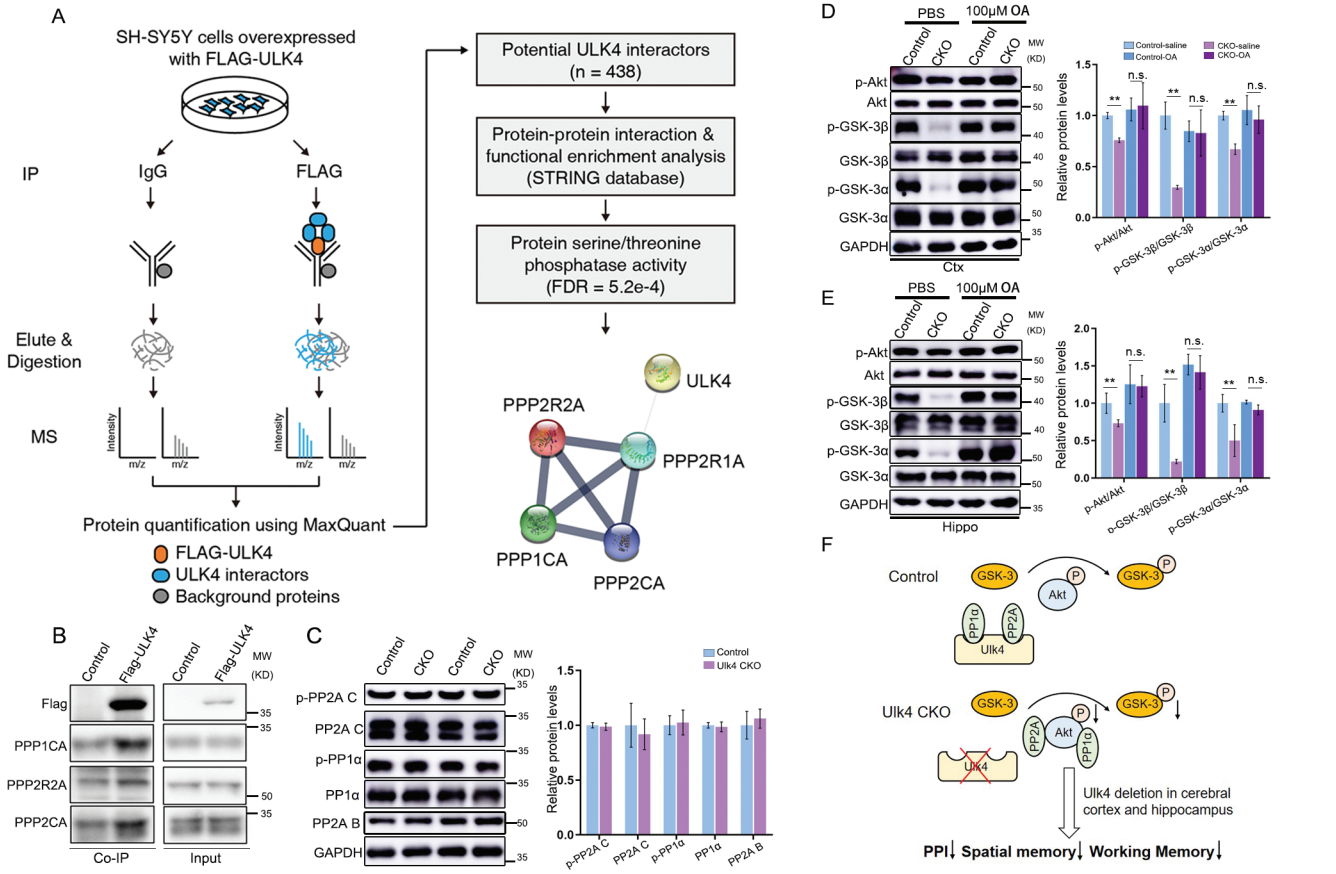


**Fig. 5.** Ulk4 regulated Akt-GSK-3 pathway via PP2A and PP1α. (A) The workflow of ULK4 interactor discovery. (B) The enrichment of PPP2R2A (PP2A B), PPP1CA (PP2A C) and PPP2R1A (PP1α) was confirmed by co-immunoprecipitation. (C) Representative blots for p-PP2A C subunit, PP2A C subunit, p-PP1α subunit, PP1α and PP2A B subunit (left). Quantitative analysis showed no difference between control and Ulk4 CKO mice (right). Student's *t* test, $N = 3$ for each group. (D, E) Representative blots (left) of p-Akt, Akt, p-GSK-3α, GSK-3α, p-GSK-3β and GSK-3β for the cortices (D) and hippocampus (E) of control and Ulk4 CKO mice before and after the administration of PBS or OA. The right panel was quantitative analysis. $N = 3$ for each group; ** $P < .01$; One-way ANOVA with Bonferroni post hoc test. n.s, no significance. Ctx, cerebral cortex; Hippo, hippocampus; OA, okadaic acid. (F) A schematic diagram depicting Ulk4-involved pathways/interactions in regulating brain functions.

mice do not show alterations of anxiety-like behavior, which seems contradictory to the report that Ulk4$^{+/tm1a}$ mice exhibit an anxiety-like behavioral phenotype.[36] This discrepancy might be explained by the following factors. First, Ulk4 was deleted only in excitatory neurons of the cerebral cortex and hippocampus in our CKO mice whereas it was halved throughout the brain of Ulk4$^{+/tm1a}$ mice. Second, defective GABAergic signaling may underlie the anxiety-like behaviors of Ulk4$^{+/tm1a}$ mice,[36] while it may not or less affected in our CKO mice. Selective deletion of Ulk4 in inhibitory neurons is required to figure out the difference between our CKO and Ulk4$^{+/tm1a}$ mice.

In the exploration of possible mechanism underlying the behavioral alterations, we found that the CKO mice presented elevated Akt-GSK-3 signaling. Critically, the blockade of Akt-GSK-3 signaling improved the alleviated capacity of pre-pulse inhibition in CKO mice. Proteome analysis showed that Ulk4 might physically bind with protein phosphatases PP2A and PP1α and pharmacological inhibition of PP2A and PP1α restored the activity of Akt-GSK-3 signaling in CKO mice. Thus, Ulk4 may form a protein interactome which comprises of phosphatases PP2A and PP1α and when defective, causes core molecular and behavioral features of SCZ in mice.

Genetic linkage analyses have revealed strong co-segregation of Akt1 haplotypes with SCZ.[37,38] GSK-3 is the main downstream substrate of Akt, and altered GSK-3 regulatory pathway has been reported in SCZ.[33] In line with these studies, we observed a decreased Akt activity which is concomitant with elevated GSK-3α/β activity in the cortex and hippocampus of Ulk4 CKO mice. Furthermore, attenuated pre-pulse inhibition in CKO mice could be partially corrected by GSK-3 inhibitor SB216763. These data suggest that disrupted Akt-GSK-3 signaling cascade primes Ulk4 CKO mice to the vulnerability of developing the core features of SCZ. Further examination of the reduced density of dendritic spines can be recovered in Ulk4 CKO mice after the treatment will provide more evidence for understanding the mechanism of Ulk4 deficiency affecting brain functions.

How Ulk4 deficiency leads to disturbed Akt-GSK-3 signaling in vivo remains ambiguous largely because the downstream substrates or interaction partners are uncertain. It is assumed that Ulk4 works as the sensor of ATP and undergo conformational changes upon the binding which subsequently promotes the roles as scaffolds for substrate recruitment.[39] Indeed, Preuss et al. have recently predicted many ULK4 interacting partners including active kinases and phosphatases which, however, need further functional validation.[40] In our study, we demonstrated that Ulk4 can physically bind PP2A and PP1α without affecting their phosphorylation. PP2A and PP1α are the mostly abundant phosphatases in eukaryotes and responsible for over 90% of total Ser/Thr dephosphorylation including Akt. PP2A and Akt work upstream of GSK-3 and tune its activity through the balance between dephosphorylation and phosphorylation. A synergic effect of PP2A and GSK-3β has been established to regulate the sensorimotor gating in mice,[41] and the dephosphorylation of Akt can be antagonized after inhibition of PP2A and PP1α.[22] Consistently, Ulk4 CKO mice have down-regulated phosphorylation of Akt1 and GSK-3, which could be reversed by OA, a nonselective inhibitor of PP2A and PP1α, which are likely to be a drug target in the treatment of mental disorders including SCZ on the basis that current antipsychotic medications restores the dysfunction of AKT/GSK3 signaling, which contributes to the clinical efficacy of antipsychotic drugs.[42–44]

In summary, we specifically deleted Ulk4 in the excitatory neurons of the cortex and hippocampus in mice which present many SCZ-like features such as impaired spatial and working memory as well as disturbed sensorimotor gating. We proposed that Ulk4 forms a protein interactome with PP2A and PP1α via physical binding. Deletion of Ulk4 caused the release of free PP2A and PP1α which subsequently leads to a cascade dephosphorylation of Akt1 and GSK-3. Our data provide novel evidence for the predisposed risk of Ulk4 deletion to SCZ, though more detailed mechanisms are still warranted for further follow-up study.

## Supplementary Material

Supplementary material is available at *Schizophrenia Bulletin* online.

## Funding

This work was supported by the National Natural Science Foundation of China (31771134 to N.-N.S.; 31671061 to L.Z.; 82071507 to B.L., 81571332 and 91232724 to Y.-Q.D.), Shanghai Pujiang Program (20PJ1413300 to L.Z.), Key Research and Development Program from Hunan Province (2018DK2011 to B.L.), Collaborative Innovation Program of Shanghai Municipal Health Commission (2020CXJQ01 to N.-N.S and Y.-Q.D), Shanghai Municipal Science and Technology Major Project (2018SHZDZX01), Zhangjiang (ZJ) Lab, and Shanghai Center for Brain Science and Brain-Inspired Technology (to N.N.S and Y.Q.D.).

## Acknowledgments

We would like to thank the Institutional Technology Center of Shanghai Institute of Materia Medica for the support of mass spectrometry technology.

## *Conflict of Interest*

The authors declare no conflicts of interest.

## References


1. Saha S, Chant D, Welham J, McGrath J. A systematic review of the prevalence of schizophrenia. *PLoS Med.* 2005;2(5):e141.
2. Marder SR, Cannon TD. Schizophrenia. *N Engl J Med.* 2019;381(18):1753–1761.
3. Schizophrenia Working Group of the Psychiatric Genomics C. Biological insights from 108 schizophrenia-associated genetic loci. *Nature* 2014;511(7510):421–427.
4. Schizophrenia Psychiatric Genome-Wide Association Study C. Genome-wide association study identifies five new schizophrenia loci. *Nat Genet.* 2011;43(10):969–976.
5. Jaaro-Peled H. Gene models of schizophrenia: DISC1 mouse models. *Prog Brain Res.* 2009;179:75–86.
6. Lang B, Pu J, Hunter I, et al. Recurrent deletions of ULK4 in schizophrenia: a gene crucial for neuritogenesis and neuronal motility. *J Cell Sci.* 2014;127(Pt 3):630–640.
7. Tassano E, Uccella S, Giacomini T, Striano P, Severino M, Porta S, Gimelli G, Ronchetto P. Intragenic microdeletion of ULK4 and partial microduplication of BRWD3 in siblings with neuropsychiatric features and obesity. *Cytogenet Genome Res.* 2018;156:14–21.
8. Ou J, Li K, Guo H, Hu Z, Zhao J, Zhang F. ULK4 genetic variants have pleiotropic effect on risk of autism, associated with brain mRNA expression and antipsychotic treatment response. *J Psychiatry Brain Sci.* 2019;4:e190010.
9. Ogura K, Wicky C, Magnenat L, Tobler H, Mori I, Muller F, Ohshima Y. *Caenorhabditis elegans* unc-51 gene required for axonal elongation encodes a novel serine/threonine kinase. *Genes Dev.* 1994;8(20):2389–2400.
10. Liu M, Guan Z, Shen Q, et al. Ulk4 regulates neural stem cell pool. *Stem Cells* 2016;34(9):2318–2331.
11. Liu M, Xu P, Guan Z, Qian X, Dockery P, Fitzgerald U, O'Brien T, Shen S. Ulk4 deficiency leads to hypomyelination in mice. *Glia.* 2018;66(1):175–190.
12. Hu L, Chen Y, Yang CP, et al. Ulk4, a newly discovered susceptibility gene for schizophrenia, regulates corticogenesis in mice. *Front Cell Dev Biol.* 2021;9:645368.
13. Vogel P, Read RW, Hansen GM, Payne BJ, Small D, Sands AT, Zambrowicz BP. Congenital hydrocephalus in genetically engineered mice. *Vet Pathol.* 2012;49(1):166–181.
14. Wang XQ, Zhang L, Xia ZY, Chen JY, Fang Y, Ding YQ. PTEN in prefrontal cortex is essential in regulating depression-like behaviors in mice. *Transl Psychiatry* 2021;11(1):185.
15. Huang Y, Zhang Q, Song NN, et al. Lrp5/6 are required for cerebellar development and for suppressing TH expression in Purkinje cells via beta-catenin. *Mol Brain* 2016;9:7.
16. Zhang L, Song NN, Zhang Q, et al. Satb2 is required for the regionalization of retrosplenial cortex. *Cell Death Different.* 2020;27(5):1604–1617.
17. Yu H, Ding J, Zhu H, et al. LOXL1 confers antiapoptosis and promotes gliomagenesis through stabilizing BAG2. *Cell Death Differ* 2020;27(11):3021–3036.
18. Zhang Q, Huang Y, Zhang L, Ding YQ, Song NN. Loss of Satb2 in the cortex and hippocampus leads to abnormal behaviors in mice. *Front Mol Neurosci.* 2019;12:33.
19. Deacon RM, Rawlins JN. T-maze alternation in the rodent. *Nat Protoc.* 2006;1(1):7–12.
20. Zhang JB, Chen L, Lv ZM, et al. Oxytocin is implicated in social memory deficits induced by early sensory deprivation in mice. *Mol Brain* 2016;9(1):98.
21. Geyer MA, Dulawa SC. Assessment of murine startle reactivity, prepulse inhibition, and habituation. *Curr Protoc Neurosci.* 2003;Chapter 8:Unit 8 17.
22. Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 2005;122(2):261–273.
23. Lang B, Zhang L, Jiang G, et al. Control of cortex development by ULK4, a rare risk gene for mental disorders including schizophrenia. *Sci Rep.* 2016;6:31126.
24. Guo HL, Hong SS, Jin XL, Chen RS, Avasthi PP, Tu YT, Ivanco TL, Li YQ. Specificity and efficiency of cre-mediated recombination in Emx1-Cre knock-in mice. *Biochem Bioph Res Commun.* 2000;273(2):661–665.
25. Kang E, Wen Z, Song H, Christian KM, Ming GL. Adult neurogenesis and psychiatric disorders. *Cold Spring Harb Perspect Biol.* 2016;8(9):a019026.
26. Sacco R, Cacci E, Novarino G. Neural stem cells in neuropsychiatric disorders. *Curr Opin Neurobiol.* 2018;48:131–138.
27. Song NN, Jia YF, Zhang L, et al. Reducing central serotonin in adulthood promotes hippocampal neurogenesis. *Sci Rep.* 2016;6:20338.
28. Garey LJ, Ong WY, Patel TS, Kanani M, Davis A, Mortimer AM, Barnes TR, Hirsch SR. Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *J Neurol Neurosurg Psychiatry* 1998;65(4):446–453.
29. Zhou Y, Kaiser T, Monteiro P, et al. Mice with Shank3 mutations associated with ASD and schizophrenia display both shared and distinct defects. *Neuron* 2016;89(1):147–162.
30. Konopaske GT, Lange N, Coyle JT, Benes FM. Prefrontal cortical dendritic spine pathology in schizophrenia and bipolar disorder. *JAMA Psychiatry* 2014;71(12):1323–1331.
31. Glantz LA, Lewis DA. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch Gen Psychiatry* 2000;57(1):65–73.
32. Freyberg Z, Ferrando SJ, Javitch JA. Roles of the Akt/GSK-3 and Wnt signaling pathways in schizophrenia and antipsychotic drug action. *Am J Psychiatry* 2010;167(4):388–396.
33. Lovestone S, Killick R, D i Forti M, Murray R. Schizophrenia as a GSK-3 dysregulation disorder. *Trends Neurosci.* 2007;30(4):142–149.
34. Gomez-Sintes R, Bortolozzi A, Artigas F, Lucas JJ. Reduced striatal dopamine DA D2 receptor function in dominant-negative GSK-3 transgenic mice. *Eur Neuropsychopharmacol* 2014;24(9):1524–1533.
35. Zhao J, Feng Y, Yan H, Chen Y, Wang J, Chua B, Stuart C, Yin D. beta-arrestin2/miR-155/GSK3beta regulates transition of 5'-azacytizine-induced Sca-1-positive cells to cardiomyocytes. *Journal of cellular and molecular medicine* 2014;18(8):1562–1570.
36. Liu M, Fitzgibbon M, Wang Y, et al. Ulk4 regulates GABAergic signaling and anxiety-related behavior. *Transl Psychiatry* 2018;8(1):43.
37. Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA. Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nature Genet.* 2004;36(2):131–137.
38. Su Z, Zhang Y, Gendron TF, et al. Discovery of a biomarker and lead small molecules to target r(GGGGCC)-associated defects in c9FTD/ALS. *Neuron* 2014;84(1):239.
39. Khamrui S, Ung PMU, Secor C, Schlessinger A, Lazarus MB. High-resolution structure and inhibition of the

schizophrenia-linked pseudokinase ULK4. *J Am Chem Soc.* 2020;142(1):33–37.

40. Preuss F, Chatterjee D, Mathea S, et al. Nucleotide binding, evolutionary insights, and interaction partners of the pseudokinase Unc-51-like kinase 4. *Structure (London, England: 1993)*. 2020;28(11):1184–1196.e1186.
41. Kapfhamer D, Berger KH, Hopf FW, Seif T, Kharazia V, Bonci A, Heberlein U. Protein Phosphatase 2a and glycogen synthase kinase 3 signaling modulate prepulse inhibition of the acoustic startle response by altering cortical M-Type potassium channel activity. *J Neurosci.* 2010;30(26):8830–8840.
42. Kang UG, Seo MS, Roh MS, Kim Y, Yoon SC, Kim YS. The effects of clozapine on the GSK-3-mediated signaling pathway. *FEBS Lett.* 2004;560(1–3):115–119.
43. Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, Caron MG. Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci U S A*. 2004;101(14):5099–5104.
44. Alimohamad H, Rajakumar N, Seah YH, Rushlow W. Antipsychotics alter the protein expression levels of beta-catenin and GSK-3 in the rat medial prefrontal cortex and striatum. *Biol Psychiatry* 2005;57(5):533–542.