

# IQSEC3 Deletion Impairs Fear Memory Through Upregulation of Ribosomal S6K1 Signaling in the Hippocampus

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## ABSTRACT

**BACKGROUND:** IQSEC3, a gephyrin-binding GABAergic (gamma-aminobutyric acidergic) synapse-specific guanine nucleotide exchange factor, was recently reported to regulate activity-dependent GABAergic synapse maturation, but the underlying signaling mechanisms remain incompletely understood.

**METHODS:** We generated mice with conditional knockout (cKO) of *Iqsec3* to examine whether altered synaptic inhibition influences hippocampus-dependent fear memory formation. In addition, electrophysiological recordings, immunohistochemistry, and behavioral assays were used to address our question.

**RESULTS:** We found that *Iqsec3*-cKO induces a specific reduction in GABAergic synapse density, GABAergic synaptic transmission, and maintenance of long-term potentiation in the hippocampal CA1 region. In addition, *Iqsec3*-cKO mice exhibited impaired fear memory formation. Strikingly, *Iqsec3*-cKO caused abnormally enhanced activation of ribosomal P70-S6K1-mediated signaling in the hippocampus but not in the cortex. Furthermore, inhibiting upregulated S6K1 signaling by expressing dominant-negative S6K1 in the hippocampal CA1 of *Iqsec3*-cKO mice completely rescued impaired fear learning and inhibitory synapse density but not deficits in long-term potentiation maintenance. Finally, upregulated S6K1 signaling was rescued by IQSEC3 wild-type, but not by an ARF-GEF (adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor) inactive IQSEC3 mutant.

**CONCLUSIONS:** Our results suggest that IQSEC3-mediated balanced synaptic inhibition in hippocampal CA1 is critical for the proper formation of hippocampus-dependent fear memory.

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Excitation-inhibition balance is critical for controlling excitability, dynamic range, input gating, and neural coding across numerous neural circuits. Diverse sets of synaptic mechanisms have been shown to contribute to modulating the fine-tuning of excitation-inhibition balance (1–3). At the single-neuron level, the balance between excitatory and inhibitory synaptic inputs is coordinated by actions of highly regulated and structurally organized molecular machinery. Decades of research have now uncovered many components of both excitatory and inhibitory synapses (3–6). In addition, substantial progress has been made in recent years in identifying a set of key molecular players that collectively build architectures of GABAergic (gamma-aminobutyric acidergic) synapses, and extensive descriptive studies have demonstrated physiological properties of a diverse range of interneuron subtypes (7–11). However, how inhibitory neural circuits are precisely dictated by diverse signaling pathways, even in well-established brain regions (e.g., hippocampus), remains unclear.

IQSEC3 (IQ Motif and SEC7 domain-containing protein 3), also known as BRAG3 or SynArfGEF, is a recently identified gephyrin-binding protein (12–14) that functions as a brain-specific ARF-GEF (adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor) for ARF6 (12,15). In addition, IQSEC3 regulates GABAergic synapse development in cultured hippocampal neurons and adult dentate gyrus granule neurons of the mouse hippocampus in an ARF-GEF activity-dependent manner (13,15). Mice with dentate gyrus-specific knockdown (KD) of IQSEC3 exhibit increased susceptibility to seizure-inducing agents, reflecting heightened network activities. Furthermore, IQSEC3 acts as a downstream factor for Npas4, an early-response transcription factor enriched in the brain, and mediates Npas4-dependent inhibitory synapse maintenance in hippocampal CA1 somatostatin-positive interneurons and anxiety-like behavior (16). However, many of these previous studies used small hairpin RNA (shRNA)-mediated KD of endogenous IQSEC3 and/or

overexpression of IQSEC3. These approaches have inherent limitations, most notably off-target effects (17), although careful control experiments were performed in each case. Thus, genetic knockout (KO) approaches should be performed to validate previous key observations derived from KD experiments.

Here, we generated mice with constitutive or conditional KO (cKO) of *Iqsec3* to examine the synaptic consequences of genetic deletion of *Iqsec3* in hippocampal CA1 synapses. We found that conditional deletion of *Iqsec3* decreased inhibitory synapse density, inhibitory synaptic transmission, and NMDA receptor (NMDAR)-triggered long-term potentiation (LTP) in the hippocampal CA1 of adult mice. Moreover, *Iqsec3*-cKO mice exhibited impaired fear memory. Notably, IQSEC3 deletion increased activity of mTOR (mechanistic target of rapamycin) and its downstream target P70-S6K1 in the hippocampus but not in the cortex. Inhibiting S6K1 activity in hippocampal CA1 neurons completely rescued the impaired inhibitory synapse density and long-term fear memory observed in *Iqsec3*-cKO mice. Collectively, these results provide evidence that IQSEC3 is a critical component in maintaining activity-dependent organization of GABAergic synapses and hippocampus-dependent fear memory that acts through mTOR signaling cascades.

## METHODS AND MATERIALS

### Animals

All mice were on a C57BL/6J background, produced by backcrossing with C57BL/6J wild-type (WT) mice (purchased from Jackson Research Laboratory). Nestin-Cre mice (003771; Jackson Research Laboratories) were a gift from Dr. Albert Chen (Duke–National University of Singapore, Singapore). Emx1-Cre mice (005628; Jackson Research Laboratories) were a gift from Dr. Eunjoon Kim (Korea Advanced Institute of Science and Technology, Korea). The mice were housed and bred in the animal facility of Daegu Gyeongbuk Institute of Science and Technology under standard, temperature-controlled laboratory conditions of a 12-hour light/dark cycle (lights on at 7 AM) and received water and food ad libitum. All mice were maintained and handled in accordance with protocols (DGIST-IACUC-19052109-00) approved by the Institutional Animal Care and Use Committee of Daegu Gyeongbuk Institute of Science and Technology. Further information on the methods is available in the [Supplement](#).

## RESULTS

### Constitutive KO of *Iqsec3* Causes Loss of Both Excitatory and Inhibitory Synaptic Transmission in Hippocampal CA1 Neurons

We first generated constitutive *Iqsec3*-KO mice using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system. Three single-guide RNAs were designed to cleave downstream of the start codon of the *Iqsec3* gene to induce early termination (Figure S1A–D). IQSEC3 proteins were undetectable in brain homogenates of constitutive *Iqsec3*-KO mice as assessed by immunoblot analysis (Figure S1E). However, constitutive *Iqsec3*-KO did not alter the expression level of other synaptic proteins residing at GABAergic

synapses, including gephyrin, collybistin, neuroligin-2, and GABA<sub>A</sub> receptor subunits (Figure S1F, G).

We next performed whole-cell electrophysiological recordings of miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) in acute brain slices from control and *Iqsec3*-KO mice (Figure S1H–M). We found that both the frequency and amplitude of mIPSCs were significantly reduced in *Iqsec3*-KO neurons (Figure S1H–J). Unexpectedly, constitutive *Iqsec3*-KO also reduced the frequency and amplitude of mEPSCs (Figure S1K–M). We have no definitive answer as to why constitutive *Iqsec3*-KO decreased excitatory synaptic transmission at hippocampal CA1 synapses, but off-target effects induced by CRISPR/Cas9-mediated genome engineering is a plausible explanation (18).

### Generation of *Iqsec3*-cKO Mice

Because some studies have reported that effects of conditional genetic deletion of certain synaptic genes differ from those obtained using constitutive KO or shRNA-mediated KD (19,20), we generated mice with cKO of *Iqsec3* by introducing flanked exon 3 with loxP sites to allow conditional deletion (Figure 1A, B). Before deciding which Cre recombinase driver line(s) to cross, we analyzed the distribution of IQSEC3 expression in the adult mouse brain. RNAscope-based in situ hybridization analyses showed that *Iqsec3* messenger RNA was detected in *CaMKIIα*-positive glutamatergic neurons and in *Gad1*-positive GABAergic neurons of adult mice brains (Figure 1C, D). Immunohistochemistry analyses similarly showed that IQSEC3 proteins were expressed in these same neurons in the adult mouse brain (Figure 1E, F). Thus we chose a Nestin-Cre driver line to cross with *Iqsec3*-floxed mice to produce transgenic mice with the deletion of *Iqsec3*. These Nestin-Cre;*Iqsec3*-cKO mice (henceforth referred to as Nestin-*Iqsec3*) were viable and fertile and bred in the expected Mendelian ratio (Figure S2A), although Nestin-*Iqsec3* mice (both male and female) weighed marginally (but significantly) less at postnatal days 30, 42, and 50, a general metabolic feature of the Nestin-Cre driver line (21) (Figure S2B). Gross morphology and neuron numbers in Nestin-*Iqsec3* mice were comparable to those of control littermates, as assessed by Nissl staining (Figure 1G) and staining for the neuron-specific marker NeuN (Figure 1H, I). In addition, semiquantitative immunoblot analyses showed that expression levels of various synaptic proteins in the hippocampus and cortex of Nestin-*Iqsec3* mice were comparable to those in littermate control mice (Figure 1J, K).

### Conditional *Iqsec3* KO Reduces the Number and Transmission of Inhibitory Synapses in Hippocampal CA1

It was previously reported that IQSEC3 is required for maintenance of GABAergic synapse density and transmission in hippocampal CA1 and dentate gyrus as assessed using an shRNA-mediated KD approach (13,15,16). Quantitative immunofluorescence analyses showed that the density and area of GABAergic synaptic markers VGAT (vesicular GABA transporter) and GABA<sub>A</sub>Rγ2 were significantly reduced in all examined hippocampal CA1 layers—stratum pyramidale, stratum oriens, stratum radiatum, and stratum lacunosum

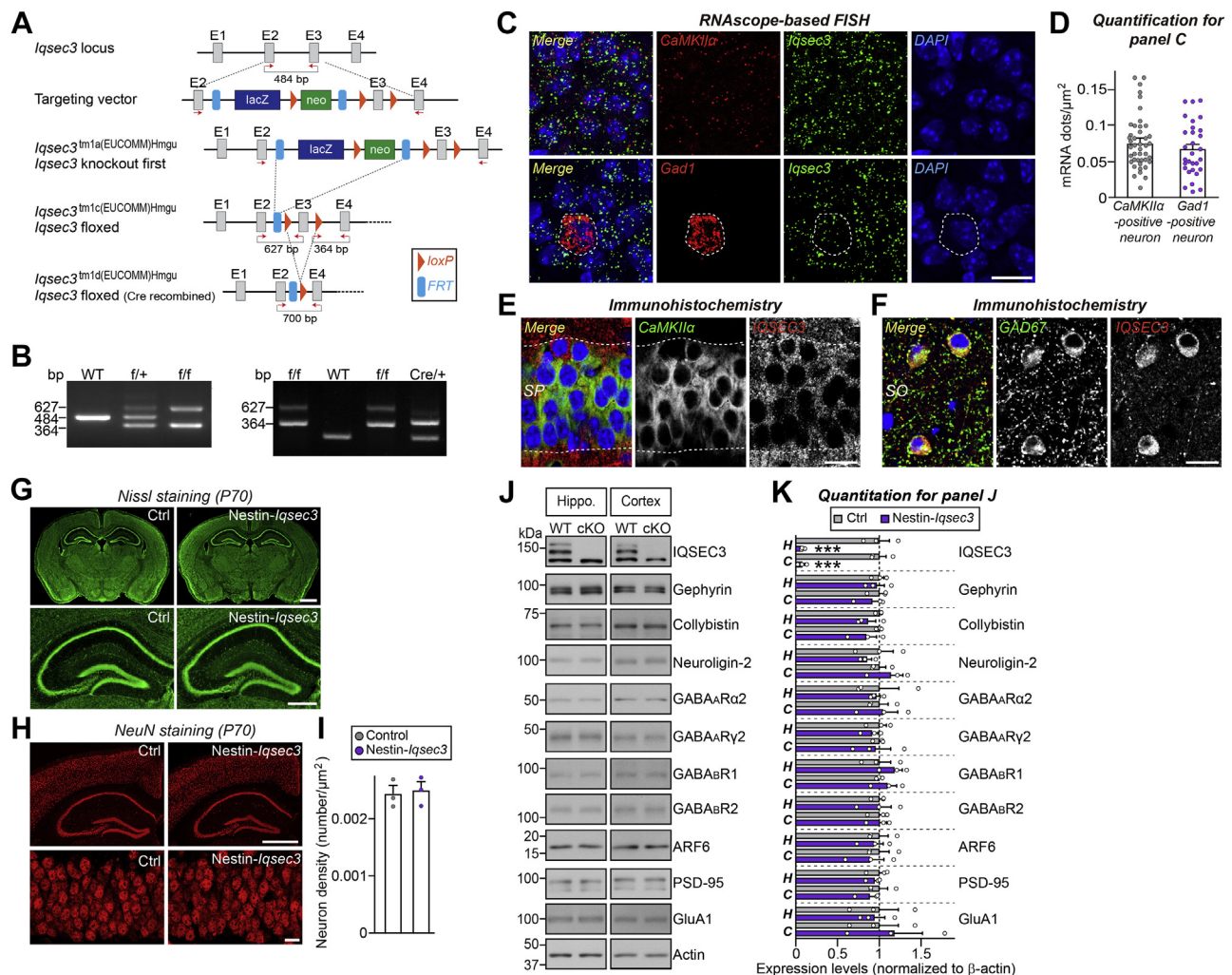
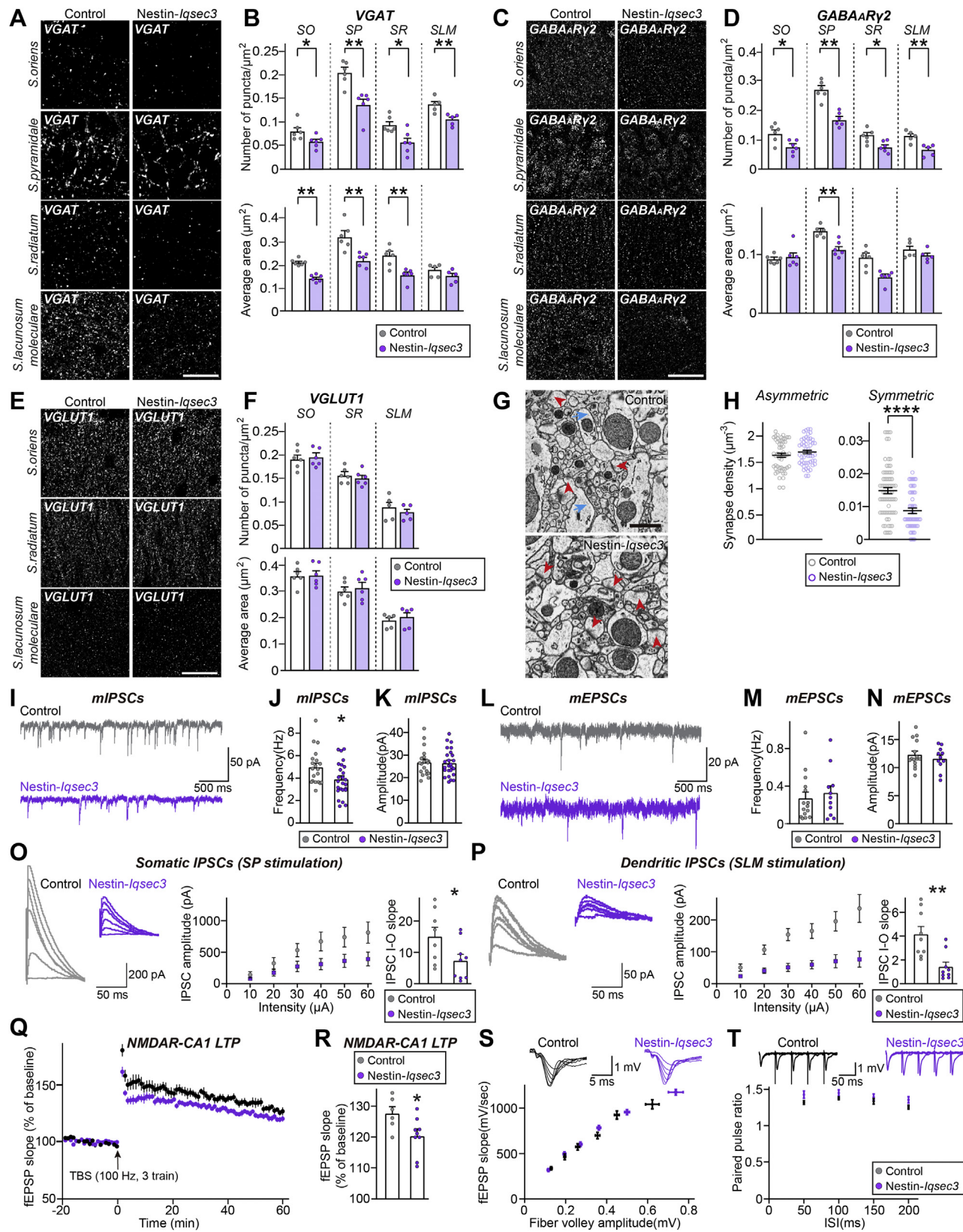


**Figure 1.** Generation of *Iqsec3* mice. (A) Strategy used to generate *Iqsec3*<sup>tm1d</sup>/tm1d cKO mice. Arrows flanking the neomycin-resistant gene and FRT or exon 3 (E3) indicate loxP sites. Red arrows indicate forward and reverse primers used for genotyping. Note that lacZ and neomycin cassettes are two separate markers. (B) Polymerase chain reaction (PCR) genotyping of Nestin-Cre; *Iqsec3*-cKO (Nestin-*Iqsec3*) mice. The 400-bp Cre-specific polymerase chain reaction product was detected only in Nestin-*Iqsec3* mice. The band sizes for the *Iqsec3* floxed allele were 627 and 364 bp. (C) Expression of *Iqsec3* mRNA (green) in *CaMKIIα*<sup>+</sup> pyramidal neurons (red) and *Gad1*<sup>+</sup> interneurons (red) of the CA1 hippocampus visualized by in situ hybridization using RNAscope technology. Nuclei were stained with DAPI (blue). Scale bar = 20 μm (applies to all images). (D) Quantitative analyses of RNAscope data measuring *Iqsec3* mRNA expression in pyramidal neurons or interneurons. Data are presented as mean ± SEM (dot indicates number of cells). (E, F) Expression of IQSEC3 protein in the CA1 hippocampus of mice at p42 (6 weeks), as visualized by immunohistochemistry using anti-IQSEC3 antibodies (red) and anti-*CaMKIIα* (E) or anti-GAD67 (F) antibodies (green). Nuclei were stained with DAPI (blue). Scale bar = 20 μm (applies to all images). (G) Normal gross morphology of the Nestin-*Iqsec3* brain at p70, as revealed by Nissl staining. Scale bar = 1 mm (top) and 500 μm (bottom). (H) Representative images of NeuN (a neuronal marker) staining in the Nestin-*Iqsec3* brain. Normal numbers of neurons in hippocampal and cortical regions (top, forebrain coronal section; bottom, hippocampal CA1 stratum pyramidale layer). Scale bar = 1 mm (top) and 20 μm (bottom). (I) Summary graphs of NeuN staining in the hippocampal CA1 pyramidal layer from panel (H). Data are mean ± SEM (n = 3 mice each after averaging data from six sections/mouse). (J, K) Representative immunoblots (J) of hippocampal and cortical lysates and summary graphs showing synaptic protein levels (K) in crude synaptosomal fractions of p42 control and Nestin-*Iqsec3* brains analyzed by semiquantitative immunoblotting. Data are mean ± SEM (\*\*p < .001; Mann-Whitney U test; n = 3 mice/group). ARF6, adenosine diphosphate ribosylation factor 6; *CaMKIIα*, calcium/calmodulin-dependent protein kinase IIα; Ctrl, control; cKO, conditional knockout; FISH, fluorescence in situ hybridization; FRT, FLP recombinant target; GABA<sub>A</sub>R, gamma-aminobutyric acid A receptor; GAD67, glutamic acid decarboxylase 67; Hippo, hippocampus; mRNA, messenger RNA; p, postnatal day; SO, stratum oriens; SP, stratum pyramidale; WT, wild-type.

molecular—in Nestin-*Iqsec3* mice (Figure 2A–D). In contrast, the density and size of VGLUT1 (vesicular glutamate transporter 1) puncta were unchanged in all the examined layers of hippocampal CA1 (Figure 2E, F). Similar results were obtained from CA1 excitatory neurons in which IQSEC3 expression was specifically suppressed by stereotactic injection of AAV-DIO-

sh*Iqsec3* (Figure S3) in Emx1-Cre mice. These findings are consistent with KD-based results (16). Furthermore, electron microscope analyses revealed that the number of GABA-positive presynaptic boutons (i.e., symmetrical/inhibitory synapse) in the stratum radiatum layer of hippocampal CA1 regions was significantly decreased in Nestin-*Iqsec3* mice



(Figure 2G, H). Collectively, both light and electron microscope analyses demonstrated that the loss of IQSEC3 significantly decreases the number of GABAergic synapses in vivo without altering their gross ultrastructure.

To corroborate these anatomical analyses, we performed whole-cell electrophysiological recordings of mEPSCs and mIPSCs in acute brain slices from Nestin-*lqsec3* and littermate WT mice (Figure 2I–N). We found that the frequency, and not the amplitude, of mIPSCs was decreased in IQSEC3-deficient hippocampal CA1 pyramidal neurons (Figure 2I–K). No alterations were observed in the frequency or amplitude of mEPSCs (Figure 2L–N). We also recorded evoked IPSCs in Nestin-*lqsec3* mice, measuring somatic IPSCs evoked by stimulation of axons in the stratum pyramidale layer or dendritic IPSCs evoked by stimulation of axons in the stratum lacunosum moleculare layer (Figure 2O, P). IQSEC3 deletion reduced the amplitudes of somatic IPSCs by approximately 51.3% and dendritic IPSCs by approximately 65.6%, suggesting that IQSEC3 is critical for regulation of both somatic and dendritic inhibition in hippocampal CA1. In addition, NMDAR-dependent LTP at Schaffer collateral-to-CA1 synapses (induced by theta burst stimulation at 100 Hz) was modestly but significantly reduced in Nestin-*lqsec3* mice, with no alterations in fiber volley size, paired-pulse facilitation of field excitatory postsynaptic potentials, or NMDAR-dependent long-term depression (Figure 2Q–T and Figure S4). Collectively, these results clearly support the idea that IQSEC3 is critical for synaptic transmission within somatic and dendritic compartments and long-term synaptic plasticity at GABAergic postsynaptic sites in hippocampal CA1 pyramidal neurons.

### Conditional *lqsec3* KO Impairs Fear Memory Maintenance

The changes in NMDAR-dependent LTP observed in Nestin-*lqsec3* mice led us to hypothesize that IQSEC3 loss could

induce certain cognitive deficits (e.g., memory formation). Home-cage monitoring using the LABORAS system showed that Nestin-*lqsec3* mice displayed comparable locomotion, climbing, rearing, grooming, drinking, and eating activities compared with WT littermate mice (Figure S5A). In addition, anxiety-like behaviors were comparable between Nestin-*lqsec3* and littermate WT mice, as assessed by open field, light and dark transition, and elevated plus maze tests (Figure 3A–D and Figure S5B, C). Moreover, there were no changes in the working memory or exploratory behavior, as assessed by Y maze, novel object recognition, and Morris water maze tests (Figure S6). Furthermore, Nestin-*lqsec3* mice exhibited normal social interaction and social novelty recognition memory, as assessed by the three-chamber test (Figure S5D–I). Fear conditioning is known to measure hippocampus- and amygdala-dependent fear memory (22). To determine whether fear conditioning was altered in Nestin-*lqsec3* mice, we exposed both littermate WT and Nestin-*lqsec3* mice to a foot shock associated with a particular environment and exposed the mice to the same environment 24 hours after memory acquisition (Figure 3E). During the training session, we observed robust freezing behavior in both Nestin-*lqsec3* and littermate WT mice (Figure 3F), suggesting that the ability to acquire fear conditioning is normal in Nestin-*lqsec3* mice. Context-dependent freezing elicited by the context 1 hour after training was comparable between Nestin-*lqsec3* mice and littermate WT mice, suggesting normal short-term fear memory in Nestin-*lqsec3* mice (Figure 3G). However, context-dependent freezing elicited by the context 24 hours after training was significantly reduced in Nestin-*lqsec3* mice (Figure 3H), suggesting impaired long-term fear memory. This result was not due to the inherent phenotypes of Nestin-Cre mice, which showed comparable fear memory as WT mice, with no differences in locomotion or anxiety levels (Figure S7). Overall, these results suggest that Nestin-*lqsec3* mice display impaired fear memory with no clear deficits in other behaviors.

**Figure 2.** *lqsec3* deletion leads to impaired GABAergic synapse maintenance in the CA1 hippocampal region. (A, C, E) Representative images of hippocampal CA1, SP, SR, and SLM regions of *lqsec3*<sup>fl/fl</sup> (control) and Nestin-*lqsec3* mice and immunostaining for the inhibitory synapse markers VGAT (A) or GABA<sub>A</sub>γ2 (C) and the excitatory synapse marker VGLUT1 (E). Scale bar = 20 μm (applies to all images). (B, D, F) Quantification of the density and area of VGAT-positive (B), GABA<sub>A</sub>γ2-positive (D), and VGLUT1-positive (F) synaptic puncta. Data are mean ± SEM (\**p* < .05; \*\**p* < .01; Mann-Whitney *U* test; *n* = 6 mice each after averaging data from three sections/mouse). (G) Representative electron microscopy image of the CA1 SR from control and Nestin-*lqsec3* mice. Red arrowheads indicate asymmetrical synapses, and blue arrows indicate symmetrical synapses. Scale bar = 1 μm (applies to all images). (H) Summary graphs showing asymmetrical and symmetrical synapse densities. Data are mean ± SEM (\*\*\**p* < .0001; Mann-Whitney *U* test; asymmetrical synapse/control, 54 sampling frames from 3 mice; asymmetrical synapse/Nestin-*lqsec3*, 54 sampling frames from 3 mice; symmetrical synapse/control, 72 sampling frames from 4 mice; symmetrical synapse/Nestin-*lqsec3*, 54 sampling frames from 3 mice). (I–K) Representative traces (I) and summary graphs (J, K) showing the frequency and amplitude of mIPSC recorded from CA1 pyramidal cells of the hippocampus of *lqsec3*<sup>fl/fl</sup> (control) and Nestin-*lqsec3* mice. Graphs show mean ± SEM (\**p* < .05; Mann-Whitney *U* test; the number of neurons analyzed are as follows: control, 20 cells from 5 mice; Nestin-*lqsec3*, 25 cells from 5 mice). (L–N) Representative traces (L) and summary graphs (M and N) showing the frequency and amplitude of mEPSCs recorded from CA1 pyramidal cells of the hippocampus of control and Nestin-*lqsec3* mice. Graphs show mean ± SEM (Mann-Whitney *U* test; the number of neurons analyzed are as follows: control, 15 cells from 3 mice; Nestin-*lqsec3*, 11 cells from 3 mice). (O) Representative somatic eIPSCs trace (left), average eIPSC I–O curve (middle), and average eIPSC I–O slope (right) for hippocampal CA1 pyramidal neurons from control and Nestin-*lqsec3* mice. Graphs show mean ± SEM (\**p* < .05; Mann-Whitney *U* test; control, nine cells from 4 mice; Nestin-*lqsec3*, nine cells from 4 mice). (P) Representative dendritic eIPSC traces (left), average eIPSC I–O curve (middle), and average eIPSC I–O slope (right) for hippocampal CA1 pyramidal neurons from control and Nestin-*lqsec3* mice. Graphs show mean ± SEM (\*\**p* < .01; Mann-Whitney *U* test; control, nine cells from 4 mice; Nestin-*lqsec3*, eight cells from four mice). (Q) Hippocampal slices prepared from control or Nestin-*lqsec3* mice were stimulated by TBS to induce LTP, measured as increases in the slopes of fEPSPs. (R) The magnitude of LTP was calculated by comparing the average slopes of fEPSPs during the last 5 minutes of recordings with that before stimulation. Data are mean ± SEM (\**p* < .05; Mann-Whitney *U* test; control, six slices from 4 mice; Nestin-*lqsec3*, nine slices from 4 mice). (S) Representative fEPSP traces from control and Nestin-*lqsec3* slices. (T) Paired-pulse facilitation of fEPSPs at various ISIs from control and Nestin-*lqsec3* slices (control, six slices from 4 mice; Nestin-*lqsec3*, eight slices from 4 mice). cKO, conditional knockout; eIPSC, evoked inhibitory postsynaptic current; fEPSP, field excitatory postsynaptic potential; GABA<sub>A</sub>R, gamma-aminobutyric acid type A receptor; ISI, interstimulus interval; LTP, long-term potentiation; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; NMDAR, NMDA receptor; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum moleculare; TBS, theta burst stimulation; VGAT, vesicular GABA transporter; WT, wild-type.

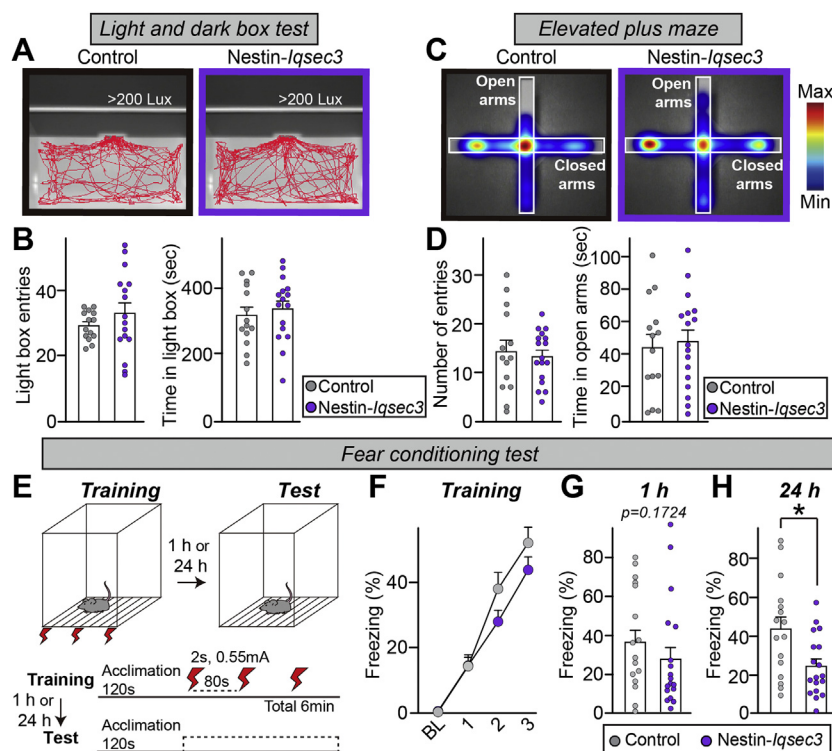


**Figure 3.** Nestin-*Iqsec3* mice exhibit impaired long-term fear memory. (A, B) Analysis of anxiety/exploration-related behavior by light and dark box test in control and Nestin-*Iqsec3* mice. (A) Representative track images in the light chamber. (B) Number of entries into the light chamber and time spent in the light chamber. Data are presented as mean ± SEM (control,  $n = 14$ ; Nestin-*Iqsec3*,  $n = 16$ ). (C, D) Analysis of anxiety/exploration-related behavior by elevated plus maze test in control and Nestin-*Iqsec3* mice. (C) Representative heat maps of the time spent in the open arms of the elevated plus maze. Red represents maximal time spent, and blue represents minimal time spent during the test. (D) Number of open arm entries and time spent in open arms. Data are presented as mean ± SEM (control,  $n = 14$ ; Nestin-*Iqsec3*,  $n = 17$ ). (E) Schematic diagram of fear conditioning. (F–H) Measurement of freezing levels of control and Nestin-*Iqsec3* mice during the training session (F), 1 hour after training (G), and 24 hours after training (H). Foot shocks were delivered at 80-s intervals. Data are mean ± SEM (\* $p < .05$ ; Mann-Whitney  $U$  test; control,  $n = 16$ ; Nestin-*Iqsec3*,  $n = 18$ ). 1, first foot shock; 2, second foot shock; 3, third foot shock; BL, baseline; Max, maximum; Min, minimum.

### Conditional *Iqsec3* KO Upregulates mTOR Signaling, Specifically in the Hippocampus

To examine the alterations in signaling pathways in Nestin-*Iqsec3* mice, we analyzed changes in mTOR-dependent signaling pathways in the hippocampus and cortex, given that altered mTOR signaling is involved in abnormalities in synaptic plasticity (23), and gephyrin, which binds IQSEC3, directly interacts with mTOR (24). To this end, we performed semiquantitative immunoblotting experiments on lysates from the cortex and hippocampus of Nestin-*Iqsec3* mice using antibodies targeting various signaling molecules that are related to mTOR-dependent signaling pathways. Notably, loss of IQSEC3 caused a significant increase in the levels of Ser2448-phosphorylated mTOR and levels of the phosphorylated targets, ribosomal P70-S6K1, ribosomal protein S6, and Akt in the hippocampus but not in the cortex (Figure 4A, B). These results suggest that IQSEC3 loss specifically hyperactivates mTOR signaling in the hippocampus, consistent with the previously advanced concept that misregulated mTOR signaling is associated with altered synaptic transmission and plasticity.

Based on the well-documented finding that activation of mTOR and S6K1 leads to increased protein synthesis, we measured the efficiency of global translation in the hippocampus of Nestin-*Iqsec3* mice using a puromycin-incorporation assay (Figure S8A–C). We found that proteins in both whole-cell lysates and synaptosome fractions could be labeled by incubating the slices with puromycin (Figure S8A). Labeling was suppressed by pretreatment with the protein synthesis inhibitor cycloheximide, suggesting that puromycin

signals represent nascent polypeptides. The intensity of puromycin labeling in whole-cell lysates and synaptosomes from the hippocampus of Nestin-*Iqsec3* mice was comparable to that in control mice, suggesting no alterations in protein synthesis in Nestin-*Iqsec3* mice (Figure S8B, C). Loss of IQSEC3 caused a significant increase in hippocampal levels of phosphorylated eEF2 (eukaryotic elongation factor 2) (Figure S8D, E), which has been reported to contribute to a decrease in global protein synthesis (25). These data suggest that hyperactivation of mTOR signaling induced by loss of IQSEC3 does not produce a net increase in protein synthesis (Figure S8F).

Gephyrin was previously reported to directly bind to mTOR, as verified by yeast-2 hybrid assays (24). In addition, mTOR mutants defective in binding to gephyrin fail to activate downstream molecules, including S6K1 (24). These observations motivated us to test the hypothesis that IQSEC3 loss might facilitate gephyrin binding to mTOR, specifically in the hippocampus, leading to enhanced mTOR signaling. To test this, we first prepared the synaptosomal fractions from the hippocampus or cortex of control and Nestin-*Iqsec3* mice, coimmunoprecipitated them using either mTOR or gephyrin antibodies, and performed immunoblotting experiments. Surprisingly, we failed to detect significant complex formation between mTOR and gephyrin in crude synaptosomal fractions of the hippocampus or cortex or in hippocampal homogenates of adult mice, despite robust complex formation between IQSEC3 and gephyrin (Figure 4C–E). These results suggest that mTOR does not form a complex with gephyrin and IQSEC3 in vivo.

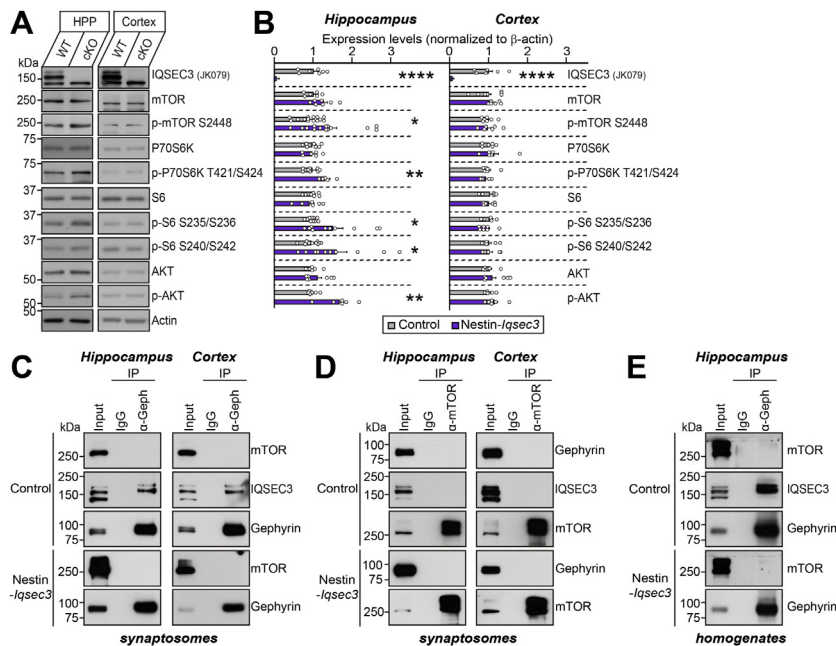


**Figure 4.** Hippocampal S6K1 signaling is abnormally elevated in *Nestin-Iqsec3* mice. **(A)** Representative Western blotting images of mTOR signaling proteins in the hippocampus and cortex. **(B)** Quantification of protein levels from panel **(A)**. Data are mean  $\pm$  SEM (\* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .0001$ ; Mann-Whitney  $U$  test). **(C, D)** Coimmunoprecipitation experiment in mouse hippocampus and cortex. Crude synaptosomal fractions of adult mouse brains were immunoprecipitated with anti-gephyrin antibodies **(C)** or anti-mTOR antibodies **(D)** and immunoblotted with the indicated antibodies. Equal amounts of mouse or rabbit IgG were used as a negative control. Input, 5%. **(E)** Coimmunoprecipitation of mouse hippocampus homogenates. Hippocampus homogenates were immunoprecipitated with anti-gephyrin and immunoblotted with the indicated antibodies. Equal amounts of mouse IgG were used as a negative control. Input, 5%. cKO, conditional knockout; Geph, gephyrin; HPP, hippocampus; IP, immunoprecipitation; mTOR, mechanistic target of rapamycin; p, phosphorylated; WT, wild-type.

### Suppressing S6K1 Activity Normalizes Impaired Inhibitory Synapse Density and Fear Memory

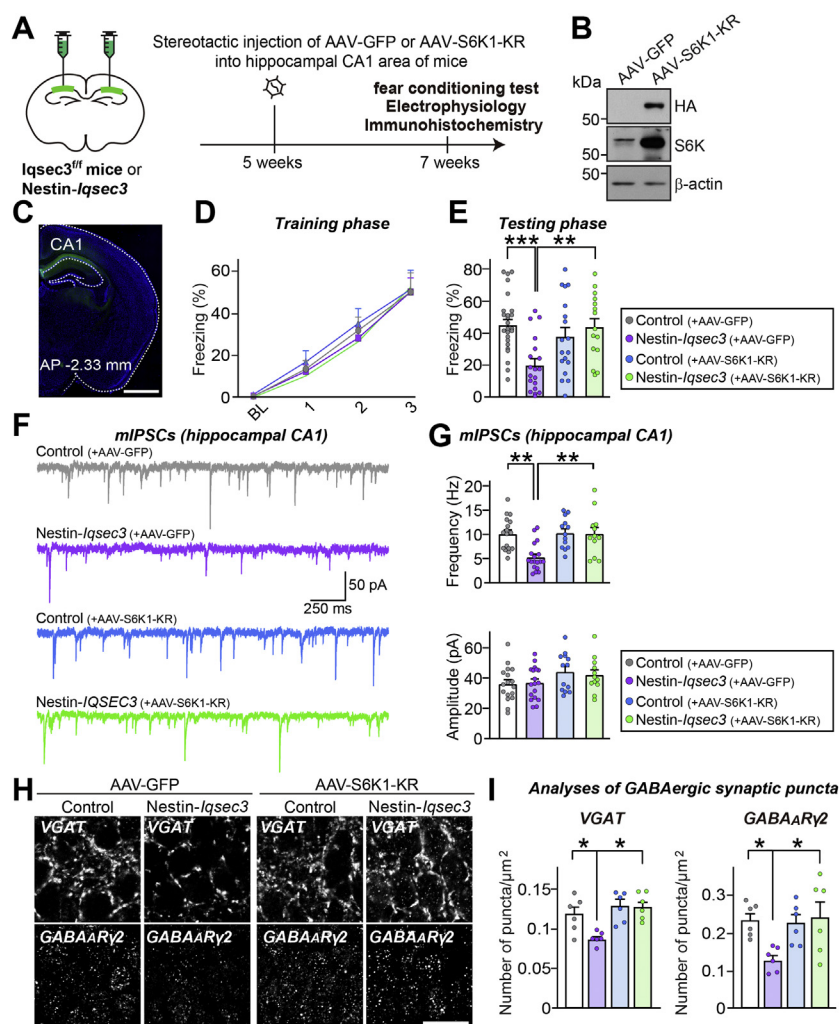
Next, we investigated whether hyperactivated mTOR signaling underlies deficits in fear memory observed in *Nestin-Iqsec3* mice. After stereotactically injecting control and *Nestin-Iqsec3* mice with AAVs (adeno-associated viruses) expressing dominant-negative S6K1 K100R (S6K1 KR), previously used to inhibit S6K1 activity in mice (26), into hippocampal CA1, we subjected mice to fear conditioning tests (Figure 5A–C). Immunofluorescence analyses verified that viral expression of S6K1 KR normalized upregulated levels of phosphorylated S6 protein in the hippocampal CA1 stratum pyramidale layer of *Nestin-Iqsec3* mice (Figure S9). Remarkably, S6K1 KR-mediated suppression of S6K1 hyperactivation rescued the impaired fear memory observed in *Nestin-Iqsec3* mice, as shown by an increase in the degree of freezing to a level similar to that observed in littermate control mice (Figure 5D, E).

We further examined whether expression of S6K1 KR similarly rescued the impaired inhibitory synapse maintenance and/or altered NMDAR LTP. We found that the decreased mIPSC frequency was rescued by expression of S6K1 KR in the hippocampal CA1 of *Nestin-Iqsec3* mice (Figure 5F, G). Notably, expression of S6K1 KR rescued the reduced VGAT and GABA<sub>A</sub>R $\gamma$ 2 puncta density in the hippocampal CA1 of *Nestin-Iqsec3* mice (Figure 5H, I), with no changes in the levels of total GABA<sub>A</sub>R $\gamma$ 2 or GABA<sub>A</sub>R $\alpha$ 2 subunits (Figure S10). However, expression of S6K1 KR failed to rescue the impaired NMDAR-dependent hippocampal CA1 LTP in *Nestin-Iqsec3* mice (Figure S11). Collectively, these results suggest that IQSEC3-mediated proper organization of inhibitory synapses formed onto hippocampal CA1 pyramidal neurons contributes to the maintenance of normal fear memory but not activity-dependent long-term synaptic plasticity.

How does IQSEC3 loss cause hyperactivation of mTOR signaling? We hypothesized that IQSEC3 regulates mTOR signaling through binding to calmodulin (CaM), given that CaM interacts with mTOR and activates its kinase activity (27,28) and that CaM also interacts with IQSEC3 through its IQ motif (14). Thus, we generated an IQSEC3 IQ mutant (I318A/Q319A) that is defective in binding to all three CaMs (Figure 6A) (14,29). First, we confirmed that IQSEC3 IQ mutant did not bind to CaM1 in HEK293T cells using coimmunoprecipitation assays (Figure 6B). We then examined whether IQSEC3 loss-induced mTOR activation could be linked to CaM signaling. To this end, we injected Cre recombinase–fused EGFP (Cre), inactive Cre recombinase–fused EGFP ( $\Delta$ Cre) into the hippocampal CA1 of *Iqsec3*-floxed mice, or coinjected Cre with AAVs expressing the indicated IQSEC3 variants (Figure 6C). Using immunostaining for phosphorylated S6 as a proxy for mTOR hyperactivation, we found that the elevation in phosphorylated S6 levels induced by IQSEC3 loss was completely rescued by expression of IQSEC3 WT as well as IQSEC3 IQ mutant (Figure 6D, E). Surprisingly, expression of IQSEC3 ARF-GEF inactive mutant (E749A) (Figure 6A) failed to rescue the elevated phosphorylated S6 levels observed in IQSEC3-deficient mice (Figure 6D, E), suggesting that dysregulation of ARF signaling, but not CaM signaling, induced by IQSEC3 deficiency likely underlies mTOR hyperactivation.

### DISCUSSION

In this study, we uncovered alterations in various synaptic parameters through biochemical, anatomical, electrophysiological, and behavioral analyses of *Iqsec3*-cKO mice, demonstrating that IQSEC3 is essential for the regulation of GABAergic synapse structure, transmission, and plasticity in hippocampal CA1 pyramidal neurons and for hippocampus-



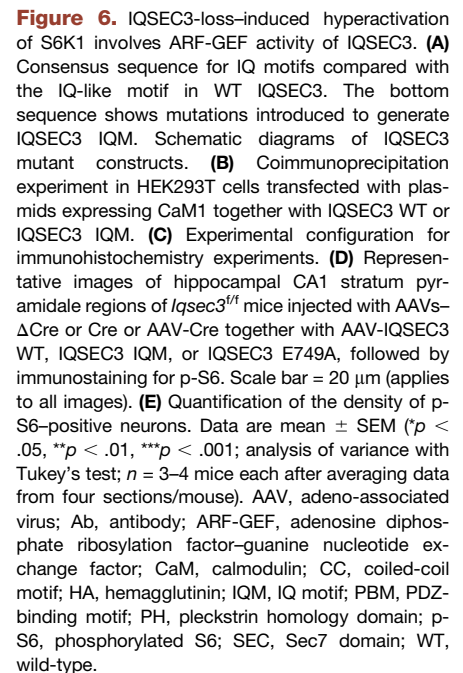
adeno-associated virus; AP, anteroposterior; BL, baseline; GABA<sub>A</sub>R, gamma-aminobutyric acid type A receptor; HA, hemagglutinin; HPP, hippocampus; mIPSC, miniature inhibitory postsynaptic current; VGAT, vesicular GABA transporter.

**Figure 5.** Suppression of S6K1 activity in the hippocampus ameliorates impaired long-term fear memory in Nestin-Iqsec3 mice. **(A)** Experimental configuration for immunohistochemistry, ex vivo electrophysiology, and behavioral experiments. **(B)** Immunoblot analyses showing S6K1 KR expression in vivo. Brain lysates from infected mice were collected after stereotactic injection of AAVs expressing S6K1 KR and immunoblotted with HA or anti-S6K1 antibodies. Anti-β-actin antibodies were used as normalization controls. **(C)** Representative images of AAV-infected neurons in hippocampal CA1 regions of mouse brain coronal sections (~2.33 mm relative to bregma), counterstained with DAPI (blue). Scale bar = 1 mm. **(D, E)** Measurement of freezing levels of control and Nestin-Iqsec3 mice injected with AAV-Control or AAV-S6K1-KR obtained during the training session **(D)** and 24 hours after training **(E)**. Foot shocks were delivered at 80-s intervals. Data are mean ± SEM (\*\**p* < .01; \*\*\**p* < .001; nonparametric analysis of variance with Kruskal-Wallis test, followed by post hoc Dunn's multiple comparison test). **(F, G)** Representative traces **(F)** and summary graphs **(G)** showing the frequency and amplitude of mIPSCs recorded from CA1 pyramidal cells of the hippocampus. Graphs show mean ± SEM (\*\**p* < .01; nonparametric analysis of variance with Kruskal-Wallis test, followed by post hoc Dunn's multiple comparison test; the number of neurons analyzed are as follows: control+AAV-GFP, 17 cells from 7 mice; Nestin-Iqsec3, 18 cells from 7 mice; control+AAV-S6K1-KR, 13 cells from 7 mice; Nestin-Iqsec3+AAV-S6K1-KR, 12 cells from 7 mice). **(H)** Representative images of hippocampal CA1 regions of *Iqsec3<sup>fl/fl</sup>* and Nestin-Iqsec3 mice and immunostaining for the inhibitory synapse markers VGAT and GABA<sub>A</sub>γ2. Scale bar = 20 μm (applies to all images). **(I)** Quantification of the density of VGAT-positive and GABA<sub>A</sub>γ2-positive synaptic puncta. Data are mean ± SEM (\**p* < .05; analysis of variance with Tukey's test; *n* = 6 mice each after averaging data from 4 to 6 sections/mouse). 1, first foot shock; 2, second foot shock; 3, third foot shock; AAV, adeno-associated virus.

dependent fear memory formation. Prior studies using shRNA-mediated KD approaches clearly showed that IQSEC3 is important for GABAergic synapses in the hippocampus (15,16). However, because the KD approach does not eliminate IQSEC3 completely and may cause off-target effects, confirming and validating the proposed synaptic function of IQSEC3 using more sophisticated genetic approaches was clearly necessary. It is possible that our CRISPR/Cas9-mediated constitutive deletion might also suffer from potential developmental effects or compensations. Indeed, CRISPR/Cas9-mediated constitutive deletion of *Iqsec3* produced severe impairment in excitatory synaptic transmission, which was not documented in previous studies (15,16) and is not consistent with an exclusive GABAergic synapse localization of IQSEC3 (12,13). Thus, we speculated that this is likely attributable to off-target effects of the CRISPR approach and focused our analyses on our newly generated *Iqsec3*-cKO model, which we believe is a more sophisticated approach for

addressing the synaptic function of IQSEC3 in mature synapses. We made three principal observations.

First, we completely reproduced previous IQSEC3 loss-of-function phenotypes in hippocampal CA1. Nestin-Iqsec3 mice exhibited decreased numbers of GABAergic synaptic puncta and a reduction in spontaneous and evoked GABAergic synaptic transmission. In addition, NMDAR LTP was slightly decreased in Schaffer collateral synapses of IQSEC3-deficient mice, with no alteration in NMDAR-long-term depression. Thus, this study demonstrated that IQSEC3 is a critical component that regulates GABAergic synapse development and plasticity at synapses in vivo. The reduced LTP observed in *Iqsec3*-cKO mice was not attributable to changes in excitatory transmission, as evidenced by the observation that our field excitatory postsynaptic potential analysis showed no significant differences in fiber volley size or paired-pulse facilitation between *Iqsec3*-cKO mice and control mice compared with control mice. We speculate that background



Third, we found that loss of IQSEC3 triggered hyperactivation of mTOR- and S6K1-related signaling cascades, specifically in the hippocampus. We further showed that S6K1 activity underlies the maintenance of GABAergic synapse density, GABAergic synaptic transmission, and fear memory but not LTP. We were unable to use rapamycin, a well-established acute inhibitor of the mTOR pathway (43) because injection of rapamycin in WT mice drastically reduced both glutamatergic and GABAergic synapse density (data not shown), hinting at its pleiotropic effects on diverse cellular properties (16). The mTOR complex is responsible for integrating a diverse set of cues and transducing divergent downstream cascades, including translational control through S6K1 and 4EBP (44). However, its functional significance at GABAergic synapse and relevant neural circuits is currently unclear. To our knowledge, our results provide the first evidence that mTOR signaling pathway plays a critical role in controlling the properties of GABAergic neural circuits. mTOR activation has been well established as a negative regulator of eEF2 kinase (25). Unexpectedly, we found that IQSEC3 loss increased phosphorylated S6K1 in association with an

increase in phosphorylated eEF2 levels, likely leading to no net changes in protein synthesis in the hippocampus. Factors other than the mTOR signaling pathway have been shown to regulate eEF2 kinase (45–47). Future studies are warranted to precisely determine how IQSEC3 integrates multiple signaling cascades in the hippocampus. A previous study showed that mTOR directly interacts with gephyrin and that gephyrin-binding-defective mutants of mTOR fail to activate S6K1 and 4EBP (24). However, another study showed that gephyrin interacts only with the inactive form of mTOR and that activated mTOR is disengaged from gephyrin (48). Puzzlingly, we failed to detect an interaction of mTOR with gephyrin in the mouse hippocampus, despite observing robust gephyrin-IQSEC3 complex formation. Multiple lines of evidence indicate that mTOR activity is finely regulated by ARF signaling pathways (49,50). In line with this notion, we found that the ARF-GEF activity of IQSEC3 is critical for rescuing the increase in phosphorylated S6 levels induced by IQSEC3 loss, suggesting that dysregulated ARF signaling in *Iqsec3*-cKO mice might primarily drive mTOR hyperactivation, although definitive evidence linking well-established mTOR pathways with known GABAergic synapse components is currently lacking. It remains to be determined whether activation of ARF signaling at GABAergic synapses directly regulates mTOR signaling cascades.

It appears that mTOR signaling in the amygdala, medial prefrontal cortex, or dorsal hippocampus is associated with the stability, reconsolidation, or extinction of fear memory (51–53). However, none of these studies elucidated inhibitory synaptic mechanisms underlying fear memory. We demonstrated that the Akt/mTOR/S6K1 pathway is a key signaling hub required for the formation of fear memory in the hippocampus. We further showed that balanced synaptic inhibition, rather than long-term synaptic plasticity, is coordinated by this signaling pathway to mediate fear memory, which has traditionally been considered to be regulated by normal LTP maintenance. Further studies are required to determine how IQSEC3, in conjunction with gephyrin and mTOR signaling pathways, controls the properties of specific GABAergic neural circuits in the hippocampus.

In sum, this study delineated a previously unidentified *in vivo* synaptic role of IQSEC3 and suggested new research avenues for evaluating whether pharmacological manipulations that favor normalizing S6K1 activity may affect fear-related cognitive impairment. Dissecting the actions of IQSEC3 in greater detail could further expand our understanding of the pathogenetic mechanisms underlying fear-related neuropsychiatric disorders, including depression and posttraumatic stress disorder.

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