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Nicotine Rescues Depressive-like Behaviors via α7-type Nicotinic Acetylcholine Receptor Activation in CaMKIV Null Mice

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Abstract

The nicotinic acetylcholine receptors (nAChRs) are essential for acetylcholine-mediated signaling. Two major functional subtypes of nAChR in the brain, α7-type and α4β2-type, have a high affinity for nicotine. Here, we demonstrated that chronic exposure to nicotine at 0.03–0.3 mg/kg for 14 days rescued depressive-like behavior in calcium/calmodulin-dependent protein kinase IV (CaMKIV) null mice. Chronic exposure to nicotine together with methyllycaconitine, an α7-type nAChR antagonist, but not with dihydro-β-erythroidine, an α4β2-type nAChR antagonist, failed to rescue the depressive-like behavior and restore the reduced number of BrdU-positive cells in the dentate gyrus (DG) of CaMKIV null mice. Furthermore, chronic exposure to nicotine enhanced the PI3K/Akt and ERK/CREB pathways and increased BDNF expression in the DG of CaMKIV null mice. Similar to chronic exposure to nicotine, both PNU-282987 and GTS-21, α7-type nAChR agonists, significantly rescued depressive-like behavior, with a reduction in the number of BrdU-positive cells in the DG of CaMKIV null mice. Both PNU-282987 and GTS-21 also enhanced the PI3K/Akt and ERK/CREB pathways and increased brain-derived neurotrophic factor (BDNF) expression in the DG of CaMKIV null mice. Taken together, we demonstrated that chronic exposure to nicotine rescues depressive-like behavior via α7-type nAChR through the activation of both PI3K/Akt and ERK/CREB pathways in CaMKIV null mice.

Keywords Nicotine · CaMKIV null mice · Depressive-like behaviors · Adult hippocampal neurogenesis · α7-type nAChR

Abbreviations

Akt	Protein kinase B
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
CREB	cAMP-responsive element-binding protein
DHβE	Dihydro-β-erythroidine
DG	Dentate gyrus
ERK	Extracellular signal-regulated kinase
MDD	Major depressive disorder
MLA	Methyllycaconitine
nAChRs	Nicotinic acetylcholine receptors
NE	Norepinephrine
PI3K	Phosphatidylinositol 3-kinase
SSRIs	Selective serotonin reuptake inhibitors
TRD	Treatment-resistant depression
WT	Wild-type
5-HT	Serotonin

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Introduction

Depression is a highly complex disorder. The monoamine hypothesis, characterized by decreased levels of catecholamines and serotonin (5-HT) in the brain of patients with depression, is the dominant theory underlying depression [1, 2]. Selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine (NE) reuptake inhibitors are typically prescribed as the first-line treatment for depression worldwide. Recently, a large portion of patients with depression have exhibited treatment-resistance to SSRIs [3]. In animal models of depression, a treatment-resistant depressive-like behavioral phenotype has been clearly identified [4, 5].

However, there is still a need for improved animal models to target the underlying mechanisms of treatment-resistant depression.

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a serine/threonine protein kinase, activated by nuclear Ca^{2+} elevation, which regulates cyclic AMP-responsive element-binding protein (CREB) at the residue Ser-133 [6, 7]. CREB phosphorylation by CaMKIV enhances brain-derived neurotrophic factor (BDNF) expression, with an essential role in learning and memory [8–10], synaptic plasticity [7], and emotional behaviors in rodents [11–13]. CaMKIV is widely distributed in the brain, associated with the anterior cingulate cortex, somatosensory cortex, cerebellum, hippocampus, and amygdala, and is primarily localized in neuronal nuclei [14]. Recently, CaMKIV null mice exhibited deficits in contextual and cued fear conditioning memory [15], impaired memory of eyeblink conditioning [16], and decreased anxiety-like behaviors [15, 17].

Interestingly, we have previously reported that CaMKIV null mice exhibit depressive-like behaviors, which do not improve following chronic treatment with the SSRI paroxetine [18]. Similarly, treatment with the common SSRI fluoxetine failed to induce hippocampal dentate gyrus (DG) neurogenesis and to exhibit anti-depressive activity in CaMKIV null mice [19]. Thus, CaMKIV null mice show treatment-resistant depressive-like behaviors.

Nicotine has been shown to influence cognitive functioning in humans [20]. This is partly due to the fact that nicotine activates neuronal nicotinic acetylcholine receptors (nAChRs) located in the brain. In the central nervous system, nAChRs are comprised of different combinations of the 17 types of nAChR subunits [21, 22], and two major isoforms, α4β2-type and α7-type, are dominantly distributed in the brain [23–25]. In the hippocampus, nicotine enhances the release of numerous neurotransmitters, including dopamine [26], NE [27], GABA [28], and glutamate [29]. Stimulation of nAChRs increases intracellular Ca^{2+} concentrations [30, 31], which can alter both intracellular signaling pathways and gene expression [32, 33].

The aim of the present study was to investigate the effect of chronic nicotine exposure in CaMKIV null mice.

Materials and Methods

Animals

All mice were housed in cages with access to food and water ad libitum at controlled temperature (23 ± 1 °C) and humidity (55 ± 5%), under a 12-h light/dark cycle (lights on at 9 am). All experimental procedures using animals were approved by the Committee on Animal Experiments at Tohoku University, based on NIH regulations.

Generation of CaMKIV Null Mice

CaMKIV null mice were established by Takao et al. [15]. Genomic DNA clones coding for the exon containing the start codon of CaMKIV were isolated from a 129/SvJ mouse genomic library (Stratagene, San Diego, CA, USA) and subcloned into the pBluescriptSK(+) vector. The targeting vector was comprised of a 14.2-kb NotI-XhoI fragment located at position 59 of the exon, a PGK-neomycin resistance gene, a 4.1-kb BstEII-EcoRI fragment, and a MC1-thymidine kinase. As a result, the exon between XhoI and BstEII sites, which contained an initial codon for CaMKIVa, was replaced with a neo cassette derived from pCM1neo-polyA (Stratagene). The linearized targeting vector was transfected into CCE ES cells derived from 129/SvJ mouse strain by electroporation. Genomic DNA from ES cells selected with G418 (250 mg/ml) and gancyclovir (5 mM) was digested with BamHI and subjected to Southern blot analysis with the 39 probe. This probe, which is a 1-kb EcoRI/BamHI fragment external to the targeting vector sequence, detects an 8.8-kb fragment and a 4.1-kb fragment in the case of wild-type and mutant alleles, respectively. A target clone was injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeric mice were bred with C57BL/6N female mice. Mutant mice were backcrossed at least for 10 generations to the C57BL/6N background. Adult (8–9 weeks old) male CaMKIV null mice were housed in cages with free access to food and water, at a constant temperature (23 ± 1 °C) and humidity (55 ± 5%), with 12-h light/dark cycles (09:00–21:00 h).

Depressive-like Behavioral Tests

We first confirmed the effect of the repeated administration (every day for 14 days) of nicotine (0.003–0.3 mg/kg of body weight, s.c.), PNU-282987 (0.5 mg/kg of body weight, i.p.), and GTS-21 (1 mg/kg of body weight, i.p.) in CaMKIV null mice compared with WT mice, using behavioral tests. The tail-suspension task was evaluated, as per a detailed protocol described by Moriguchi et al. [34]. This test is based on the fact that normally, animals exhibit immobile postures when subjected to short-term, inescapable stress by tail-suspension. The forced-swim task was also conducted, as per a protocol described by Moriguchi et al. [34]. Mice are placed individually in glass cylinders (height, 20 cm; diameter, 15 cm) filled with 25 °C water, and the duration of immobile periods within 5 min are quantified.

Biochemical Analysis

We obtained the hippocampal dentate gyrus (DG) brain tissues from both CaMKIV null and WT mice. Western blotting was performed as described by Moriguchi et al. [35]. We used

the following antibodies: anti-phospho-calcium/calmodulin-dependent protein kinase II (CaMKII) (1:5000, Fukunaga et al. [36]), anti-CaMKII (1:5000, Fukunaga et al. [37]), anti-phospho-MAP kinase (diphosphorylated ERK 1/2; Thy-202/Tyr-204) (1:2000, Sigma-Aldrich, St. Louis, MO, USA), anti-MAP kinase (1:2000, Millipore, Billerica, MA, USA), anti-phospho-CREB (Ser-133) (1:1000, Millipore), anti-CREB (1:1000, Millipore), anti-phospho-protein kinase B (Akt) (Ser-473) (1:1000, Millipore), anti-Akt (1:1000, Millipore), anti-BDNF (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-β-tubulin (1:5000, Sigma-Aldrich). Bound antibodies were visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK) and analyzed semi-quantitatively using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

We followed the methods described by Moriguchi et al. [34]. Mice at 10–12 weeks of age were anesthetized with sevoflurane and perfused via the ascending aorta with phosphate-buffered saline (PBS; pH 7.4) until the outflow became clear. The perfusate was then switched to phosphate buffer (pH 7.4) containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. The brain was removed, post-fixed in the same solution for 2 h at 4 °C, embedded in 2% agarose, and sliced (50 μm sections) using a vibratome (Dosaka EM Co. Ltd., Kyoto, Japan, or Leica VT1000S, Nußloch, Germany). Coronal brain sections were permeabilized with 0.3% Triton X-100 in PBS and blocked with 5% normal goat or donkey serum (Abcam, Cambridge, MA, USA) for 3 h before an overnight incubation step with mouse anti-NeuN monoclonal antibody (1: 500) (Millipore) and rat anti-BrdU polyclonal antibody (1:500) (Accurate Chemical and Scientific, Oxford Biotechnology, Oxfordshire, UK) in blocking solution at 4 °C. After thorough washing with PBS, sections were incubated for 3 h with Alexa 594-labeled anti-mouse IgG (anti-NeuN) or Alexa 488-labeled anti-rat IgG (anti-BrdU). After several PBS washes, sections were mounted on slides with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence images were analyzed using a confocal laser-scanning microscope (Nikon EZ-C1, Nikon, Tokyo, Japan or LSM 710, Zeiss, Oberkochen, Germany). To count BrdU and NeuN double-positive cells after immunohistochemistry, six hippocampal sections were cut (50 μm sections) beginning at 1.7 to 2.2 mm caudal to the bregma. The number of BrdU/NeuN or BrdU/β-catenin double-positive cells in the DG region was determined in a 300 × 300-μm area of each section. In the DG, the GCL (approximately 50-μm wide) and the SGZ (defined as a zone two cell bodies wide (5 μm) along the border of the GCL and hilus) were quantified together. The number of BrdU/NeuN double-positive cells counted per mouse was expressed as the number of double-positive cells per 300 × 300-μm area. Six sections per mouse and six mice per condition were used. The researcher responsible for the cell counts was blinded to all experimental conditions.

BDNF mRNA Quantification Using Real-Time PCR

Analysis was performed in 48-well plates (MiniOpticon Real-Time PCR System, Bio-Rad) using the iQ SYBR Green Supermix 2× (Bio-Rad). The primers used (and the respective sequences) were as follows: BDNF-IF (CCTGCATC TGTTGGGGAGAC), BDNF-IR (GCCTTGTCCGTGGA CGTTTA), BDNF-IVF (CAGAGCAGCTGCCTTGATGT T), BDNF-IVR (GCCTTGTCCGTGGACGTTTA), GAPDH-F (TGTGTCCGTCGTGGATCTGA), and GADPH-R (CACCACCTTCTTGATGTCATCATAC). Quantification of the relative changes in mRNA expression was calculated using the ΔΔCt method. Briefly, the ΔCt values are calculated as per the CT of the target gene minus that of the reference gene. Then, the ΔΔCt values are given by the ΔCt of the experimental sample minus that of the control sample. Finally, the fold differences in the target gene expression are equal to $2^{-\Delta\Delta Ct}$. Product purity and specificity were confirmed in the absence of the DNA template, and using a standard melting curve analysis, respectively.

Other Chemicals

Nicotine, PNU-282987, GTS-21, dihydro-β-erythroidine (DHβE), and BrdU were purchased from Sigma-Aldrich (Tokyo, Japan). Methyllycaconitine (MLA) was purchased from Funakoshi (Tokyo, Japan).

Data Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism 6 (GraphPad Software, San Diego, CA, USA). Comparisons between two experimental groups were made using the unpaired Student's *t* test. Statistical significance for differences among groups (more than two) was assessed using the one-way or two-way analysis of variance (ANOVA), followed by a post hoc Bonferroni's multiple comparisons' test between the control and the remaining groups. Asterisks ($*p < 0.05$, $**p < 0.01$) denote statistical significance in graphs.

Results

Chronic Exposure to Nicotine Rescues Depressive-like Behaviors in CaMKIV Null Mice

We first tested if chronic exposure to nicotine (0.003–0.3 mg/kg, s.c., for 14 days) rescued depressive-like behaviors in CaMKIV null mice, as per the tail-suspension and forced-swim tasks. In the tail-suspension task, the immobility time was significantly increased in CaMKIV null mice versus wild-type (WT) mice (WT mice, 114.6 ± 13.4 s, $n = 6$; CaMKIV null mice, 168.0 ± 9.9 s, $n = 6$), indicative of depressive-like behaviors in the null mice (Fig. 1a). Interestingly, chronic treatment with nicotine at doses of 0.03~0.3 mg/kg significantly decreased the immobility time of null mice in the tail-suspension task in a dose-dependent manner (0.3 mg/kg, 119.4 ± 15.5 s, $n = 6$; 0.03 mg/kg: 131.6 ± 10.6 s, $n = 6$) (Fig. 1a). Interestingly, the pretreatment of CaMKIV null mice with the selective α7-type nAChR antagonist, MLA (3 mg/kg, i.p.), eliminated the effects of nicotine (MLA, 159.1 ± 11.6 s, $n = 6$) (Fig. 1a). In contrast, treatment with the α4β2-type nAChR antagonist, DhβE (1 mg/kg, i.p.), did not alter the immobility time of CaMKIV null mice. Of note, in WT mice, chronic nicotine treatment at a dose of 0.3 mg/kg had no effect on the immobility time (versus non-treated WT mice; Fig. 1a). Both antagonists were administered 1 h prior to nicotine treatment.

Similarly, when we assessed the immobility time in the forced-swim task, CaMKIV null mice showed a significantly increased immobility time compared to WT mice (WT mice, 63.5 ± 6.9 s, $n = 6$; CaMKIV null mice, 135.5 ± 11.9 s, $n = 6$) (Fig. 1b). Once more, the chronic treatment with nicotine at a dose of 0.3 mg/kg significantly decreased the immobility time of CaMKIV null mice (0.3 mg/kg, 71.1 ± 9.0 s, $n = 6$). Moreover, pretreatment of CaMKIV null mice with MLA (3 mg/kg, i.p.), but not DHβE (1 mg/kg, i.p.), significantly increased their immobility time relative to that of mice that had undergone repeated nicotine treatment (MLA, 136.5 ± 11.1 s, $n = 6$) (Fig. 1b). In the WT mice, chronic nicotine treatment at a dose of 0.3 mg/kg had no effect on the immobility time (versus untreated WT mice) in the forced-swim task (Fig. 1b).

Chronic Exposure to Nicotine Ameliorates Adult Hippocampal Neurogenesis in CaMKIV Null Mice

To address mechanisms underlying the anti-depressive effects of chronic exposure to nicotine, we evaluated adult hippocampal neurogenesis in CaMKIV null mice. Mice were injected with BrdU at a dose of 50 mg/kg, i.p., on the first day of drug treatment, and in the next four consecutive days. Two weeks after nicotine treatment, animals were euthanized. To identify BrdU-positive cells, hippocampal slices were double-stained with antibodies anti-BrdU and the neuronal marker NeuN. Compared with the hippocampal slices of WT mice, the number of BrdU-positive neuronal cells significantly decreased in the hippocampal slices of CaMKIV null mice (WT mice, 108.0 ± 6.9 cells, $n = 8$; CaMKIV null mice, 62.4 ± 4.4 cells, $n = 8$) (Fig. 2a, b). Importantly, chronic treatment with nicotine at a dose of 0.3 mg/kg significantly increased the number of BrdU-positive cells in the hippocampal slices of CaMKIV null mice (chronic treatment with nicotine in CaMKIV null mice, 114.6 ± 5.1 cells, $n = 8$) (Fig. 2a, b). Once again, pretreatment of CaMKIV null mice with MLA (3 mg/kg, i.p.),

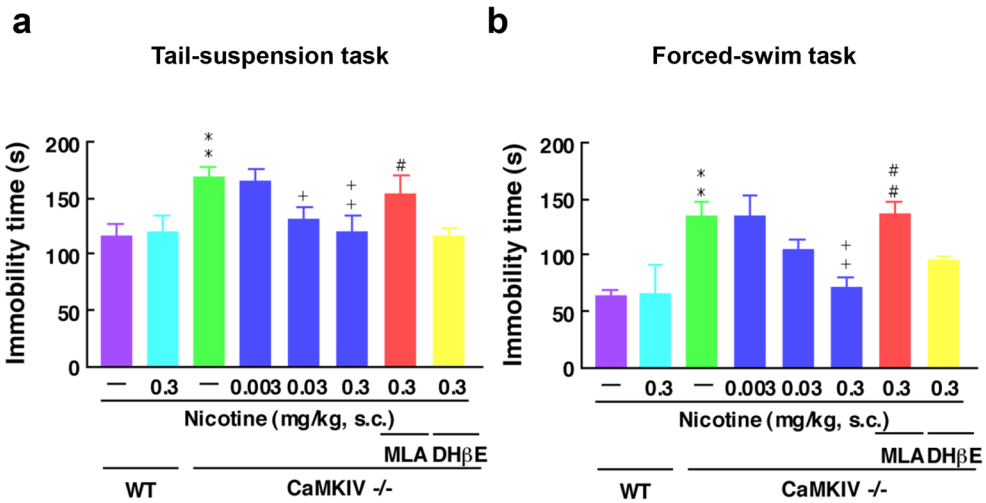

Fig. 1 Chronic exposure to nicotine rescues depressive-like behaviors in CaMKIV null mice. **a**, **b** Immobility time as determined by the tail-suspension (**a**) or forced-swim (**b**) tasks was measured following chronic exposure to nicotine at 0.003–0.3 mg/kg s.c. for 14 days. Chronic exposure to nicotine significantly rescued the increased immobility time seen in CaMKIV null mice, an effect blocked by pretreatment with methyllycaconitine (MLA) at 3 mg/kg i.p. but not with dihydro-β-erythroidine (DHβE) at 1 mg/kg i.p. (Mann-Whitney *U* test, $n = 6$ per group). Vertical lines show the SEM. $^{**}p < 0.01$ versus the wild-type mice. $^{+}p < 0.05$, $^{++}p < 0.01$ versus CaMKIV null mice. $^{\#} < 0.05$, $^{\#\#} < 0.01$ versus nicotine-treated CaMKIV null mice

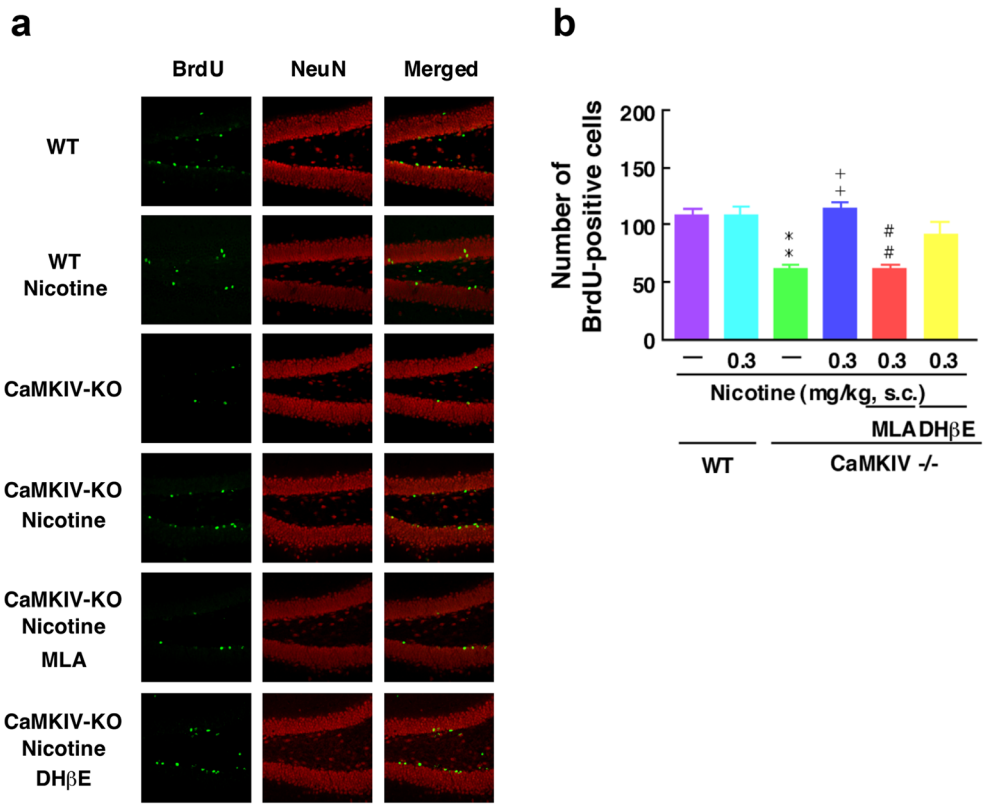

Fig. 2 Chronic exposure to nicotine enhances adult hippocampal neurogenesis in CaMKIV null mice. **a** Confocal microscopy images showing staining for BrdU (green), NeuN (red), and merged signals in hippocampal slices from wild-type mice, nicotine-treated wild-type mice, CaMKIV null mice, nicotine-treated CaMKIV null mice, methyllycaconitine (MLA) plus nicotine-treated CaMKIV null mice, and dihydro-β-erythroidine (DHβE) plus nicotine-treated CaMKIV null mice. Mice were injected with BrdU from the first day of drug treatment, for 5 consecutive days. Mice were treated with nicotine, MLA, or DHβE for 2 weeks. **b** Quantitative analyses of the number of BrdU/NeuN double-positive cells in the DG ($n = 8$). Vertical lines show the S.E.M. $^{**}p < 0.01$ versus the wild-type mice. $^{++}p < 0.01$ versus CaMKIV null mice. $^{\#\#} < 0.01$ versus nicotine-treated CaMKIV null mice

but not DHβE (1 mg/kg, i.p.), eliminated the effects of chronic treatment with nicotine observed in the slices of CaMKIV null mice (MLA plus nicotine-treated CaMKIV null mice, 62.3 ± 3.3 cells, $n = 8$) (Fig. 2a, b). Of note, in the DG of WT mice, chronic nicotine treatment at a dose of 0.3 mg/kg showed no change in the number of BrdU-positive cells relative to untreated WT animals (Fig. 2a, b).

Chronic Exposure to Nicotine Improves ERK, CREB, and Akt Phosphorylation, CaMKIIα Autophosphorylation, and BDNF Expression in the DG of CaMKIV Null Mice

Activation of phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) signal pathways plays an essential role in proliferation and maturation [38]; we assessed those activities following chronic treatment in the context of nicotine-induced hippocampal neurogenesis in the DG of CaMKIV null mice. In CaMKIV null mice, phosphorylation of Akt (Ser-473), ERK (Thr-202/Tyr-204), and CREB (Ser-133) in the DG was markedly decreased compared to that of WT mice (pAkt (Ser-473): 70.7 ± 3.0% of WT, $n = 6$; pERK (Thr-202/Tyr-204): 64.2 ± 6.0% of WT, $n = 6$; pCREB (Ser-133): 58.0 ± 1.8% of WT, $n = 6$) (Fig. 3a, b). Chronic treatment with nicotine at a dose of 1 mg/kg, i.p., significantly restored the phosphorylation of Akt (Ser-473), ERK (Thr-202/Tyr-204), and CREB (Ser-133) in the DG of null mice (pAkt (Ser-473): 96.2 ± 6.1% of WT, $n = 6$; pERK (Thr-202/Tyr-204): 100.6 ± 4.1% of WT, $n = 6$; pCREB (Ser-133): 97.4 ± 8.6% of WT, $n = 6$) (Fig. 3a, b). Pretreatment with MLA (3 mg/kg, i.p.), but not DHβE (1 mg/kg, i.p.), completely abrogated the increased phosphorylation of Akt (Ser-473), ERK (Thr-202/Tyr-204), and CREB (Ser-133) in the DG of null mice (pAkt (Ser-473): 72.3 ± 3.7% of WT, $n = 6$; pERK (Thr-202/Tyr-204): 71.4 ± 7.6% of WT, $n = 6$; pCREB (Ser-133): 57.0 ± 4.0% of WT, $n = 6$) (Fig. 3a, b). Of note, in the WT mice, chronic treatment with nicotine at a dose of 0.3 mg/kg had no effect on phosphorylation of Akt (Ser-473) and CREB (Ser-133) in the DG (Fig. 3a, b).

In contrast, we previously reported that CaMKIIα (Thr-286) autophosphorylation regulates BDNF transcription in the DG of CaMKIV null mice via CREB (Ser-133) phosphorylation [18]. Interestingly, CaMKIIα (Thr-286) autophosphorylation was significantly decreased in the DG of null mice (pCaMKIIα (Thr-286): 66.3 ± 3.2% of WT, $n = 6$) (Fig. 3a, b). Remarkably, the chronic treatment with nicotine at a dose of 1 mg/kg, i.p., significantly ameliorated autophosphorylation of CaMKIIα (Thr-286) in the DG of null mice (pCaMKIIα (Thr-286): 95.5 ± 5.7% of WT, $n = 6$) (Fig. 3a, b). Additionally, and in line with all of the above data, pretreatment with MLA (3 mg/kg, i.p.), but not DHβE (1 mg/kg, i.p.), completely prevented the increase in the autophosphorylation of CaMKIIα (Thr-286) in the DG of null mice (pCaMKIIα (Thr-286): 67.4 ± 2.9% of WT, $n = 6$)

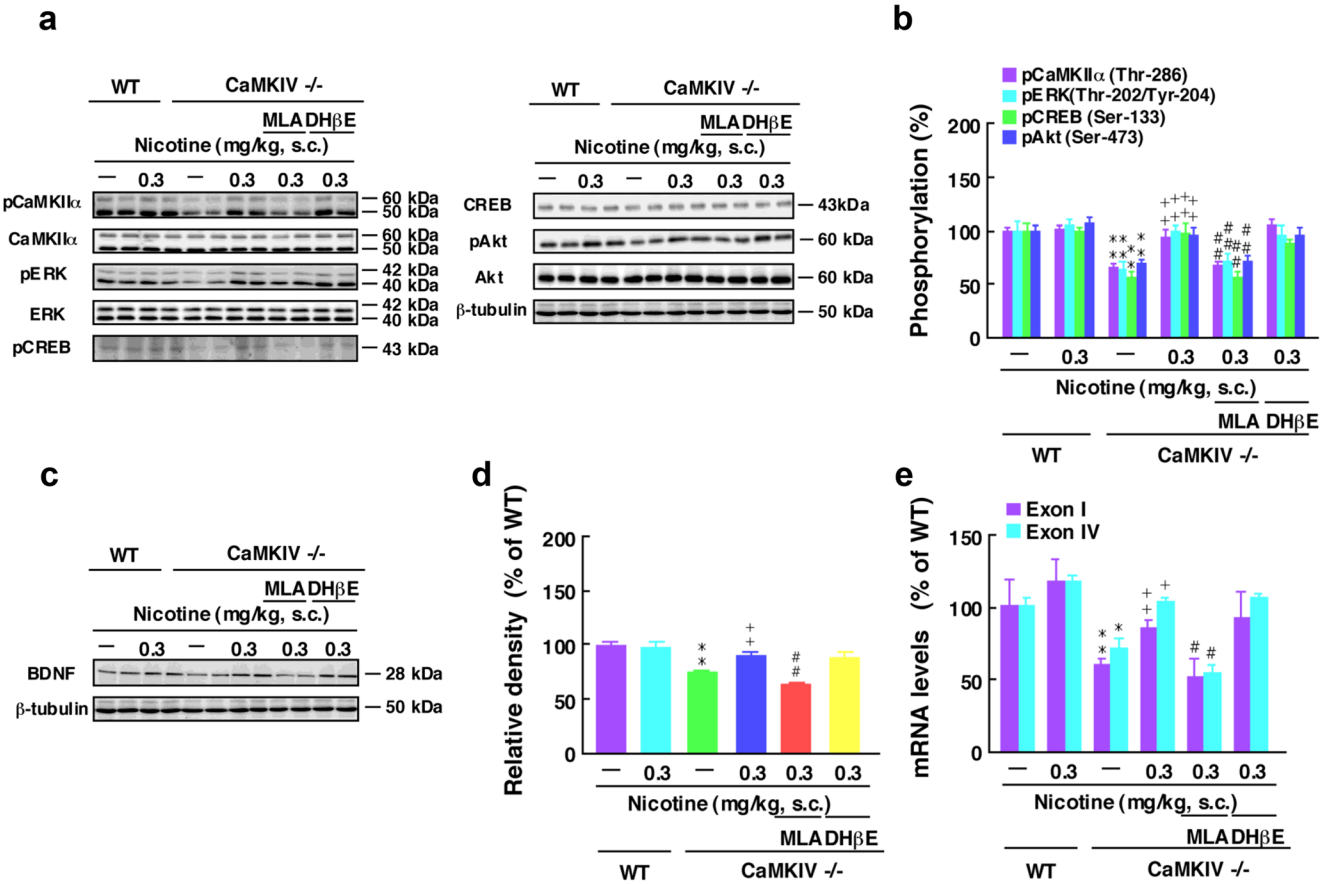

Fig. 3 Chronic exposure to nicotine rescues the phosphorylation of ERK (Thr-202/Tyr-204), CREB (Ser-133), Akt (Ser-473), the autophosphorylation of CaMKIIα (Thr-286), and BDNF protein/mRNA expression in the DG of CaMKIV null mice. **a**, **c** Representative images of immunoblots using antibodies against autophosphorylated CaMKIIα (Thr-286), CaMKIIα, phosphorylated ERK (Thr-202/Tyr-204), ERK, phosphorylated CREB (Ser-133), CREB, phosphorylated Akt (Ser-473), Akt, BDNF, and β-tubulin. **b**, **d** Quantitative analyses of autophosphorylated CaMKIIα (Thr-286), phosphorylated ERK (Thr-202/Tyr-204), phosphorylated CREB (Ser-133), phosphorylated Akt (Ser-473), and BDNF. **e** Quantitative analyses of BDNF mRNA containing exon I or exon IV. Vertical lines show the SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the wild-type mice. $^{+}p < 0.05$, $^{++}p < 0.01$ versus CaMKIV null mice. $^{\#} < 0.05$, $^{\#\#} < 0.01$ versus nicotine-treated CaMKIV null mice

(Fig. 3a, b). In the WT mice, the chronic treatment with nicotine at a dose of 0.3 mg/kg had no effect on autophosphorylation of CaMKIIα (Thr-286) in the DG (Fig. 3a, b), also in line with the above results.

Since enhanced adult hippocampal neurogenesis is associated with activation of CREB/BDNF pathways [34, 39], we next examined protein expression levels and/or the levels of transcripts encoding BDNF in the DG. Particularly, we assessed the levels of BDNF transcripts considering exons I and IV, because these BDNF exons are associated with Ca^{2+} signaling [40, 41]. CaMKIV null mice showed significantly decreased protein levels of BDNF in the DG, compared with those of WT mice (75.2 ± 1.3% of WT, $n = 6$) (Fig. 3c, d). Chronic treatment with nicotine at a dose of 1 mg/kg, i.p., significantly increased BDNF in the DG of null mice (90.7 ± 2.2% of WT, $n = 6$) (Fig. 3c, d). Pretreatment with MLA (3 mg/kg, i.p.), but not DHβE (1 mg/kg, i.p.), completely eliminated the nicotine-induced increase in BDNF protein levels in the DG of null mice (63.2 ± 2.5% of WT, $n = 6$) (Fig. 3c, d). On the other hand, in WT mice, chronic treatment with nicotine at a dose of 0.3 mg/kg had no effect on BDNF protein levels in the DG (Fig. 3c, d). Similar to BDNF protein levels, BDNF mRNA levels (exons I and IV) were significantly decreased in the DG of null versus WT mice (exon I: 51.7 ± 0.6% of WT, $n = 4$; exon IV: 69.5 ± 10.1% of WT, $n = 4$) (Fig. 3e). Chronic treatment with nicotine at a dose of 1 mg/kg, i.p., significantly restored them (exon I: 92.2 ± 3.2% of WT, $n = 4$; exon IV: 100.4 ± 9.3% of WT, $n = 4$), unless null mice were retreated with MLA (3 mg/kg, i.p.), but not DHβE (1 mg/kg, i.p.) (exon I: 47.3 ± 9.1% of WT, $n = 4$; exon IV: 55.0 ± 12.6% of WT, $n = 4$) (Fig. 3e).

Chronic Exposure to α7-type nAChR Agonists Rescues Depressive-like Behaviors in CaMKIV Null Mice

Since we reproducibly observed that the α7-type nAChR antagonist, MLA, eliminated the effects of chronic exposure to

nicotine in CaMKIV null mice, we next examined whether chronic exposure to the α7-type nAChR agonists, PNU-282987 (0.5 mg/kg, i.p.) and GTS-21 (1 mg/kg, i.p.), could rescue the depressive-like behaviors in CaMKIV null mice as per the tail-suspension and forced-swim tasks. In the tail-suspension task, chronic treatment with PNU-282987 or GTS-21 significantly reduced the immobility time in CaMKIV null mice (PNU-282987: 114.5 ± 10.3 s, $n = 6$; GTS-21: 103.7 ± 11.3 s, $n = 6$) (Fig. 4a). On the other hand, chronic treatment with PNU-282987 or GTS-21 had no effect on the immobility time of WT mice (Fig. 4a). Similarly, chronic treatment with PNU-282987 or GTS-21 significantly decreased the immobility time in CaMKIV null mice in the forced-swim task (PNU-282987: 75.5 ± 10.7 s, $n = 6$; GTS-21: 74.2 ± 9.6 s, $n = 6$)(Fig. 4b). Conversely, chronic treatment with PNU-282987 or GTS-21 had no effect on the immobility time relative to WT mice in the context of the forced-swim task (Fig. 4b).

Chronic Exposure to α7-Type nAChR Agonists Ameliorates Adult Hippocampal Neurogenesis in CaMKIV Null Mice

As chronic exposure to nicotine enhanced adult hippocampal neurogenesis in the DG of CaMKIV null mice, we investigated whether the α7-type nAChR agonists, PNU-282987 (0.5 mg/kg, i.p.) and GTS-21 (1 mg/kg, i.p.), would induce the same effect. Importantly, the chronic treatment of CaMKIV null mice with PNU-282987 or GTS-21 significantly increased the number of BrdU-positive neuronal cells in their hippocampal slices, compared with those of non-treated null animals, and even of WT animals (PNU-282987: 102.9 ± 6.3% of WT $n = 8$; GTS-21: 115.8 ± 6.9% of WT, $n = 8$) (Fig. 5a, b). Of note, chronic treatment with PNU-282987 or GTS-21 had no effect on the number of BrdU-positive neuronal cells in WT mice (Fig. 5a, b).

Chronic Exposure to α7-type nAChR Agonists Improves ERK, CREB, and Akt Phosphorylation, CaMKIIα Autophosphorylation, and BDNF Expression in the DG of CaMKIV Null Mice

Finally, we investigated whether chronic exposure to α7-type nAChR agonists could regulate ERK (Thr-202/Tyr-204), CREB (Ser-133), and Akt (Ser-473) phosphorylation, CaMKIIα (Thr-286) autophosphorylation, and BDNF expression in the DG of CaMKIV null mice. In hippocampal DG slices of CaMKIV null mice, chronically exposed to the α7-type nAChR agonists PNU-282987 (0.5 mg/kg, i.p.) and GTS-21 (1 mg/kg, i.p.), ERK (Thr-202/Tyr-204), CREB (Ser-133), and Akt (Ser-473) phosphorylation significantly increased compared with that of WT mice (ERK (Thr-202/Tyr-204), PNU-282987: 120.5 ± 12.8% of WT $n = 6$; GTS-21: 122.6 ± 13.9% of WT, $n = 6$; CREB (Ser-133), PNU-282987: 107.2 ± 7.2% of WT $n = 6$; GTS-21: 111.9 ± 4.2% of WT, $n = 6$; Akt (Ser-473), PNU-282987: 90.7 ± 3.8% of WT $n = 6$; GTS-21: 99.6 ± 2.2% of WT, $n = 6$) (Fig. 6a, b). On the other hand, chronic exposure to the α7-type nAChR agonists PNU-282987 and GTS-21 had no effect on the phosphorylation of ERK (Thr-202/Tyr-204), CREB (Ser-133), and Akt (Ser-473) in WT mice (Fig. 6a, b).

Similarly, CaMKIIα (Thr-286) autophosphorylation was significantly restored in the DG of CaMKIV null mice after chronic exposure to the α7-type nAChR agonists (pCaMKIIα (Thr-286), PNU-282987: 94.9 ± 5.5% of WT, $n = 6$; GTS-21: 104.9 ± 3.4% of WT, $n = 6$) (Fig. 6a, b), while no effect was observed in treated WT mice (Fig. 6a, b).

These results were reproduced, in the context of BDNF protein levels in the DG of CaMKIV null versus WT mice.

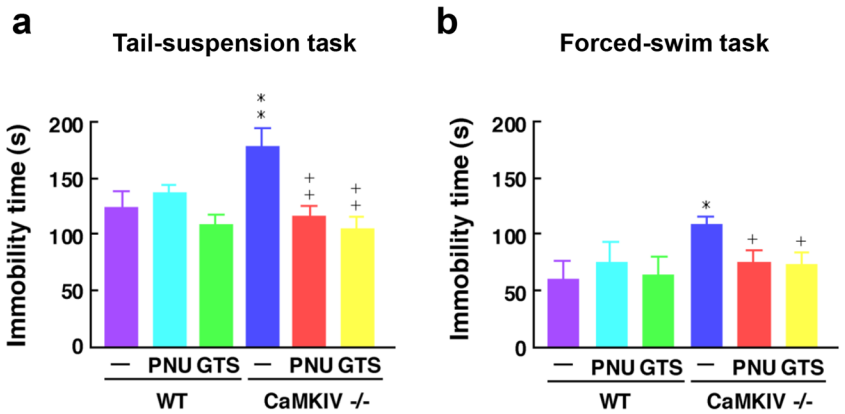

Fig. 4 Chronic exposure to PNU-282987 or GTS-21 rescues depressive-like behaviors in CaMKIV null mice. **a**, **b** Immobility time as determined by the tail-suspension (**a**) or forced-swim (**b**) tasks was measured following chronic exposure to PNU-282987 at 0.5 mg/kg i.p. or GTS-21 at 1 mg/kg i.p. for 14 days. Chronic exposure to PNU-282987 or GTS-21 significantly rescued the increased immobility time seen in CaMKIV null mice (Mann-Whitney U test, $n = 6$ per group). Vertical lines show the SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the wild-type mice. $^{+}p < 0.05$, $^{++}p < 0.01$ versus CaMKIV null mice. $^{\#} < 0.05$, $^{\#\#} < 0.01$ versus nicotine-treated CaMKIV null mice

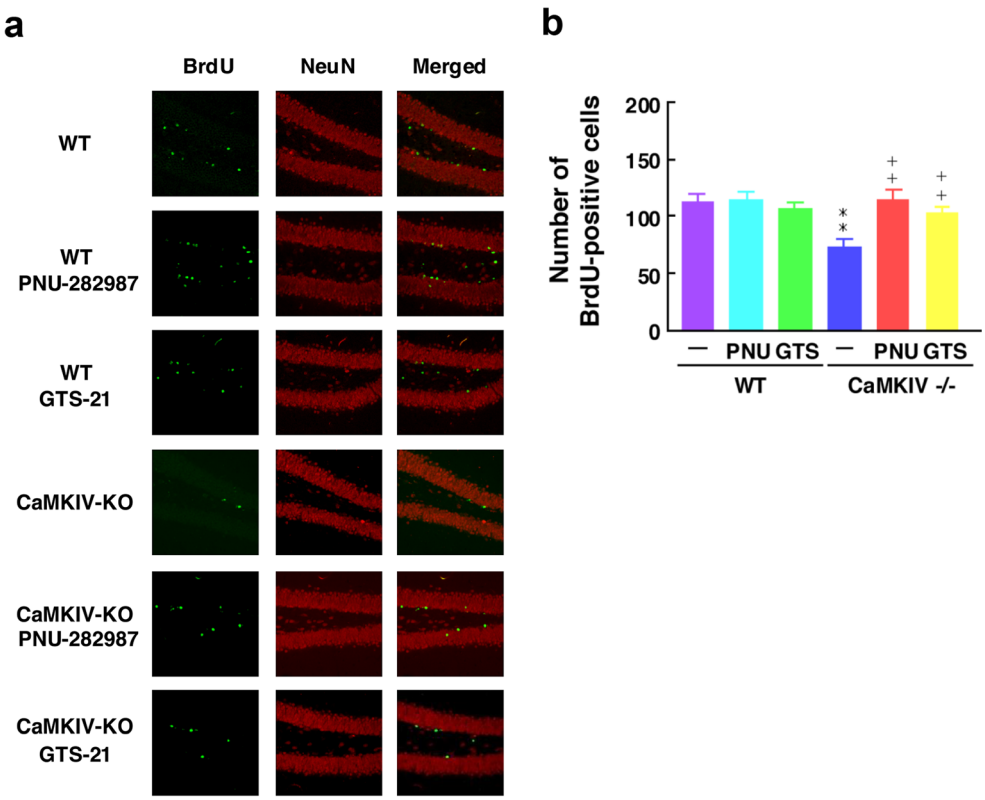

Fig. 5 Chronic exposure to PNU-282987 or GTS-21 enhances adult hippocampal neurogenesis in CaMKIV null mice. **a** Confocal microscopy images showing staining for BrdU (green), NeuN (red), and merged signals in hippocampal slices from wild-type mice, PNU-282987-treated wild-type mice, GTS-21-treated wild-type mice, CaMKIV null mice, PNU-282987-treated CaMKIV null mice, and GTS-21-treated CaMKIV null mice. Mice were injected with BrdU from the first day of drug treatment for 5 consecutive days. Mice were treated with PNU-282987 or GTS-21 for 2 weeks. **b** Quantitative analyses of the number of BrdU/NeuN double-positive cells in the DG ($n = 8$). Vertical lines show the SEM. $^{**}p < 0.01$ versus the wild-type mice. $^{++}p < 0.01$ versus CaMKIV null mice. $^{\#\#} < 0.01$ versus nicotine-treated CaMKIV null mice

The BDNF protein levels were significantly restored in the DG of CaMKIV null mice (BDNF, PNU-282987: 95.9 ± 5.1% of WT, $n = 6$; GTS-21: 111.7 ± 5.0% of WT, $n = 6$), while no change was observed in WT mice (Fig. 6c, d).

Discussion

The DG of the hippocampus is essential for spatial and episodic memory formation [42, 43]. Notably, depressive-like behaviors are associated with injured adult hippocampal neurogenesis in the subgranular zone of the hippocampal DG in rodents [44]. Restricted exposure to X-irradiation to the hippocampus has been shown to block adult hippocampal neurogenesis in the DG and impair the ability of anti-depressants to rescue depressive-like behaviors [45]. SSRIs, such as fluoxetine or fluvoxamine, improve injured adult hippocampal neurogenesis in the DG of rodents [45, 46]. In post-mortem brain studies in humans, chronic treatment with anti-depressants, such as imipramine, showed an increase in hippocampal cell proliferation and neurogenesis in the DG of the depressed individual [47, 48]. Thus, anti-depressants that function as SSRIs are useful in most cases of major depressive disorder (MDD).

Recently, treatment-resistant depression (TRD) has emerged in a substantial fraction of patients with MDD who respond poorly to anti-depressants, including SSRIs [49]. Interestingly, SSRIs, such as fluoxetine and paroxetine, fail to rescue the adult hippocampal neurogenesis and lack anti-depressive activity in CaMKIV null mice [18, 19]. Tiraboschi et al. [50] previously reported that fluoxetine increases CREB activation via phosphorylation of its residue Ser-133 activation by both CaMKIV and MAP kinase cascades. These previous reports indicate that CaMKIV null mice are useful animal models to study TRD.

Here, we demonstrated that chronic exposure to nicotine rescues depressive-like behaviors and increases adult hippocampal neurogenesis via the α7-type nAChR activation in CaMKIV null mice (Fig. 1). In the hippocampus, the most common nAChR subtypes expressed include α7-type and α4β2-type subunits [51–54]. Distribution of α7-type and α4β2-type nAChRs has been observed in granule cells in the DG [55]. Campbell et al. [56] previously reported that activation of α7-type nAChR was required for the maturation and synaptic integration of adult-born neurons in the DG of the hippocampus. Similar to our results, Tizabi et al. [57] reported that chronic administration of nicotine at a dose of 0.4 mg/kg, s.c., for 14 days, significantly rescued depressive-like behaviors, as assessed by the forced-swim task. In addition, the α7-type nAChR agonist, TC-7020, has been shown to reactivate adult hippocampal neurogenesis in mice [58]. In the present study, we similarly demonstrated that treatment with the α7-type nAChR agonists PNU-282987 (0.5 mg/kg, i.p.) and GTS-21 (1 mg/kg, i.p.) for 14 days rescued

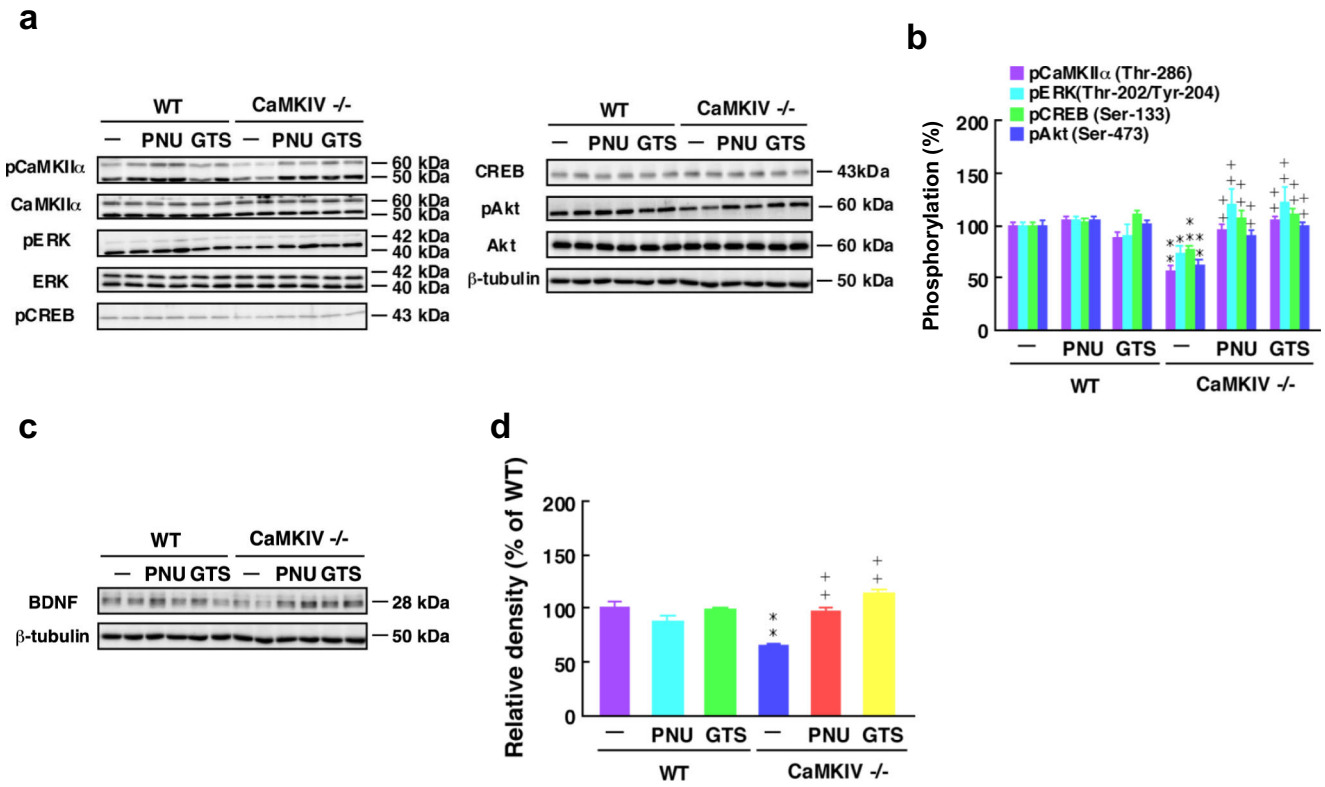

Fig. 6 Chronic exposure to PNU-282987 or GTS-21 rescues the phosphorylation of ERK (Thr-202/Tyr-204), CREB (Ser-133), and Akt (Ser-473), the autophosphorylation of CaMKIIα (Thr-286), and BDNF protein, or mRNA expression in the DG of CaMKIV null mice. **a**, **c** Representative images of immunoblots using antibodies against autophosphorylated CaMKIIα (Thr-286), CaMKIIα, phosphorylated ERK (Thr-202/Tyr-204), ERK, phosphorylated CREB (Ser-133), CREB, phosphorylated Akt (Ser-473), Akt, BDNF, and β-tubulin. **b**, **d** Quantitative analyses of autophosphorylated CaMKIIα (Thr-286), phosphorylated ERK (Thr-202/Tyr-204), phosphorylated CREB (Ser-133), phosphorylated Akt (Ser-473), and BDNF. Vertical lines show the SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the wild-type mice. $^{++}p < 0.01$ versus CaMKIV null mice

depressive-like behaviors in CaMKIV null mice, as assessed by the tail-suspension and the forced-swim tasks (Fig. 4). Thus, the activation of α7-type nAChR may serve as a novel strategy to improve TRD.

In contrast, long-term exposure to tobacco at low intensity inhibits adult hippocampal neurogenesis in mice [59]. In addition, chronic administration of nicotine at doses of 0.08 mg/kg by intravenous self-administration impairs the survival of newborn cells and CREB activation in the DG; this has been used to model the relatively unlimited daily nicotine intake in regular smokers [60]. Thus, low doses of nicotine injure adult hippocampal neurogenesis.

This said, here, we confirmed that chronic exposure to nicotine increases phosphorylation of Akt at residue Ser-473 and ERK at residue Thr-202/Tyr-204 in the DG of CaMKIV null mice (Fig. 3). Li et al. [38] previously reported that activation of these pathways in neuronal progenitors plays a critical role in cell proliferation and maturation. We demonstrated that chronic exposure to nicotine enhances adult hippocampal neurogenesis via the PI3K/Akt and ERK/CREB pathways and potentiates synaptic efficacy through CaMKII activation (Fig. 3). Enhanced PI3K/Akt signaling and ERK activation are essential for adult hippocampal neurogenesis [34, 61]. Furthermore, these pathways increase CREB phosphorylation at residue Ser-133 and BDNF expression [34, 39, 62]. Son et al. [63] previously reported that chronic neonatal nicotine exposure increases the BDNF mRNA levels in the hippocampus. In the present study, we confirmed that chronic exposure to nicotine increases both mRNA and protein expression levels of BDNF in the DG of CaMKIV null mice (Fig. 3). In contrast, CaMKII also likely accounts for CREB phosphorylation and BDNF expression. Indeed, Sun et al. [64] reported that CaMKII regulates CREB activity via additional phosphorylation of CREB at the residue Ser-142. We also detected that overexpression of CaMKII increases BDNF transcription levels (exon IV) in NG108-15 cells [65]. In contrast, the activation of α7-type nAChR directly increases calcium influx [66] and regulates the PI3K/Akt and ERK pathways [67–69]. Nicotine treatment at 0.5 μM increases Akt phosphorylation in rat primary neurons, and its effects are antagonized by

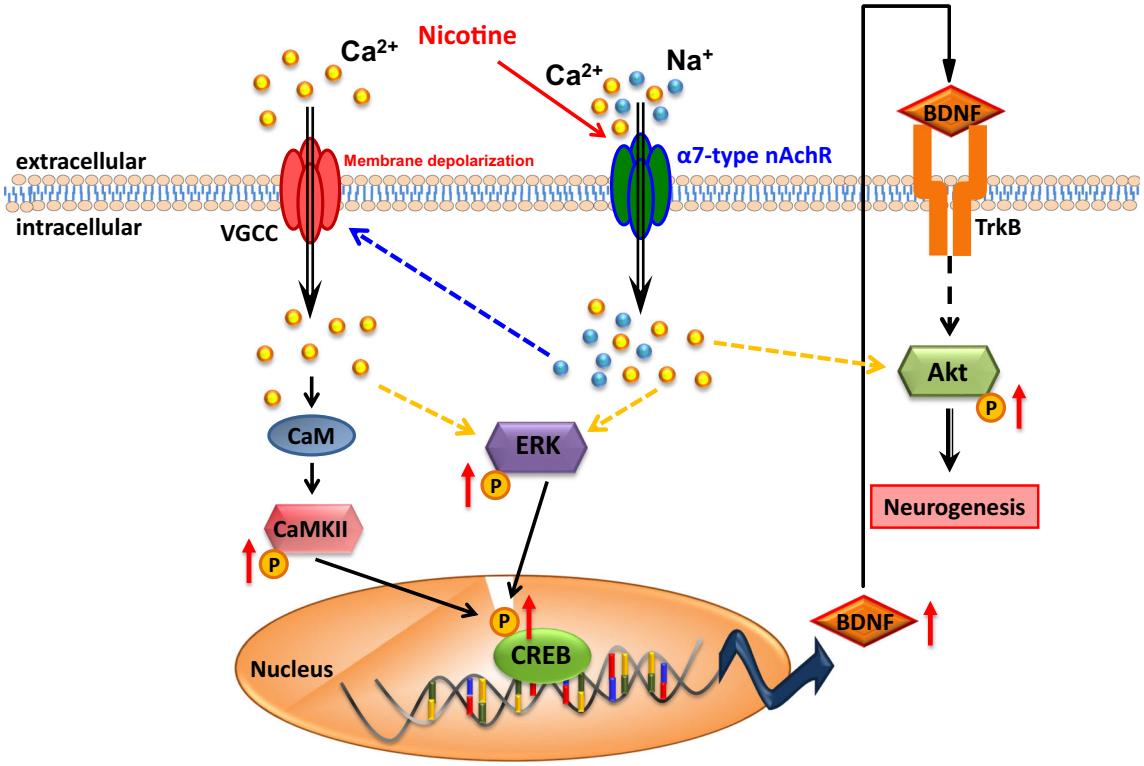

Fig. 7 Schematic representation of altered adult hippocampal neurogenesis in the DG. Chronic exposure to nicotine stimulates α7-type nAChR and enhances intracellular calcium signaling. Chronic exposure to nicotine increases CaMKIIα, ERK, and Akt phosphorylation. Increased CaMKIIα and ERK phosphorylation induces the CREB/BDNF pathway, which in turn promotes adult hippocampal neurogenesis

α7-type nAChR inhibitor [70]. In addition, nicotine promotes the ERK phosphorylation in human nasopharyngeal carcinoma cells [71]. Thus, activation of α7-type nAChR by nicotine regulates PI3K/Akt and ERK pathways via calcium influx, directly. In the future, further experiments are required to elucidate the detailed mechanisms of adult hippocampal neurogenesis enhanced by chronic exposure to nicotine in CaMKIV null mice.

In conclusion, this study demonstrates that chronic exposure to nicotine via α7-type nAChR rescues depressive-like behaviors in CaMKIV null mice and increases adult hippocampal neurogenesis in the DG via PI3K/Akt and ERK/CREB pathways' activation. Chronic exposure to nicotine also improves hippocampal synaptic efficacy in the DG by activating CaMKII (Fig. 7). Thus, activation of α7-type nAChR may serve as a novel therapeutic target for TRD and warrants further research.

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Authors' Contributions S.M., R.I., L.Y., and M.S. performed the experiments. H.S. and K.F. provided critical advice. H.S. provided the CaMKIV null mice. S.M. designed the study and wrote the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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