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ARTICLE

Protein kinase Mζ is involved in the modulatory effect of fluoxetine on hippocampal neurogenesis *in vitro*

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Abstract

The efficacy of chronic selective serotonin reuptake inhibitors (SSRIs) on depression is paralleled by the recovery of deficits in hippocampal neurogenesis related to sustained stress and elevated glucocorticoids. Previous studies have shown that atypical protein kinase C (aPKC) is implicated in the regulation of neurogenesis and the antidepressant response. Whether the specific aPKC isoforms (PKCζ, PKMζ and PKCι) are involved in SSRI-induced hippocampal neurogenesis and the underlying mechanisms is unknown. The present study shows that PKMζ and PKCι but not PKCζ are expressed in rat embryonic hippocampal neural stem cells (NSCs), whereas PKMζ but not PKCι expression is increased by the SSRI fluoxetine both in the absence and presence of the glucocorticoid receptor agonist dexamethasone. PKMζ shRNA significantly decreased neuronal proliferation and neuron-oriented differentiation, increased NSC apoptosis, and blocked the stimulatory effect of fluoxetine on NSC neurogenesis. Fluoxetine significantly increased PKMζ expression in hippocampal NSCs in a 5-hydroxytryptamine-1A (5-HT1A) receptor-dependent manner in both the absence and presence of dexamethasone. The PKMζ peptide blocker ZIP and MEK inhibitor U0126 significantly inhibited the increase in extracellular signal-regulated kinase 1/2 and cyclic adenosine monophosphate response element binding protein phosphorylation in the mitogen-activated protein kinase (MAPK) pathway and hippocampal NSC neurogenesis in response to fluoxetine and the 5-HT1A receptor agonist 8-OH DPAT. Collectively, our results suggest that the SSRI fluoxetine increases hippocampal NSC neurogenesis via a PKMζ-mediated mechanism that links 5-HT1A receptor activation with the phosphorylation of the downstream MAPK signaling pathway.

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Key words: Fluoxetine, hippocampal neurogenesis, protein kinase Mζ.

Introduction

Depression is a stress-related psychiatric illness that has been consistently reported to be associated with deficits in hippocampal neurogenesis (Boldrini et al., 2009). Chronic stress can attenuate the negative feedback effect of the hippocampus on a hyperactive hypothalamic–pituitary–adrenal axis and lead to the disinhibitory secretion of glucocorticoids in the adrenal cortex, which further impairs neurogenesis in the hippocampal dentate gyrus (DG) (Pariante and Lightman, 2008). Selective serotonin reuptake inhibitors (SSRIs) can significantly reverse glucocorticoid-induced deficits in hippocampal neurogenesis (Xi et al., 2011). The effects of SSRI antidepressants are paralleled by an increase in hippocampal neurogenesis (Malberg et al., 2000), both of which usually take several weeks to fully develop. Importantly, the inhibition of hippocampal neurogenesis has been reported to block antidepressant efficacy in rodents (Santarelli et al., 2003). The mechanisms that underlie antidepressant-related neurogenesis have not yet been clearly elucidated.

Protein kinase C (PKC) comprises a multigene family that includes conventional, novel and atypical isozyme groups and has been widely implicated in the pathophysiology of mood disorders (Pandey and Dwivedi, 2005). PKC activity is decreased in platelets in depressed patients (Pandey et al., 1998) and the frontal cortex in depressed suicide victims (Pandey et al., 1997). Atypical PKCs (aPKCs) consist of three members (PKCι, PKCζ, and PKMζ) with high amino acid homology. Both PKCζ and PKMζ are encoded by the PRKCZ gene. Evidence suggests that aPKCs might be involved in the regulation

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of neurogenesis. PKCζ and PKCι have been shown to have essential and distinct roles in controlling cortical neurogenesis during embryonic development (Wang et al., 2010, 2012). PKCζ promotes the differentiation of radial precursors into neurons by activating cyclic adenosine monophosphate response element binding protein (CREB), whereas PKCι inhibits the transition from radial precursors to basal progenitors (Wang et al., 2012). PKMζ is also implicated in the protective effect of aspirin on the *N*-methyl-D-aspartate (NMDA)-induced apoptosis of cortical neurons (Crisanti et al., 2005). However, the role of aPKCs in hippocampal neurogenesis has not yet been reported. Differential expression of aPKCs was found in the adult mouse DG, which contains high PKCι and PKMζ transcripts but not PKCζ, indicating specific roles for individual aPKC isozymes in hippocampal neurogenesis (Oster et al., 2004). Intriguingly, our recent pharmacogenetic study indicated that genetic variants of the PRKCZ gene (rs2280272 polymorphism in exon 5) were significantly associated with the antidepressant response in patients with major depression (Shi et al., 2012). This raises the possibility that antidepressants might activate hippocampal neurogenesis through aPKCs, mainly PKMζ and PKCι, thus mediating their therapeutic efficacy.

The 5-hydroxytryptamine-1A (5-HT1A) receptor is essential for the SSRI-induced increase in hippocampal neurogenesis. 5-HT1A receptor agonists stimulate neurogenesis in the hippocampal DG (Santarelli et al., 2003), whereas 5-HT1A receptor antagonists decrease this effect (Radley and Jacobs, 2002). 5-HT1A receptor agonists upregulate the mitogen-activated protein kinase (MAPK) pathway in hippocampal HN2-5 cells, which further inhibits the proapoptotic protease caspase-3 (Adayev et al., 2003). Activation of the MAPK pathway results in changes in the downstream cascade of genes involved in neurogenesis, such as CREB (Ying et al., 2002). Interestingly, upstream regulators, including various PKC isotypes, have been found to increase the activation of the MAPK extracellular signal-regulated kinase (ERK) cascade (Ueda et al., 1996; Schonwasser et al., 1998). However, whether aPKCs are involved in SSRI-induced MAPK activation is still unknown. We hypothesized that antidepressants modulate hippocampal neurogenesis via an aPKC-mediated mechanism, in which aPKCs are stimulated by 5-HT1A receptor activation, which further elevates the phosphorylation of the MAPK signaling pathway.

Materials and methods

Chemical reagents

The PKMζ blocker peptide ZIP (myr-SIYRRGARRWRKL-OH) was purchased from ENZO Biochem (USA). N2 and B27 were purchased from Invitrogen (USA). Epidermal grown factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Peprotech (UK). All other drugs and reagents were purchased from Sigma-Aldrich (USA). ZIP and the selective MAPK kinase (MEK) inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126) were dissolved in dimethylsulfoxide (DMSO). The other reagents were prepared with stock solutions in distilled phosphate-buffered saline (PBS) and stored at −20 °C until use.

Isolation and culture of rat embryonic hippocampal neural stem cells

Hippocampal tissues were isolated from embryonic day 14.5 foetal Sprague–Dawley rats and then washed with ice-cold PBS. After the tissues were mechanically dissected into single-cell suspensions, the dissociated cells were passed through a 70 μm nylon cell strainer (BD Biosciences, USA), followed by centrifugation at 1000 *g* for 6 min at 4 °C. The pellets were resuspended in Dulbecco's modified Eagle's medium-F12 supplemented (DMEM/F12) with 20 ng/ml of bFGF, 20 ng/ml EGF, 2% B27, 1% N2 supplement, 2 mmol/l glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were incubated in a humidified incubator at 37 °C with 5% CO_2. The procedures used were approved by Jiang Su Animal Care and Use Committee.

Small-interfering RNA transfection

Four small-interfering RNA (siRNA) duplexes that target PKMζ were used: siRNA1 (5′-GCCAGTGCAGCGAAAGGATAT-3′), siRNA2 (5′-GCAAGCTGCTTGTCCATAAAC-3′), siRNA3 (5′-GGCATATGGATTCTGTCATGC-3′) and siRNA4 (5′-GCTGGGTGTCCTTATGTTTGA-3′). The negative control (NC) sequence 5′-TTCTCCGAACGTGTCACGT-3′ exhibited no homology with any rat genes. For transfection, lentiviral shRNA plasmids (pLKD. UBC, GFP.U6.shRNA, Neuron Biotech, China) were mixed with NSCs in growth medium. Transfection efficiency was evaluated 24, 48, and 72 h after transfection using a fluorescence microscope. The knockdown efficiency of different lentiviral shRNA clones in NSCs was determined by real-time PCR and Western blot.

5-HT enzyme-linked immunosorbent assay

The NSCs were plated in 24-well plates in growth medium in either the absence or presence of fluoxetine (1 μM) for 48 h. The levels of 5-HT in the culture media were then measured using a serotonin enzyme-linked immunosorbent kit (DRG Instruments, Germany) according to the manufacturer's instructions. The optical density was read at 450 nm using a microplate reader (Bio-Rad, USA).

Immunocytochemistry of 5-HT system markers in NSCs

After being fixed in 4% paraformaldehyde (PFA) for 30 min, NSCs on poly-L-lysine-coated coverslips were

blocked in a buffer with 5% bovine serum albumin and 0.1% Triton X-100 in PBS for 20 min. The cells were incubated overnight at 4 °C with one of the following primary antibodies diluted in blocking buffer: rabbit anti-nestin (1:200; Chemicon, USA), rabbit anti-SERT (1:100; Santa Cruz Biotechnology, USA), rabbit anti-serotonin (1:100; Sigma-Aldrich, USA), rabbit anti-5HT1AR (1:100; Sigma-Aldrich), and rabbit anti-tryptophan hydroxylase (TPH; 1:100; Sigma-Aldrich). The cells were then incubated with secondary antibodies (Alexa Fluor 488 or Alexa Fluor 594 goat anti-rabbit, 1:1000; Invitrogen, USA) diluted in PBS for 2 h at room temperature. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 15 min at room temperature. Images of four independent fields were captured for each well using a fluorescence microscope (Olympus, Japan). The numbers of total and immunoreactive cells were counted and normalized to DAPI-stained cells.

Neural stem cell proliferation assay

Neural stem cells were plated on 24-well plates, then treated with fluoxetine (1 μM), the 5-HT1A receptor agonist 8-OH DPAT (5 μM), the 5-HT1A receptor antagonist WAY-100635 (50 μM), the glucocorticoid receptor agonist dexamethasone (1 μM), ZIP (10 μM), or U0126 (10 μM). The ZIP- and U0126-treated cells were pretreated for 30 min and 1 h, respectively, when co-administered with the other intervention reagents. After 1 d of intervention reagent treatment, 5′-bromodeoxyuridine (BrdU; 10 μM, Sigma, USA) was added for an additional 24 h incubation. After being plated onto poly-L-lysine-coated glass coverslips, the NSCs were fixed for 30 min with 4% PFA and then sequentially incubated with 2 N HCl for 30 min, blocking solution for 2 h at room temperature, primary antibody (rat anti-BrdU, 1:100; Abcam, UK) at 4 °C overnight, and secondary antibody (Alexa Fluor 546 goat anti-rat, 1:1000; Invitrogen, USA) for 2 h at room temperature. The proportion of the number of BrdU-positive cells to the total number of DAPI-positive cells was determined to describe the effects of the various reagents on cell proliferation.

Neural stem cell differentiation assay

NSCs were plated on poly-L-lysine-coated 24-well plates and cultured in DMEM/F12 medium supplemented with 10% foetal bovine serum (FBS) for 5 d. The NSCs were then treated with the intervention reagents as described above. After being fixed in 4% PFA and incubated with the blocking buffer, the cells were assayed for the immunocytochemical detection of neuronal and glial antigens using the following primary antibodies overnight at 4 °C: mouse anti-βIII-tubulin (1:500; Sigma, USA) and rabbit anti-glial fibrillary acidic protein (GFAP; 1:400; Sigma, USA). The cells were then incubated with secondary antibody (Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit, 1:1000; Invitrogen, USA) for 2 h at room temperature. After counterstaining with DAPI, the relative number of neurons and astrocytes was determined as percentages of the total DAPI-stained cell population.

Neural stem cell apoptosis assay

NSC apoptosis in response to drug treatment was detected during the proliferation stage. The NSCs were plated in 24-well plates, and treated with intervention reagents as described above. The detection of apoptotic cells was performed using flow cytometry with an apoptosis detection kit (BIPEC, USA). The cells were stained with annexin V-FITC and propidium iodide (PI), and analysed by fluorescence-activated cell sorting (FACS; BD Biosciences, USA).

Real-time PCR

Total RNA was extracted from cells using the Trizol reagent kit (TaKaRa, Japan) and converted to cDNA with reverse transcriptase (Takara, Japan). The PCR amplifications were performed using an ABI 7300 PCR instrument (Applied Biosystems, USA) using SYBR Premix Ex TaqTM (TaKaRa, Japan). The following PCR primers were used: CREB1:5′-AGTGACTGAGGAGCTTGTACCA-3′ and 5′-TGTGGCTGGGCTTGAAC-3′; PKMζ: 5′-TCTATTAGATGCCTGCTCTCCA-3′ and 5′-CGGTATAGCTTCCTCCATCTTC-3′; PKCζ: 5′-TGAAGGTGACCCTTGTACTGTG-3′ and 5′-CGGTATAGCTTCCTCCATCTTC-3′; PKC*ι*: 5′-GGAGAAGCAGATTCGCATACCG-3′ and 5′-TCCCAATCCACATTACGGAAGAA-3′; SERT: 5′-TTTTCCAATACAACTATCCCCA-3′ and 5′-ATCCGCTCCTTAAGTGTCCCCG-3′; 5-HT1AR: 5′-TTCAGAGCCGCACGCTTCCGAA-3′ and 5′-TCACCCTGCCTCACTGCCCCAT-3′; TPH1: 5′-CTTGAAGAATGAAGTTGGAGGA-3′ and 5′-ACAGAGAGGACCGTGGTGTGGG-3′; TPH2: 5′-GGTTACTTTCCTCCATCGGAGA-3′ and 5′-GAAGGTGGTGATTAGGCATTCC-3′); β-actin: 5′-GGAGATTACTGCCCTGGCTCCTA-3′ and 5′-GACTCATCGTACTCCTGCTTGCTG-3′. The cycling conditions were the following: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension for 31 s at 60 °C. All mRNA values of each target gene were normalized to β-actin mRNA. Additionally, several PCR products of each gene were analysed by electrophoresis on 1.2% agarose gels and visualized with ethidium bromide under ultraviolet light to confirm the product sizes.

Western blot

Neural stem cells were collected and solubilized in ice-cold lysis buffer. The cell lysates were centrifuged at 14 000 *g* for 15 min at 4 °C to remove debris. The BCA method was used to detect the protein concentration. Approximately 15 μg protein was separated by SDS-PAGE and transferred to PVDF membrane

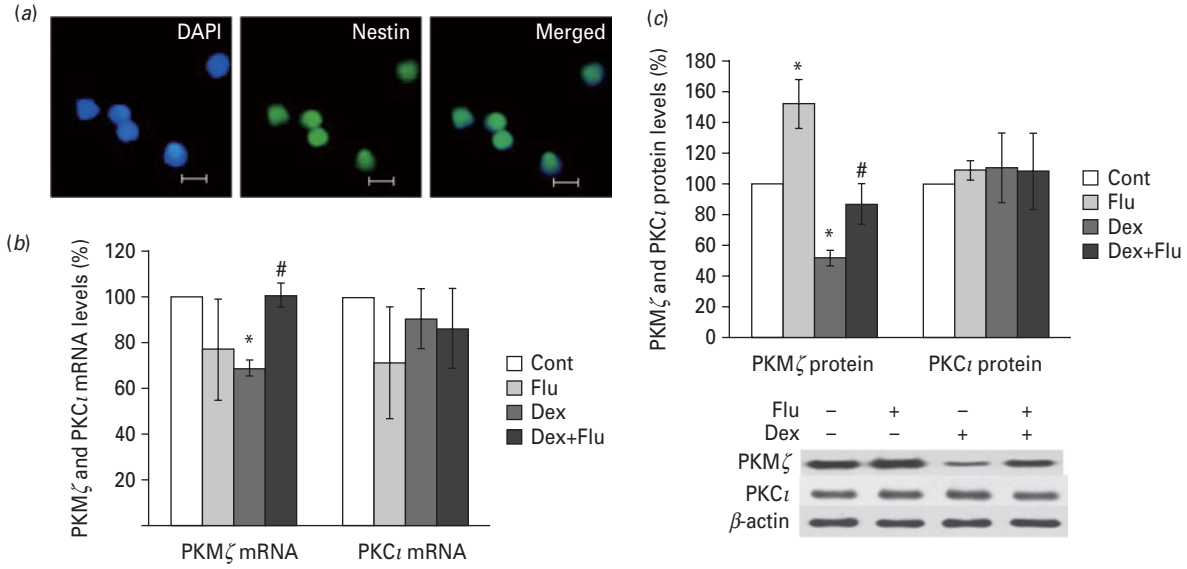

Fig. 1. Effect of fluoxetine on PKMζ and PKCι expression in isolated hippocampal neural stem cells (NSCs). (*a*) Double-staining of Nestin (green) and DAPI (blue) in NSCs. Scale bars = 50 μm. (*b*) mRNA expression in NSCs following fluoxetine (1 μM, 48 h) and dexamethasone (1 μM, 48 h) administration ($n=3$). *$p<0.05$ *vs.* control; #$p<0.05$ *vs.* dexamethasone. The data were normalized to controls. (*c*) Protein expression in NSCs following fluoxetine (1 μM, 48 h) and dexamethasone (1 μM, 48 h) administration ($n=3$). *$p<0.05$ *vs.* control; #$p<0.05$ *vs.* dexamethasone. The data were normalized to controls. Cont, control; Flu, fluoxetine; Dex, dexamethasone.

(Millipore, USA). After blocking with 5% nonfat milk for 2 h at room temperature, the membranes were incubated overnight at 4 °C with the following primary antibodies diluted in blocking solution: anti-CREB (1:500; Cell Signaling Technology, USA), anti-pCREB (Ser133) (1:500; Cell Signaling Technology), anti-ERK1/2 (1:500; Santa Cruz Biotechnology, USA), anti-pERK1/2 (1:500; Santa Cruz Biotechnology), anti-PKMζ (1:500; Santa Cruz Biotechnology), and anti-PKCι (1:500; Cell Signaling Technology). After washing, the membranes were incubated with secondary antibodies coupled to horseradish peroxidase (1:2000; Cell Signaling Technology) at room temperature for 2 h and then detected using the enhanced chemiluminescence (ECL) method. The density of the immunoblot bands was detected using a Bio-Rad Calibrated Densitometer. β-actin was used as an internal control.

Statistical analysis

All of the data are expressed as mean ± S.D. Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test was performed for either selected or multiple comparisons. Statistical significance was set at $p<0.05$.

Results

Fluoxetine enhances the expression of PKMζ in isolated hippocampal NSCs

Isolated rat embryonic hippocampal NSCs exhibited a small round shape, the typical morphological feature of neurospheres. Almost all of the NSCs showed strong immunoreactivity to nestin, a specific marker of undifferentiated neuroepithelial cells (Fig. 1*a*). Real-time PCR and Western blot showed that PKMζ and PKCι but not PKCζ mRNA and protein were expressed in cultured NSCs. The expression of the PKMζ protein but not mRNA in NSCs was increased by fluoxetine ($p<0.05$) compared with the vehicle control (Fig. 1*b*, *c*). Dexamethasone significantly decreased the expression levels of both PKMζ mRNA ($p<0.001$) and protein ($p<0.05$), which were robustly reversed by fluoxetine ($p<0.05$; Fig. 1*b*, *c*). Neither fluoxetine nor dexamethasone affected PKCι mRNA and protein expression.

Regulation of hippocampal neurogenesis by PKMζ

Real-time PCR and Western blot showed that PKMζ shRNA4 exhibited the strongest knockdown of PKMζ expression among the four PKMζ shRNAs, with an efficiency of 80% compared with PKMζ-NC shRNA (data not shown). PKMζ shRNA4 was used in further NSCs neurogenesis experiments.

The effect of PKMζ knockdown on NSC proliferation was detected using BrdU labeling (Fig. 2*a*, *b*). The proportion of BrdU-positive cells was significantly decreased by PKMζ shRNA-4 and dexamethasone (both $p<0.05$). Fluoxetine significantly increased the proportion of BrdU-positive cells ($p<0.05$) and reversed the dexamethasone-induced decrease in the proportion of BrdU-positive cells ($p<0.05$). The fluoxetine-induced enhancement of NSC proliferation was significantly blocked by PKMζ shRNA-4 in the absence and

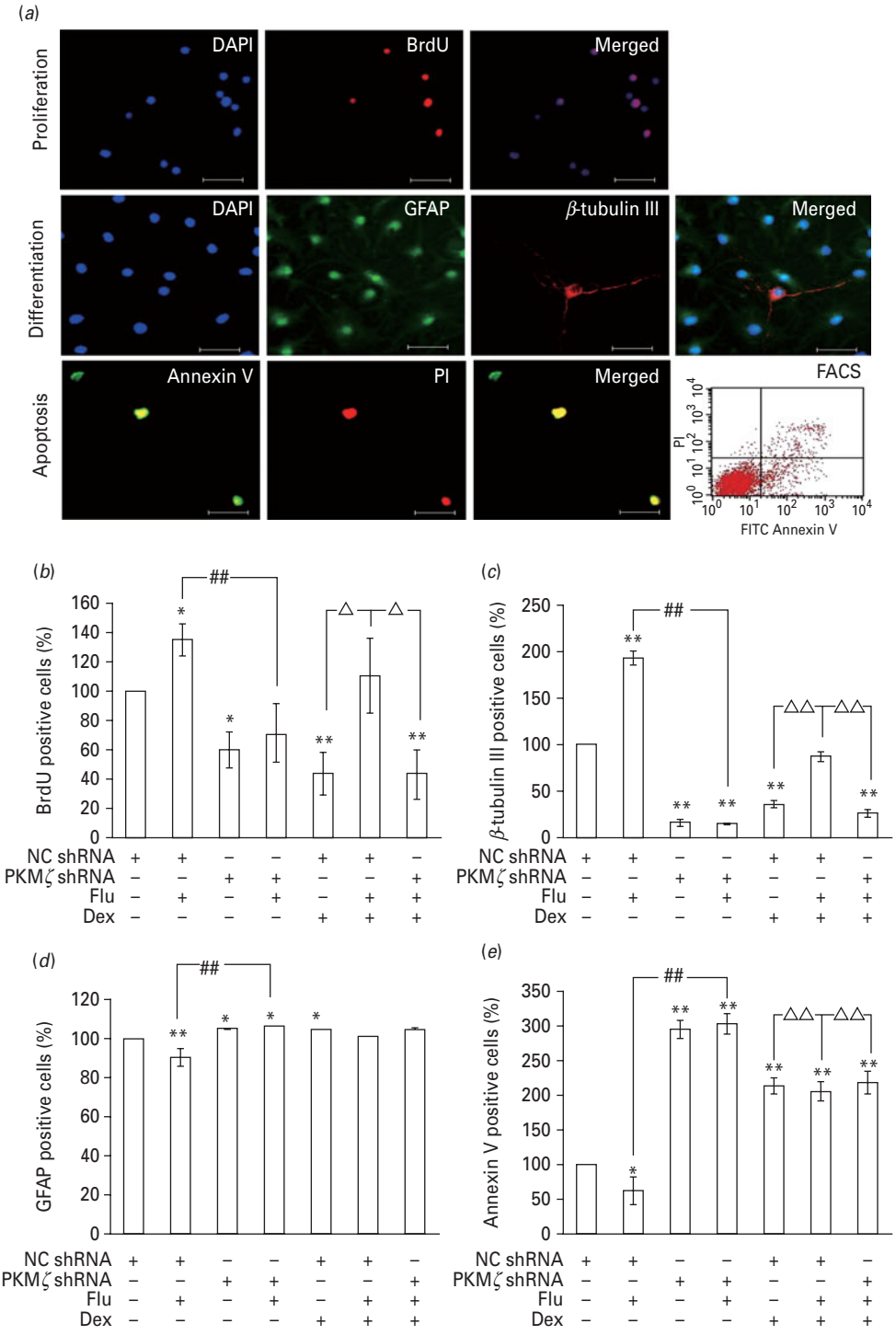

Fig. 2. PKMζ regulates hippocampal neurogenesis. (*a*) Morphology of neural stem cell (NSC) neurogenesis. NSC proliferation was detected using BrdU immunostaining. β-tubulin III and GFAP incorporation were used to assess neuronal and astrocyte differentiation, respectively. The apoptosis of NSCs was detected using Fluorescence Activated Cell Sorter (FACS) counting of annexin V/PI-stained cells. Scale bars=50 μm. (*b–e*) PKMζ shRNA4 attenuated the effect of fluoxetine on NSC neurogenesis in the absence and presence of dexamethasone (n=3). For NSC proliferation and apoptosis, NSCs were treated with PKMζ siRNA4 for 3 d before fluoxetine (1 μM, 48 h) and dexamethasone (1 μM, 48 h) administration. For NSC differentiation, NSCs were treated with PKMζ siRNA4 for 3 d followed by 5 d treatment with 10% foetal bovine serum. Neural stem cells were then treated with fluoxetine (1 μM) and dexamethasone (1 μM) for an additional 48 h. Quantitative analyses of (*b*) BrdU, (*c*) β-tubulin III, (*d*) GFAP, and (*e*) annexin V/PI positive cells are shown. The data were normalized to the control (NC shRNA). *p<0.05, **p<0.001 *vs.* control; $^{\#}p$<0.05 *vs.* fluoxetine; △p<0.05, △△p<0.001 *vs.* fluoxetine+dexamethasone. Flu, fluoxetine; Dex, dexamethasone.

presence of dexamethasone ($p<0.001$ and $p<0.05$, respectively).

The NSCs spontaneously differentiated into neurons and gliocytes, which were labeled by β-tubulin III and GFAP, respectively (Fig. 2*a, c, d*). The percentages of NSCs that differentiated into neurons was significantly decreased by PKMζ shRNA-4 and dexamethasone (both $p<0.001$) and increased by fluoxetine ($p<0.001$). Fluoxetine also significantly reversed the dexamethasone-induced decrease in the proportion of β-tubulin III-positive cells ($p<0.001$). PKMζ shRNA-4 significantly inhibited the stimulatory effect of fluoxetine on neuron-oriented differentiation in the absence and presence of dexamethasone. The percentage of gliocytes was significantly increased by PKMζ shRNA-4 and dexamethasone (both $p<0.05$) and decreased by fluoxetine ($p<0.05$). The effect of fluoxetine on gliocyte-oriented differentiation was significantly blocked by PKMζ shRNA-4 ($p<0.05$). Fluoxetine showed a tendency toward attenuating the effect of dexamethasone on gliocyte-oriented differentiation, which was also slightly blocked by PKMζ shRNA-4.

Annexin V immunostaining was performed to determine the effect of PKMζ knockdown on NSC apoptosis (Fig. 2*a, e*). The proportion of annexin V-positive cells was significantly increased by PKMζ shRNA-4 and dexamethasone (both $p<0.001$) and decreased by fluoxetine ($p<0.05$). Fluoxetine also significantly reversed the pro-apoptotic effect of dexamethasone ($p<0.001$). The effect of fluoxetine on NSC apoptosis was significantly blocked by PKMζ shRNA-4 in the absence and presence of dexamethasone (both $p<0.001$).

Fluoxetine-induced enhancement of PKMζ expression depends on 5-HT1A receptors

Immunohistochemical staining with specific antibodies revealed the expression of 5-HT, 5-HT1A receptors, the serotonin transporter (SERT), and tryptophan hydroxylase (TPH) in NSCs (Fig. 3*a*). Real-time PCR was used to determine the mRNA expression of 5-HT1A receptors, SERT, and TPH1/2 in cultured NSCs (Fig. 3*b*). Furthermore, the ELISA showed that fluoxetine (1 μM) caused significant 47% and 11% increases in 5-HT concentration in the culture medium during the proliferation and differentiation phases of NSCs, respectively (both $p<0.05$; Fig. 3*c*).

PKMζ protein levels in NSCs were increased by both fluoxetine ($p<0.05$) and 8-OH DPAT ($p<0.001$) but decreased by WAY-100635 ($p<0.05$) (Fig. 3*e*). The stimulatory effects of fluoxetine ($p<0.001$) and 8-OH DPAT ($p<0.05$) on PKMζ protein expression were significantly reversed by WAY-100635. PKMζ mRNA expression was not significantly changed by fluoxetine, 8-OH DPAT, or WAY-100635 NSCs (Fig. 3*d*). Both fluoxetine and 8-OH DPAT robustly reversed the inhibitory effect of dexamethasone on PKMζ mRNA and protein expression ($p<0.05$ and $p<0.001$, respectively), whereas these protective effects were significantly blocked by WAY-100635 (all $p<0.05$; Fig. 3*f, g*).

The effects of fluoxetine and 5-HT1A receptor activation on MAPK pathway phosphorylation are mediated by PKMζ and MEK

When hippocampal NSCs were cultured in the absence of dexamethasone, fluoxetine and 8-OH DPAT significantly increased pERK1/2 and pCREB protein levels in isolated NSCs ($p<0.05$ and $p<0.001$, respectively), whereas WAY-100635 significantly decreased pERK1 levels ($p<0.05$) and showed a tendency toward reducing pERK2 and pCREB levels. WAY-100635 counteracted the stimulatory effects of fluoxetine and 8-OH DPAT on both pERK1/2 and pCREB ($p<0.05$ and $p<0.001$; respectively; Fig. 4*a*). Dexamethasone significantly decreased the phosphorylation of both ERK1/2 and CREB ($p<0.05$), which were entirely reversed by fluoxetine and 8-OH DPAT ($p<0.05$ and $p<0.001$, respectively). Interestingly, the counteracting effect of fluoxetine on ERK1/2 and CREB phosphorylation induced by dexamethasone was further significantly abolished by WAY-100635 (all $p<0.001$; Fig. 4*b*). Moreover, fluoxetine, 8-OH DPAT, and WAY-100635 in the absence of dexamethasone had no significant effects on the total levels of either tERK1 or tCREB proteins (data not shown).

Both ZIP and U0126 significantly decreased the levels of pERK1/2 and pCREB (all $p<0.05$). The stimulatory effects of fluoxetine and 8-OH DPAT on both pERK1/2 and pCREB levels were robustly reversed by ZIP and U0126, respectively (all $p<0.001$; Fig. 4*c, d*).

The effects of antidepressants and 5-HT1A receptor activation on neurogenesis are mediated by PKMζ and MEK

We investigated whether 5-HT1A receptors are involved in the antidepressant-induced alteration of hippocampal NSC neurogenesis (Fig. 5*a–d*). Similar to fluoxetine, the 5-HT1A receptor agonist 8-OH DPAT significantly increased NSC proliferation according to the proportion of BrdU-positive cells (both $p<0.05$) and decreased NSC apoptosis, reflected by the proportion of annexin V-positive cells during the proliferation stage (both $p<0.05$). In contrast to fluoxetine, the 5-HT1A receptor antagonist WAY-100635 significantly decreased NSC proliferation ($p<0.001$) and increased the level of apoptosis ($p<0.001$), whereas WAY-100635 also reversed the effects of fluoxetine and 8-OH DPAT on NSC proliferation and apoptosis (both $p<0.05$). β-tubulin III-positive cells were increased by fluoxetine ($p<0.05$). WAY-100635 significantly decreased β-tubulin III-positive cells ($p<0.05$) and also abolished the increase in β-tubulin III-positive cells induced by fluoxetine ($p<0.05$). The ANOVA revealed no significant difference in the proportion of GFAP-positive cells among the six groups.

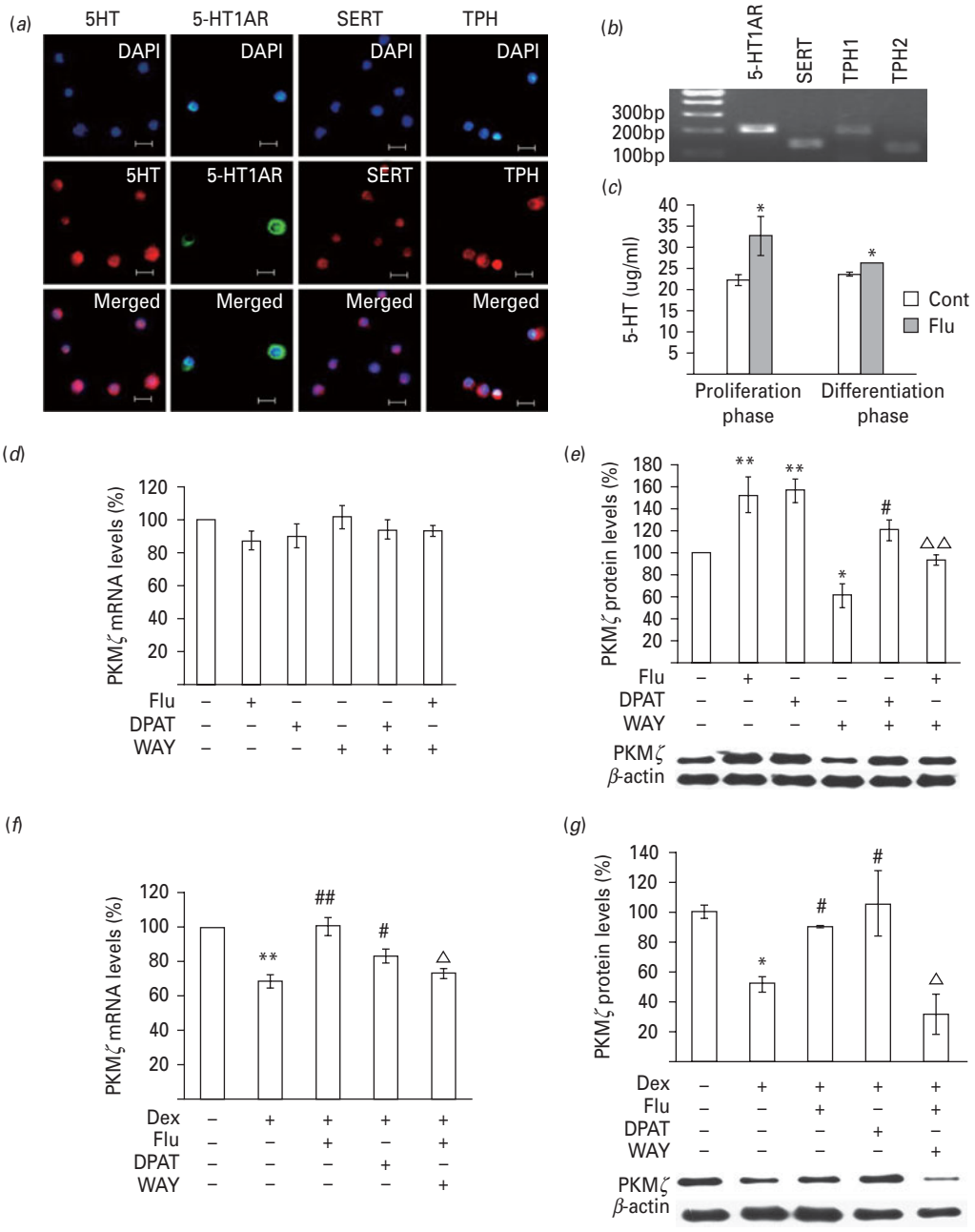

Fig. 3. Fluoxetine-induced enhancement of PKMζ expression depends on 5-HT1A receptors. (*a*) Immunoreactivity of nestin, 5-HT, 5-HT1A receptors, the serotonin transporter (SERT), and tryptophan hydroxylase (TPH) in NSCs. Scale bars=20 μm. (*b*) Gene expression of 5-HT1AR (200 bp), SERT (130 bp), TPH1 (198 bp), and TPH2 (110 bp) in NSCs. (*c*) The ELISA showed that fluoxetine (1 μM, 48 h) increased 5-HT concentrations in the culture media in both the NSC proliferation and differentiation phases (n=3 each). *p<0.05 *vs.* control. (*d*) The mRNA expression of PKMζ in NSCs was unchanged by fluoxetine (1 μM, 48 h), the 5-HT1A receptor agonist 8-OH DPAT (5 μM, 48 h), and the 5-HT1A receptor antagonist WAY-100635 (50 μM, 48 h) (n=3 each). (*e*) Fluoxetine enhanced PKMζ protein expression via 5-HT1A receptors (n=3). *p<0.05, **p<0.001, *vs.* control; $^{\#}p$<0.05 *vs.* 8-OH DPAT; △△p<0.001 *vs.* fluoxetine. (*f*) Fluoxetine enhanced PKMζ mRNA expression via 5-HT1A receptors in the presence of dexamethasone (n=3). **p<0.001 *vs.* control; $^{\#}p$<0.05, $^{\#\#}p$<0.001 *vs.* dexamethasone; △p<0.05 *vs.* dexamethasone+fluoxetine. (*g*) Fluoxetine enhanced PKMζ protein expression via 5-HT1A receptors in the presence of dexamethasone (n=3). *p<0.05 *vs.* control; $^{\#}p$<0.05 *vs.* dexamethasone; △p<0.05 *vs.* dexamethasone+fluoxetine. The mRNA and protein values were normalized to β-actin levels and are expressed as a percentage of control. Flu, fluoxetine; Dex, dexamethasone. DPAT, 8-OH DPAT; WAY, WAY-100635.

We further tested whether 5-HT1A receptors are involved in the protective effects of antidepressants against glucocorticoid-induced impairments in NSC neurogenesis (Fig. 5*e–h*). Dexamethasone significantly decreased the proportion of BrdU-positive cells to 38% compared with the vehicle-treated control (p<0.001), whereas fluoxetine (p<0.05) and 8-OH DPAT (p<0.001) reversed the inhibitory effects of dexamethasone, increasing the

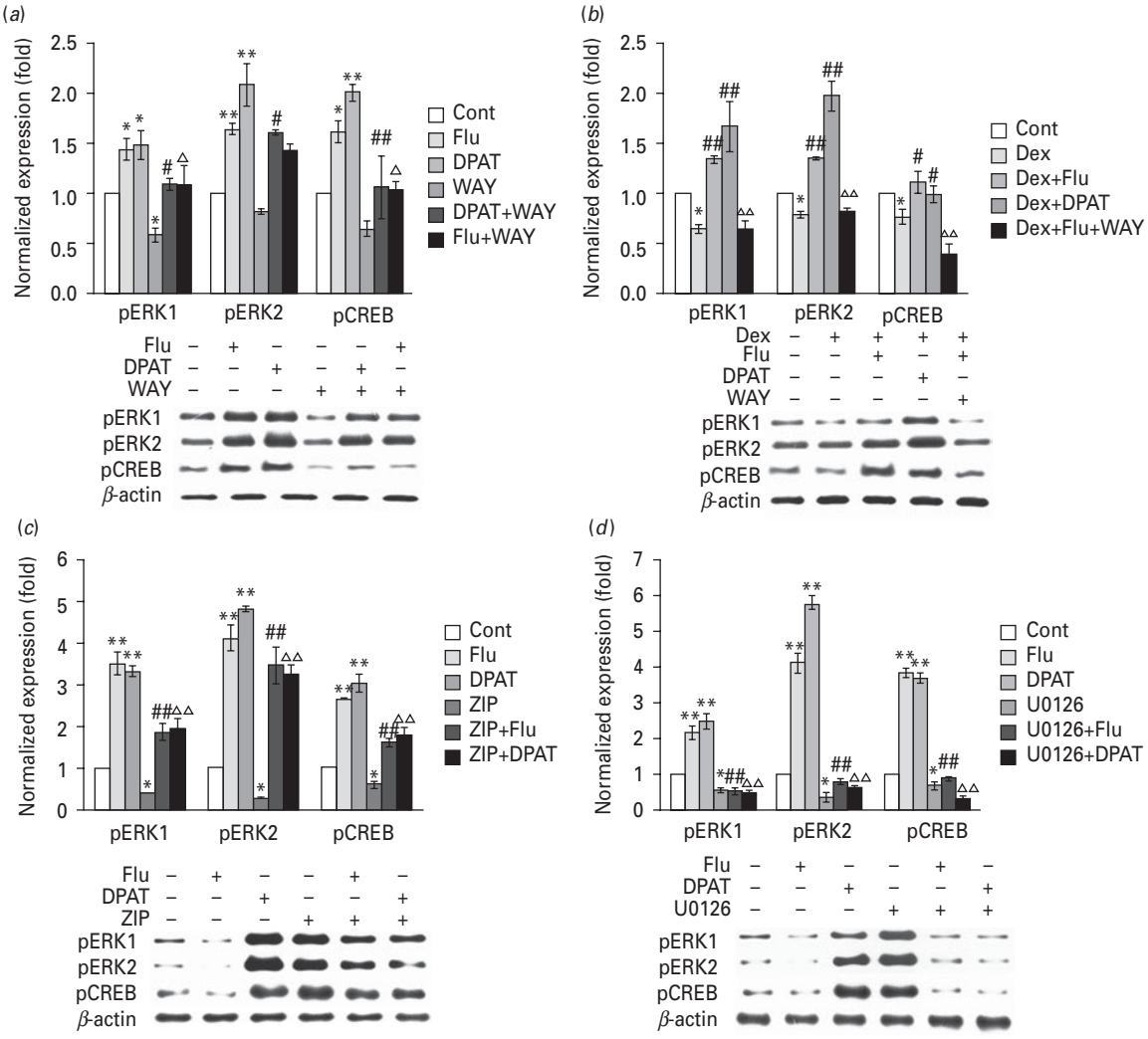

Fig. 4. The effects of fluoxetine and 5-HT1A receptor activation on MAPK pathway phosphorylation are mediated by PKMζ and MEK. (*a*) Fluoxetine (1 μM, 48 h) and the 5-HT1A receptor agonist 8-OH DPAT (5 μM, 48 h) enhanced the protein expression of pERK1, pERK2 and pCREB in NSCs, which were blocked by the 5-HT1A receptor antagonist WAY-100635 (50 μM, 48 h). *$p<0.05$, **$p<0.001$ *vs.* control; $^{\#}p<0.05$, $^{\#\#}p<0.001$ *vs.* 8-OH DPAT; $\triangle p<0.05$ *vs.* fluoxetine. (*b*) Fluoxetine (1 μM, 48 h) and the 5-HT1A receptor agonist 8-OH DPAT (5 μM, 48 h) increased the protein expression of pERK1, pERK2 and pCREB in the presence of dexamethasone (1 μM, 48 h). The 5-HT1A receptor antagonist WAY-100635 (50 μM, 48 h) reversed the effect of fluoxetine. *$p<0.05$ *vs.* control; $^{\#}p<0.05$, $^{\#\#}p<0.001$ *vs.* dexamethasone; $\triangle\triangle p<0.001$ *vs.* dexamethasone+fluoxetine. (*c*) The PKMζ blocker peptide ZIP (10 μM, 48 h) reversed the stimulatory effects of fluoxetine and 8-OH DPAT on pERK1, pERK2 and pCREB. *$p<0.05$, **$p<0.001$ *vs.* control; $^{\#\#}p<0.001$ *vs.* fluoxetine; $\triangle\triangle p<0.001$ *vs.* 8-OH DPAT. (*d*) The MEK inhibitor U0126 (10 μM, 48 h) reversed the stimulatory effects of fluoxetine and 8-OH DPAT on pERK1, pERK2 and pCREB. *$p<0.05$, **$p<0.001$ *vs.* control; $^{\#\#}p<0.001$ *vs.* fluoxetine; $\triangle\triangle p<0.001$ *vs.* 8-OH DPAT. The protein values were normalized to β-actin protein. The data are expressed as the mean±S.D. percentage of control ($n=3$). Flu, fluoxetine; Dex, dexamethasone. DPAT, 8-OH DPAT; WAY, WAY-100635.

percentage of BrdU-positive cells to 87% and 115%, respectively. Dexamethasone significantly decreased the proportion of β-tubulin III-positive cells approximately two-fold ($p<0.05$) and significantly increased the proportion of GFAP-positive cells ($p<0.001$) compared with the control. The effects of dexamethasone on both β-tubulin III- and GFAP-positive cells were significantly reversed by both fluoxetine and 8-OH DPAT (both $p<0.05$). The proportion of annexin V-positive cells was significantly increased by dexamethasone up to 11-fold compared with the vehicle control ($p<0.001$). This effect was significantly reversed by fluoxetine and 8-OH DPAT (both $p<0.001$). The effects of fluoxetine on NSC neurogenesis impairments induced by dexamethasone were completely abolished by the 5-HT1A receptor antagonist WAY-100635 ($p<0.05$ and $p<0.001$, respectively).

We then sought to identify the possible role of PKMζ and the MAPK pathway on antidepressant and 5-HT1A receptor activation-related neurogenesis (Fig. 5*i–l*). Both the PKMζ blocker peptide ZIP and selective MEK inhibitor U0126 significantly decreased the proportion of BrdU-positive cells (both $p<0.05$) and increased annexin

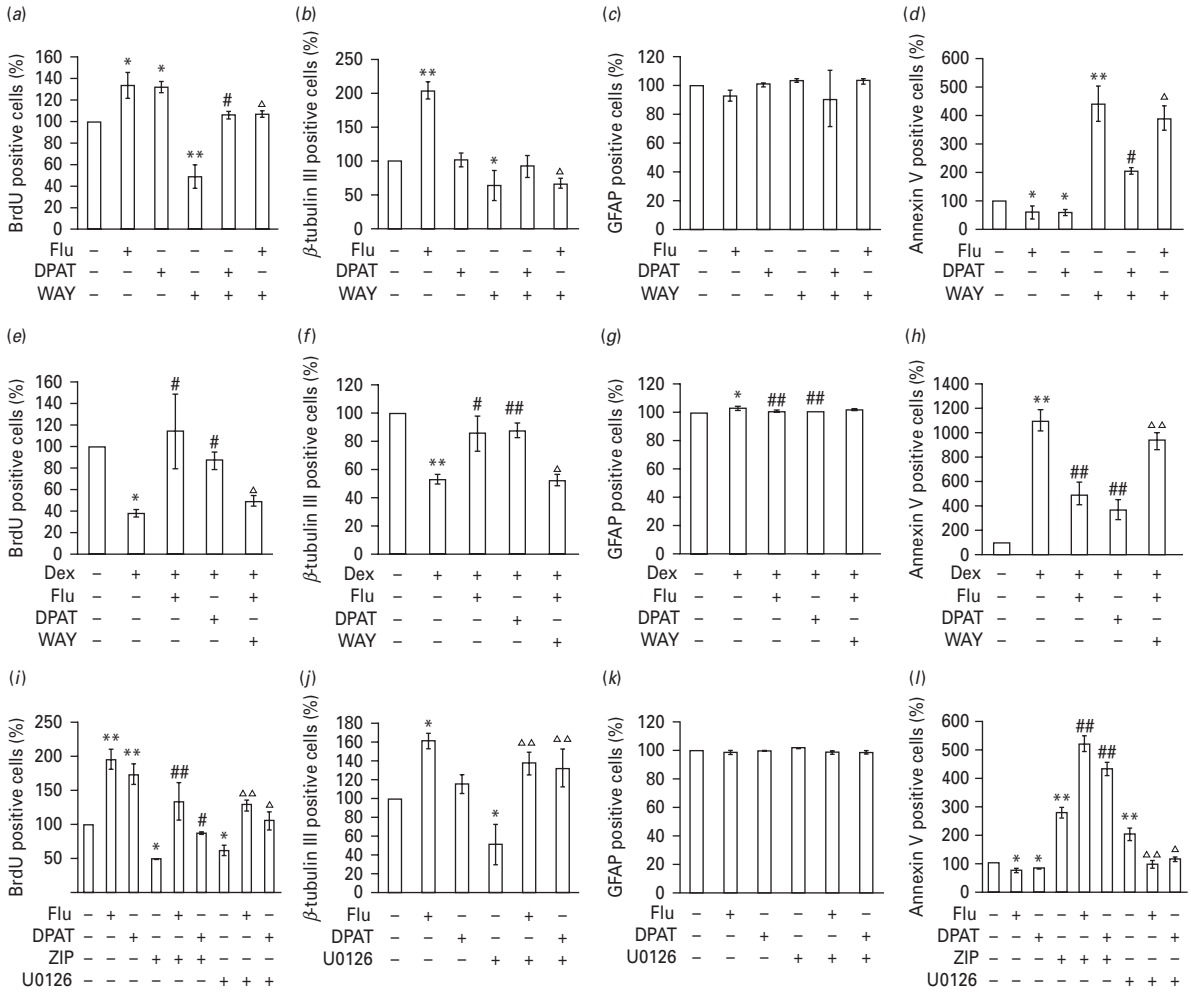

Fig. 5. The effects of fluoxetine and 5-HT1A receptor activation on neural stem call (NSC) neurogenesis are mediated by PKMζ and MEK. (*a–d*) The role of 5-HT1A receptors on NSC neurogenesis induced by fluoxetine was revealed by the proportion of (*a*) BrdU, (*b*) β-tubulin III, (*c*) GFAP and (*d*) annexin V positive cells. For NSC proliferation and apoptosis, NSCs were treated with fluoxetine (1 μM), 8-OH DPAT (5 μM), and WAY-100635 (50 μM) for 48 h. For NSC differentiation, NSCs were treated with fluoxetine (1 μM), 8-OH DPAT (5 μM), and WAY-100635 (50 μM) for 48 h after 5 d treatment with 10% foetal bovine serum. *$p<0.05$, **$p<0.001$ *vs.* control; $^{\#}p<0.05$ *vs.* 8-OH DPAT; $\triangle p<0.05$ *vs.* fluoxetine. (*e–h*) The role of 5-HT1A receptors in the regulatory effects of fluoxetine on NSC neurogenesis in the presence of dexamethasone was revealed by the proportion of (*e*) BrdU, (*f*) β-tubulin III, (*g*) GFAP and (*h*) annexin V positive cells. For NSC proliferation and apoptosis, NSCs were treated with dexamethasone (1 μM) for 48 h. For NSC differentiation, NSCs were treated with dexamethasone (1 μM) for 48 h after 5 d treatment with 10% foetal bovine serum. *$p<0.05$, **$p<0.001$ *vs.* control; $^{\#}p<0.05$, $^{\#\#}p<0.001$ *vs.* dexamethasone; $\triangle p<0.05$, $\triangle\triangle p<0.001$ *vs.* dexamethasone+fluoxetine. (*i*) The role of PKMζ and MEK in NSC proliferation induced by fluoxetine and 5-HT receptor activation was revealed by the proportion of BrdU-positive cells. Neural stem cells were treated with the PKMζ blocker peptide ZIP (10 μM) and MEK inhibitor U0126 (10 μM) for 48 h. (*j–k*) The role of MEK in NSC differentiation induced by fluoxetine and 5-HT1A receptor activation was revealed by the proportion of (*j*) β-tubulin III and (*k*) GFAP positive cells. (*l*) The role of PKMζ and MEK in NSC apoptosis induced by fluoxetine and 8-OH DPAT was revealed by the proportion of annexin V-positive cells. (*i–l*) *$p<0.05$, **$p<0.001$ *vs.* control; $^{\#}p<0.05$, $^{\#\#}p<0.001$ *vs.* ZIP; $\triangle p<0.05$, $\triangle\triangle p<0.001$ *vs.* U0126. The data are expressed as the mean±S.D. percentage of control ($n=3$). Flu, fluoxetine; Dex, dexamethasone; DPAT, 8-OH DPAT; WAY, WAY-100635.

V-positive cells (both $p<0.001$) in the proliferation phase of NSCs. Both ZIP and U0126 significantly decreased the stimulatory effects of fluoxetine (both $p<0.001$) and 8-OH DPAT (both $p<0.05$) on NSC proliferation assessed by BrdU-positive cell staining. Fluoxetine ($p<0.001$) and 8-OH DPAT ($p<0.05$) significantly reversed the increases in NSC apoptosis caused by U0126, demonstrated by the proportion of annexin V-positive cells ($p<0.05$ and $p<0.001$, respectively). However, fluoxetine and 8-OH DPAT increased the NSC apoptotic effects of ZIP. U0126 significantly decreased the proportion of β-tubulin III-positive cells in the NSC differentiation phase ($p<0.05$), whereas U0126 reversed the activational effect of fluoxetine on the proportion of β-tubulin III-positive cells ($p<0.001$). The NSCs lost adherence and their differentiation ability after ZIP treatment. Data on

the effects of ZIP on NSC differentiation are not available. The ANOVA revealed no significant difference in the proportion of GFAP-positive cells among the six groups.

Discussion

The present study demonstrated that PKMζ mediates hippocampal neurogenesis related to antidepressant administration. Specifically, the SSRI fluoxetine increased the proliferation and differentiation of embryonic hippocampal NSCs *in vitro* via a PKMζ-mediated mechanism, in which PKMζ expression was increased by 5-HT1A receptor stimulation and in turn elevated the phosphorylation of the ERK-CREB signaling pathway. Interestingly, this effect could be observed in both the absence and presence of the glucocorticoid receptor agonist dexamethasone, which mimics stress-induced glucocorticoid secretion (Xi et al., 2011).

Growing evidence indicates that the enhancement of hippocampal neurogenesis is crucial in the mechanisms of antidepressant efficacy (Yan et al., 2011). Protein kinase C plays a potential role in mood disorders and regulates various neurobiological functions, including neurogenesis (Bright et al., 2004; Apostolatos et al., 2012; Lim and Alkon, 2012; Abrial et al., 2013), neurotransmitter release, and receptor desensitization (Tanaka and Nishizuka, 1994). Protein kinase C has recently been shown to dual-directionally regulate depressive-like and mania-like behavior in rats. The chronic inhibition of PKC resulted in a reduction of cell proliferation in the hippocampal DG (Abrial et al., 2013). Specific members of the PKC family, such as the conventional PKCα, novel PKCδ and PKCε, and aPKCζ, have been implicated in neural proliferation, differentiation, cell survival, apoptosis and synaptic plasticity (Kaasinen et al., 2002; Bright et al., 2004; Schwamborn and Puschel, 2004; Yoshii et al., 2011; Korulu et al., 2013). Previous studies have shown that aPKCs (ι or ζ) regulate cell polarity in neuronal precursors, neurons and astrocytes (Schwamborn and Puschel, 2004). Atypical PKCs (PKCζ and ι) play distinct roles in the regulation of cortical neurogenesis and gliogenesis during embryonic development (Wang et al., 2010, 2012). These findings provided the rationale for further investigating the involvement of specific aPKC isoforms in hippocampal neurogenesis relevant to antidepressants. The present results show that two aPKCs, PKMζ and PKCι, but not PKCζ are expressed in rat embryonic hippocampal NSCs. PKMζ has been shown to be the major ζ form in the adult rodent forebrain, especially in the hippocampus, where there is a near absence of full-length PKCζ (Hernandez et al., 2003; Oster et al., 2004). Moreover, our results reveal an increase in PKMζ but not PKCι expression following fluoxetine administration in both the absence and presence of dexamethasone. To date, the reported effects of SSRIs on PKC activity have been inconsistent, ranging from an inhibitory effect in the rat cerebral cortex and hippocampus to an increase in rat cortical synaptoneurosomes (Mann et al., 1995; Giambalvo and Price, 2003). These distinct distribution and regulation patterns indicate that PKMζ may be the specific aPKC isozyme that responds to antidepressants and glucocorticoids in hippocampal NSCs. This hypothesis is further supported by PKMζ knockdown experiments, which have shown that PKMζ shRNA significantly decreases the proliferation and differentiation of NSCs and increases NSC apoptosis, which also significantly inhibits the stimulatory effect of fluoxetine on NSC neurogenesis in both the absence and presence of dexamethasone. These findings suggest that PKMζ, a constitutively active aPKC isoform, specifically mediates the hippocampal neurogenesis response to SSRI treatment.

The mechanism by which SSRIs regulate PKMζ is unknown. The primary action of chronic SSRI is based on the inhibition of serotonin reuptake to elevate synaptic 5-HT concentrations, thereby activating postsynaptic 5-HT receptors and triggering downstream intracellular signaling cascades. The present RT-PCR and immunostaining findings demonstrate the expression of TPH, 5-HT, SERT, and 5-HT1A receptors in hippocampal NSCs. These molecular effects have also been observed in postnatal rat cerebellar neural progenitors (Zusso et al., 2008). This phenotypic characterization indicates the existence of a complete circuit for antidepressants that regulates the neurobiological effect of 5-HT in an *in vitro* NSC system. Hippocampal NSCs may be capable of synthesizing and self-secreting serotonin. Interestingly, our results further show that fluoxetine significantly increases 5-HT concentrations in culture solution, suggesting that fluoxetine is able to inhibit serotonin reuptake and consequently increase 5-HT receptor stimulation in hippocampal NSCs. Importantly, the present study has found that both fluoxetine and 8-OH DPAT significantly increase PKMζ protein expression in the absence and presence of dexamethasone. The effects of both fluoxetine and 8-OH DPAT can be blocked by the 5-HT1A receptor antagonist WAY-100635. Among 12 isoforms of the PKC family, only PKCα has been previously reported to be stimulated by 5-HT1A receptor activation, playing a regulatory role in hippocampal NSC proliferation, synaptic plasticity and apoptosis in hippocampal HN2-5 cells (Adayev et al., 2003; Mehta et al., 2007; Mogha et al., 2012). The present study provides evidence of the ability of fluoxetine to regulate PKMζ expression via 5-HT1A receptors, possibly indicating the molecular mechanism that underlies SSRI-related neurogenesis and its antidepressant effect.

Atypical PKCs, both PKCζ and PKMζ, have been implicated in the regulation of the ERK/MAPK cascade in several cell types (Berra et al., 1995; Schonwasser et al., 1998; Fernandez et al., 2000; Monick et al., 2000). Activation of the MAPK pathway may consequently stimulate the transcription factor CREB, which is a critical

regulator of neural development and plasticity (Davis et al., 2000; Schafe et al., 2000; Ying et al., 2002). Thus, investigating whether the MAPK-CREB pathway may act as a downstream factor following PKMζ upregulation related to antidepressant treatment and 5-HT1A receptor activation is important. However, several animal studies have reported that the systemic administration of the 5-HT1A receptor agonist 8-OH DPAT induced pERK inhibition in the hippocampus (Chen et al., 2002; Sullivan et al., 2005; Crane et al., 2007; Buritova et al., 2009). A possible explanation is that 8-OH DPAT more efficiently binds 5-HT1A autoreceptors in the raphe nuclei, which decreases hippocampal 5-HT release and indirectly leads to pERK inhibition in postsynaptic hippocampal neurons (Chen et al., 2002). Interestingly, 8-OH DPAT does not inhibit ERK activity in cultured hippocampal neurons (Cowen et al., 2005) but inhibits pERK in RN46A cells, a model of raphe neurons (Kushwaha and Albert, 2005). The present study provides direct evidence of 5-HT1A receptor activation within the ERK/MAPK cascade in cultured hippocampal NSCs. The results show that pERK1/2 and pCREB are significantly increased following fluoxetine and 8-OH DPAT administration in both the absence and presence of dexamethasone, which can be reversed by the 5-HT1A receptor antagonist WAY-100635. More importantly, the present study has also found that both the PKMζ blocker ZIP and MEK inhibitor U0126 significantly decrease pERK1/2 and pCREB in response to fluoxetine and 5-HT1A receptor activation. These results raise the possibility that fluoxetine might activate MAPK-CREB signaling pathway phosphorylation via a 5-HT1A receptor-mediated increase in PKMζ in hippocampal NSCs.

One strength of the present study is that we have for the first time demonstrated the involvement of PKMζ in the activational effects of 5-HT1A receptors on hippocampal NSC neurogenesis following fluoxetine administration. Previous studies have consistently reported a critical role for 5-HT1A receptors in the proliferative activity of neural progenitors in both neuronal cell cultures and the mammalian brain (Djavadian, 2004; Benninghoff et al., 2010). However, the involvement of 5-HT1A receptors in NSC differentiation and survival has not been fully understood. 5-HT1A receptor activation has been found to promote the development of neural precursors into adult neurons (Banasr et al., 2004), but negative results have also been reported (Sahay and Hen, 2007). Our results show that fluoxetine and 8-OH DPAT increase the proliferation, neuron-oriented differentiation, and apoptosis of hippocampal NSCs in the absence and presence of dexamethasone, all of which can be abolished by WAY-100635. Thus, the present study confirms a powerful serotonergic impact on NSC differentiation and survival *in vitro* that can be mediated by 5-HT1A receptors. Furthermore, the present study shows that the enhancement of NSCs proliferation in response to fluoxetine and 8-OH DPAT is markedly inhibited by the PKMζ blocker ZIP and selective MAP kinase inhibitor U0126. U0126 can also reverse the increase in neuron-oriented differentiation induced by fluoxetine and 8-OH DPAT, whereas ZIP completely blocks the differentiation of hippocampal NSCs. Combined with the aforementioned findings that PKMζ mediates the activation effects of fluoxetine and 8-OH DPAT on MAPK-CREB phosphorylation, the present results suggest that PKMζ might be involved in fluoxetine- and 5-HT1A receptor-related hippocampal NSC neurogenesis. Wang et al. (2012) recently reported that aPKCs play important roles in regulating the differentiation of cortical neural progenitors. This study did not differentiate the effects of specific isoforms of PKCζ and PKMζ on cortical neural differentiation. Interestingly, PKMζ has been shown to be abundant in most regions of the mouse cortex, whereas PKCζ is restricted to the lateral olfactory tract in forebrain areas. Therefore, these findings suggest that PKMζ might be the predominant isoform involved in both cortical and hippocampal NSC neurogenesis.

We acknowledge that the current studies on rat foetal NSCs do not necessarily extrapolate either to NSCs in the adult animal or to the human, and only fluoxetine has been studied here; further work is needed to determine if the findings generalize to all SSRIs or even other antidepressant drugs. Clearly, further studies in adult animals will provide one step towards confirming the relevance of our findings to understanding mechanisms underlying the treatment of depression.

In summary, the present study has found that the SSRI fluoxetine increase hippocampal NSC neurogenesis via a PKMζ-mediated mechanism that links postsynaptic 5-HT1A receptor activation and the phosphorylation of the downstream ERK-CREB signaling pathway. Our data indicate a specific PKMζ-related intracellular molecular signaling cascade involved in SSRI-induced hippocampal neurogenesis. These results may have important implications for enhancing our understanding of the etiological mechanisms that underlie neurogenesis deficits in depression and improving clinical treatments for this stress-related disease.

Acknowledgments

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Statement of Interest

None.

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