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ORIGINAL ARTICLE

Influence of Tacrolimus on Depressive-Like Behavior in Diabetic Rats Through Brain-Derived Neurotrophic Factor Regulation in the Hippocampus

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Abstract

The neurotoxicity of immunosuppressive agents and diabetes mellitus are known risk factors of neurological complications in kidney transplant recipients. The aim of the present study was to investigate the influence of tacrolimus on brain-derived neurotrophic factor (BDNF), the critical protein for maintenance of neuronal functions, in the hippocampus in a diabetic condition. A diabetic rat model was established by a single streptozotocin injection (60 mg/kg). Control and diabetic rats then received daily tacrolimus (1.5 mg/kg per day) injections for 6 weeks. BDNF expression in the hippocampus was examined in the dentate gyrus (DG) and CA3 region using immunohistochemistry. There was a significant decrease of BDNF expression in the DG and CA3 region in tacrolimus-treated and diabetic rats compared with that of the control group injected with vehicle only. However, there was no difference in BDNF expression between the two experimental groups. Tacrolimus treatment in diabetic rats further decreased the BDNF expression level in the DG and CA3 region. Interestingly, mossy fiber sprouting, demonstrated by prominent punctate immunolabeling of BDNF with synaptoporin, was observed in the diabetic group treated with tacrolimus, which localized at the stratum oriens of the CA3 region. These data suggest that tacrolimus treatment or a diabetic condition decreases BDNF expression in the hippocampus, and that tacrolimus treatment in the diabetic condition further injures the CA3 region of the hippocampus. In addition to BDNF expression, decreased locomotor activity and evident depressive behavior were observed in tacrolimus-treated diabetic rats. Moreover, there were significant decreases of the mRNA levels of γ-aminobutyric acid and serotonin receptors in the diabetic hippocampus with tacrolimus treatment. This finding suggests that tacrolimus treatment may cause further psychiatric and neurological complications for patients with diabetes, and should thus be used with caution.

Keywords Brain-derived neurotrophic factor · Tacrolimus · Diabetes · Hippocampal dysfunction · Interneuron · Mossy fiber

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Introduction

Approximately one-third of organ transplant recipients experience neurologic alterations as a complication (Zivkovic 2007; Zivkovic and Abdel-Hamid 2010). Tacrolimus (TAC) is an immunosuppressant that is widely used in organ transplantation to prevent fatal graft-versus-host disease or to suppress rejection symptoms (Kahan 1992; Powles et al. 1980). Despite its high immunosuppressive potency, it is well-known that TAC induces multiple organ toxicity (Adu et al. 1983; Gupta et al. 1991; Hamilton et al. 1981) and new-onset diabetes after transplantation (NODAT) (Bee et al. 2011; Cosio et al. 2002; Hjelmesaeth et al. 2006; Israni et al. 2014; Larson et al. 2006; Luan et al. 2011; Montori et al. 2002).

The neurological complications after kidney transplantation in patients under long-term TAC treatment have been widely reported in many clinical studies (Bechstein 2000; Cadavid-Aljure et al. 2012; Chegounchi et al. 2006; Kastrup et al. 2012, 2015; Mayer et al. 1997; Neylan et al. 1998; Veroux et al. 2002; Wu et al. 2013). Diabetes mellitus (DM) is proven to be the main etiological factor contributing to neurological complications after kidney transplantation (Bruno and Adams 1988; Lee and Raps 1998; Ponticelli and Campise 2005; Yardimci et al. 2008). However, the multifactorial pathogenesis of diabetic neurological deficits associated with TAC treatment is not yet completely understood.

Previous reports have shown that the hippocampus is the most sensitive region to DM among the central nervous system regions (Ahmadpour et al. 2010). The hippocampus plays a pivotal role in memory formation, as well as in shaping emotional, adaptive, and reproductive behaviors (Squire 1992; Witter and Amaral 2004). New memory formation and the consolidation process of events by the hippocampus largely depend on the integrity of the internal trisynaptic circuit (Lewis 2012; Turgut and Turgut 2011). Neurological alterations in the hippocampus are mediated by brain-derived neurotrophic factor (BDNF) that regulates synaptic plasticity (Korte et al. 1995, 1998; Patterson et al. 1996). Thus, BDNF is one of the critical proteins maintaining neuronal functions, playing essential roles in physiological and morphological processes related to learning and memory, as well as mood disorder. Indeed, reduction of BDNF expression in humans or experimental animals was shown to lead to cognitive impairment/dementia (Connor et al. 1997; Krabbe et al. 2007; Peng et al. 2005; Tsai 2003; Zhen et al. 2013). In addition, several studies reported that the serum levels of BDNF are significantly decreased in patients with depression (Karege et al. 2002; Shimizu et al. 2003), and that antidepressant treatment can reverse this effect (Aydemir et al. 2005; Gervasoni et al. 2005; Gonul et al. 2005). A clinical study showed that bilateral hippocampal injury was induced by calcineurin inhibitors after bone marrow transplantation (Lee et al. 2008). In addition, chronic administration of cyclosporine A induced the suppression of BDNF and its receptor TrkB in the hippocampus and midbrain (Chen et al. 2010). Therefore, we hypothesized that BDNF might play a role in the diabetes-related neurotoxicity of TAC.

Accordingly, the aim of this study was to elucidate the influence of TAC on the hippocampal dysfunction in diabetic rats. To this end, we investigated the spatial regulation and characteristic of BDNF expression in the hippocampus of diabetic rats with and without TAC treatment. Moreover, the depressive behavior and related neurotransmitter receptor expression were examined in the TAC-treated diabetic rats.

Materials and Methods

Animal Care

The Animal Care and Use Committee of the Catholic University of Korea approved the experimental protocol (CUMC-2018-0038-02), and all procedures performed in this study were in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experiments provided by the Institutional Animal Care and Use Committee (IACUC) at the School of Medicine, the Catholic University of Korea. Eight-week-old male Sprague-Dawley rats (Charles River Technology, Seoul, Korea) that initially weighed 300–400 g were housed in social groups with 2 animals/cages (Nalge, Rochester, NY, USA) in a controlled-temperature (20–26 °C), controlled-humidity (50 ± 10%), and controlled-light (12-h light-dark cycle) environment at the Catholic University of Korea's animal care facility.

Establishment of the Experimental Model

An experimental model of DM was induced by single injecting streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 60 mg/kg via the tail vein; STZ was diluted in 0.1 M citrate buffer (pH 4.5) (Park et al. 2003). Subsequent development of DM was confirmed according to the fasting blood glucose (FBG) level at 1 week after injection (Park et al. 2003). Only rats with a basal FBG level above 200 mg/dL in the STZ-treated group were confirmed to have DM and were used in subsequent experiments. The rats received a low-salt diet (0.05% sodium chloride, Research Diets, New Brunswick, NJ, USA). After acclimation for 1 week, weight-matched rats were randomized into the following four groups ($n = 9$ per group): (1) VH group, in which the rats received a single tail vein injection of normal saline at the same volume as STZ, and treatment with olive oil at the same volume as TAC via subcutaneous injection daily for 6 weeks; (2) STZ group, in which DM was induced by a single tail vein injection of STZ (60 mg/kg body weight); (3) TAC

group, in which the rats received a daily subcutaneous injection of TAC (1.5 mg/kg body weight; Prograft, Astellas Pharma Inc., Ibaraki, Japan) diluted in olive oil (Sigma-Aldrich, St. Louis, MO, USA) for 6 weeks; and (4) TAC + STZ group, in which TAC (1.5 mg/kg body weight) was administered by subcutaneous injection daily after DM was established for 6 weeks.

The dose and duration of TAC treatment were based on our previous findings that TAC treatment at therapeutic levels (1.5 mg/kg per day for 6 weeks) caused organ injury such as nephrotoxicity or islet cell injury (Hwang et al. 2012; Jin et al. 2017, 2018; Song et al. 2009).

The rats were pair-fed and their body weight was monitored daily. To evaluate the optimal induction of diabetes, the blood glucose levels of the STZ-injected animals were checked using a glucose analyzer (Accu-Check; Roche Diagnostics, Basel, Switzerland) during a fasting state. After the treatment period, the animals were housed individually in metabolic cages (Tecniplast, Gazzada, Italy) for the evaluation of metabolites, and their urine volume and water intake were measured over 24 h.

Tissue Preparation

The experimental animals were anesthetized with tiletamine-zolazepam (10 mg/kg, intraperitoneal injection; Zoletil 50, Virbac Laboratories, Carros, France) and xylazine (15 mg/kg, intraperitoneal; Rompun®, Bayer, Leverkusen, Germany), and killed by transcardial perfusion with a fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH = 7.4) for 30 min or by decapitation. For histological evaluation, the brain was post-fixed in 4% PFA for 4 h and then embedded in paraffin. For reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, the hippocampus was separated and immediately frozen in liquid nitrogen. The samples were stored at − 70 °C until further processing.

Immunohistochemistry and Double Labeling

Dewaxed sections were incubated in retrieval solution (pH 6.0) and then washed in phosphate-buffered saline. After blocking with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h, the sections were incubated with a mouse monoclonal antibody against BDNF (ab205067; Abcam, Cambridge, UK) overnight at 4 °C. Primary antibody binding was visualized using peroxidase-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA) with 0.01% H_2O_2 as the substrate. For double-immunofluorescence histochemistry, the sections were incubated at 4 °C overnight with a mixture of monoclonal mouse anti-BDNF antibody (Abcam) and one of the following antibodies: polyclonal rabbit antibody against parvalbimin (PV27; Swant, Bellinzona, Switzerland) and synaptoporin (102 002; Synaptic System, Göttingen, Germany). Antibody staining was visualized using the following secondary antibodies: peroxidase-conjugated secondary antibodies with DAB for BDNF antibody, and Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch) for the others. Slides were viewed with a confocal microscope (LSM 700; Carl Zeiss Co. Ltd., Oberkochen, Germany). Images were converted to the TIFF format, and contrast levels were adjusted using Adobe Photoshop v. 13 (Adobe System, San Jose, CA, USA).

RT-qPCR

Total RNA from the rat hippocampus was extracted using RNA isolation reagent (RNA-Bee; Tel Test, Inc., TX, USA). Five micrograms of each purified RNA was reverse-transcribed into first-strand complementary DNA with a Dyne 1st-Strand cDNA Synthesis Kit (DyneBio Inc., Seong-Nam, Korea). RT-qPCR amplification was conducted using SYBR Green PreMiX in a LightCycler 480 system (Roche, Rotkreuz, Switzerland). The mRNA expression level was normalized to that of *Gapdh* using the change in cycle threshold method. The following primers were used for qPCR: 5′-GCA GAA GTT GTC TAT GAG TGG AC-3′ and 5′-TCC AGA GTC AAC TGT TTG CCC-3′ for rat $GABA_{A1}$ receptor, 5′-TCA GTG CTC GAA ATT CCC TTC CCA-3′ and 5′-TAC ACT CTT TCC ATC CCA AGC CCA-3′ for rat $GABA_{A2}$ receptor, 5′-AGC TGA CCA GAC CTT GGT CAT T-3′ and 5′-AAC TGG CTT CTC CCT ATG TGG-3′ for rat $GABA_{B1}$ receptor, 5′-GAT GGG TGT GAA CCA CGA GAA AT-3′ and 5′-ACG GAT ACA TTG GGG GTA GGA A-3′ for rat $GABA_{B2}$ receptor, 5′-CTC CTT GGC GGT TAC TGA T-3′ and 5′-GTA GAT GGT GTA GCC GTG GT-3′ for rat 5-HT_{1A} receptor, and 5′-AGG TCG GTG TGA ACG GAT TTG-3′ and 5′-GGG GTC GTT GAT GGC AAC A-3′ for rat *Gapdh*. All experiments were performed in triplicate on the individual rats ($n = 4$).

Behavior Tests

Open-Field Test

The open-field test (OFT) was performed to evaluate general locomotor activities in the rats, as previously described with a slight modification (Damián et al. 2014; Lee et al. 2014). Each of the rats ($n = 7$) was placed in the center area of the OFT apparatus (50 × 50 × 30 cm) and its motility was observed during a 5-min period. The locomotor activities were indicated by the total distance moved (cm), movement time (s), and velocity (cm/s), and analyzed by a computerized video–tracking system using the SMART program (PanLab Co., Barcelona, Spain). After behavioral monitoring, the surface

of the apparatus was cleaned with 70% ethanol solution and left to dry before testing the next animal.

Tail Suspension Test

The tail suspension test (TST) was based on the method of Chermat et al. (1986). The rats ($n = 7$) were suspended upside down in a tail suspension box placed 50 cm above the floor. An adhesive tape was used to fix the rats approximately 2 cm from the tip of the tail. All animals were suspended for a 6-min period, and monitored by a computerized video–tracking system using the SMART program (PanLab Co., Barcelona, Spain). The animals were considered to be immobile only when they hung passively and remained completely motionless.

Quantitative and Statistical Analysis

BDNF-labeled profiles were evaluated by counting approximately 20 randomly selected areas (50 × 50 μm per field) of each of the stained tissue sections at × 400 magnification using a color image analyzer (TDI Scope Eye version 3.0 for Windows). All data are presented as the mean ± standard error, and one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was used to compare the data among groups. Differences with P values of less than 0.05 were considered statistically significant. All statistical analyses were conducted with GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of TAC on Basic Parameters in Diabetic Rats

Table 1 summarizes the basic parameters of the experimental groups. The increase in body weight was significantly decreased in the TAC group compared with that in the VH group, and was further decreased in the STZ and STZ + TAC groups compared with that in the TAC group. In addition, TAC significantly increased the water intake, urine volume, and hemoglobin A1c and glucose levels compared with those of the VH group, although these levels were still lower than those of the STZ group. The STZ + TAC group showed the highest values of all parameters above, indicating that TAC treatment negatively affects functional parameters in a diabetic condition.

BDNF Expression in the Rat Hippocampus

The distribution and cellular localization of BDNF protein in the CA1, dentate gyrus (DG), and CA3 region of the hippocampus of the experimental rats were examined using immunohistochemistry. In the CA1 region, no specific BDNF immunolabeling was observed in the pyramidal cell layer (pcl) and stratum radiatum (sr) in all four groups (Fig. 1b, d, f, h). However, in the DG, prominent signals were detected in the granule cell layer and hilar region in the VH group, and BDNF immunoreactivity was obviously detected in some cells in the granular cell layer (GCL) (arrowheads in Fig. 1c) in agreement with previous findings (Nam et al. 2014). The distribution pattern and morphology of these cells suggested that they might represent interneurons. The labeling intensity of BDNF in the DG was remarkably decreased in the TAC group (labeling area of the GCL, 2.3 ± 0.2 μm^2; labeling area of the hilus, 12.4 ± 0.9 μm^2), STZ group (labeling area of the GCL, 2 ± 0.2 μm^2; labeling area of the hilus, 12.1 ± 0.9 μm^2), and STZ + TAC group (labeling area of the GCL, 1.2 ± 0.2 μm^2; labeling area of the hilus, 8.9 ± 0.6 μm^2) (Fig. 1e, g, i; Fig. 2g–h) compared with that of the VH group (labeling area of the GCL, 6.4 ± 0.4 μm^2; labeling area of the hilus, 30.7 ± 0.9 μm^2), suggesting an influence of both diabetes and TAC on BDNF expression in the hippocampus.

Table 1 The basic parameters in each group

	VH	TAC	STZ	STZ + T
Δ Body weight (g)	71 ± 4	53 ± 3[1]	− 49 ± 12[1,2]	− 96 ± 22[1,2]
Water intake (mL/day)	39 ± 6	85 ± 6[1]	97 ± 1[1,]	161 ± 9[1,2,3]
Urine volume (mL/day)	19 ± 6	57 ± 1[1]	94 ± 2[1,2]	162 ± 12[1,2,3]
HbA1c (%)	4 ± 1	5.6 ± 1[1]	10 ± 1[1,2]	14 ± 2[1,2,3]
Blood glucose level (mg/dL)	102 ± 3	204 ± 6[1]	226 ± 18[1,]	433 ± 31[1,2,3]

VH vehicle, *TAC* tacrolimus, *STZ* streptozotocin, *n* number of animals, *BW* body weight, *UV* urine volume, *WI* water intake, *HbA1c* hemoglobin A1c. The values shown are the mean ± SE ($n = 8$)

[1] $P < 0.05$ vs. VH

[2] $P < 0.05$ vs. TAC

[3] $P < 0.05$ vs. STZ

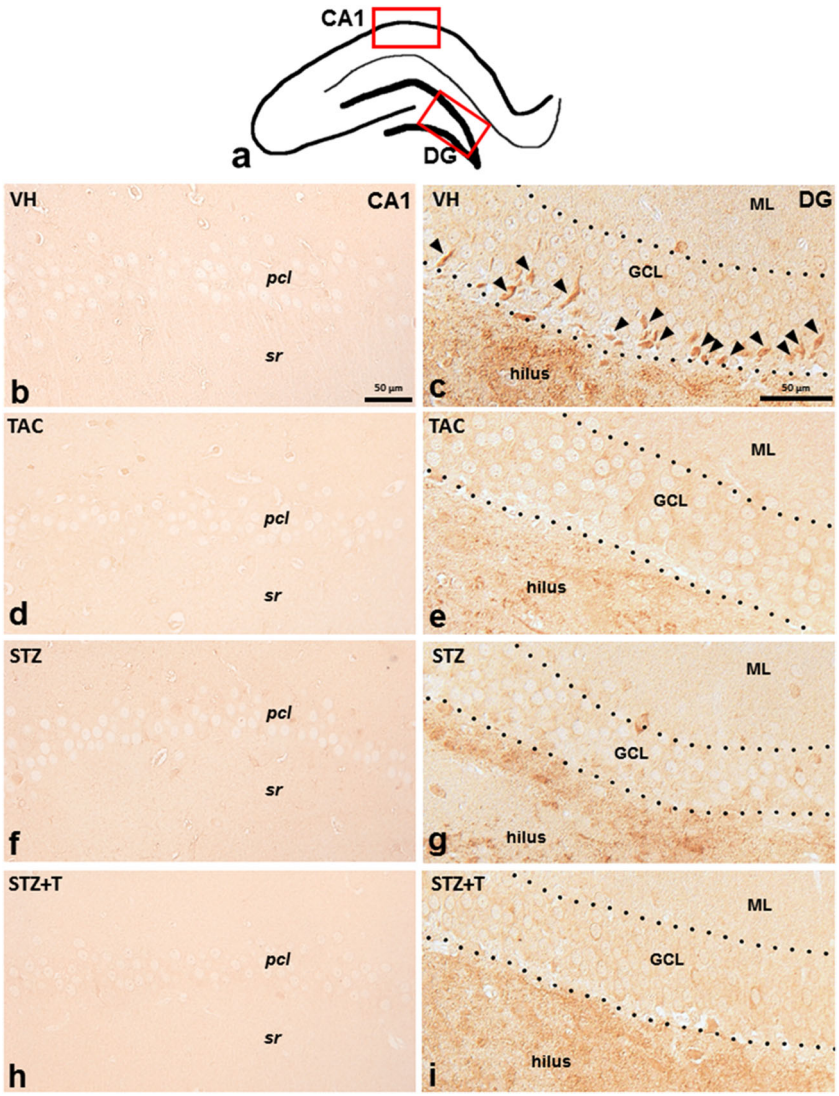

Fig. 1 Spatial expression of brain-derived neurotrophic factor (the BDNF) in the CA1 and dentate gyrus of the diabetic hippocampus. **a** Schematic diagram indicating the areas of observation for BDNF immunolabeling. Boxed areas denote the CA1 and dentate gyrus in the hippocampus. (**b**, **d**, **f**, **h**) In the CA1 region, immunoreactivity for BDNF was not detectable in the pyramidal cell layer and stratum radiatum in all groups. (**c**, **e**, **g**, **i**) In the dentate gyrus, BDNF expression was detected in the granular cell layer and the dentate hilar region. Note evident immunoreactivity for BDNF in neuron-like cells in the granular cell layer and the labeling intensity was more prominent in the VH group (**c**) compared with that of the other groups (**e**, **g**, **i**). DG, dentate gyrus; ML, molecular layer; GCL, granular cell layer; pcl, pyramidal cell layer; sr, stratum radiatum. Scale bars = 50 μm for **b**–**i**

Identification of BDNF-Expressing Cells in the DG

To define the phenotype of cells expressing BDNF in the DG in the VH group, double labeling was performed using the cell type–specific marker parvalbumin, a calcium-binding albumin protein that is specifically expressed in the neurons of the brain. Most of the BDNF-immunoreactive cells in the GCL of the DG in the VH group were confirmed to be parvalbumin-positive interneurons (asterisks in Fig. 2d–f).

Influence of TAC Treatment on BDNF Expression in the Diabetic Hippocampus

Immunohistochemistry showed spatial differences of BDNF expression in the stratum lucidum (*sl*), stratum pyramidale (*sp*), and stratum oriens (*so*) of the CA3 region, resulting in clear demarcation of all areas in this region (Fig. 3b–e). In the *sl* of the CA3 region, BDNF immunoreactivity showed a punctate structure that surrounded the dendrite-like profiles of pyramidal cells (asterisks in Fig. 4a–d). BDNF protein signals were particularly evident with a punctate appearance in the VH group, and the labeling intensity was more prominent than that of the other groups (labeling area, $50 \pm 2.4\ \mu m^2$). In the TAC or STZ group, the labeling pattern was similar to that observed in the VH group, but with significantly decreased intensity. Moreover, the BDNF labeling intensity in the STZ + TAC group (labeling area, $6.5 \pm 0.8\ \mu m^2$) was further decreased compared with that of the TAC group (labeling area, $14.2 \pm 1.6\ \mu m^2$) or STZ group (labeling area, $13.4 \pm 1.6\ \mu m^2$) (Fig. 3e–g, Fig. 4e).

Effects of TAC Treatment on BDNF Expression in Mossy Fiber Sproutings in the Diabetic Hippocampus

In the *so* of the CA3 region, prominent BDNF-immunoreactive punctate structures were observed

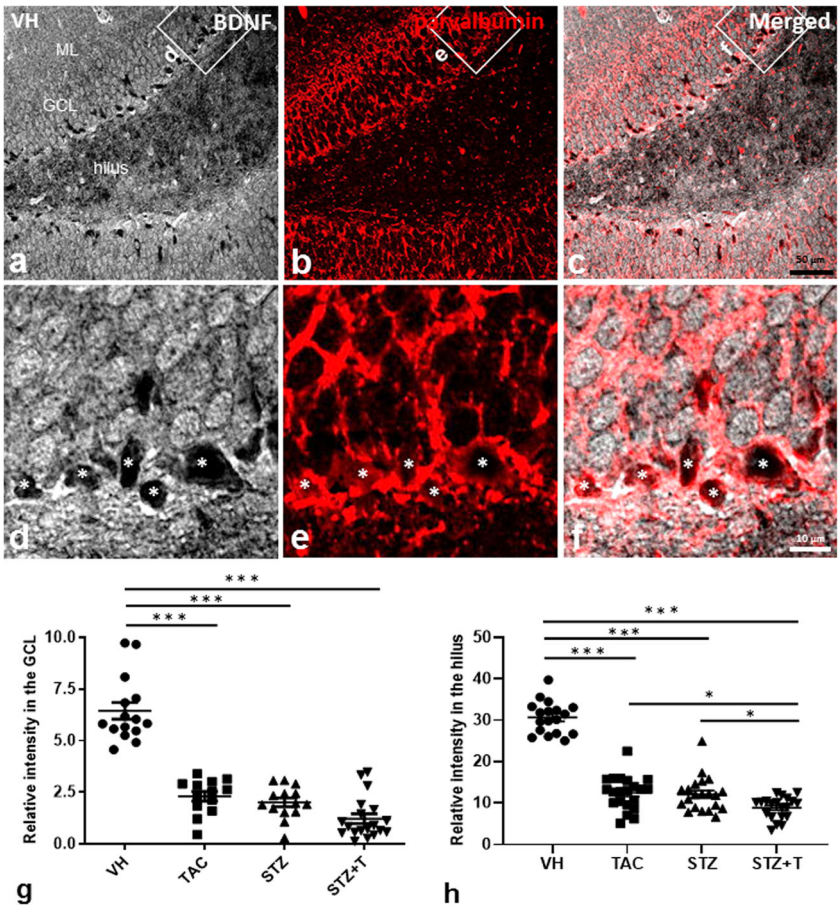

Fig. 2 BDNF-expressing cells were parvalbumin-positive interneurons of the dentate gyrus (DG) in the VH group. **a**–**c** Double labeling for BDNF and parvalbumin, a neuronal marker, showing that the majority of BDNF-labeled cells were the parvalbumin-positive interneurons of the dentate gyrus in the VH group. **d**–**f** High-magnification images of the boxed areas in **a**–**c**, respectively. Asterisks denote the double-labeled cells in the granular cell layer of the dentate gyrus. **g**–**h** Quantification of the relative intensity for BDNF staining in the GCL and hilus of the DG. Data are presented as means ± standard errors of the mean. $^{*}P < 0.05$; $^{***}P < 0.001$. ML, molecular layer; GCL, granular cell layer. Scale bars = 50 μm for **a**–**c**; 10 μm for **d**–**f**

exclusively in the STZ + TAC group, with very weak or negligible expression observed in the other groups (Fig. 3f–i, Fig. 4f–j). In the *sp*, no significant induction of BDNF expression was detected in any of the groups.

To identify the phenotypes of BDNF-positive profiles in the CA3 region, we performed double labeling with BDNF and the mossy fiber–specific marker synaptoporin. The majority of BDNF protein was co-localized with synaptoporin-positive mossy fiber projections (Fig. 5a–f). Higher-magnification confocal images (Fig. 5g–i) revealed prominent BDNF expression in a subset of synaptoporin-positive mossy fiber sproutings only in the STZ + TAC group, with no prominent BDNF expression detected in the other groups (Fig. 5a–d).

Effects of TAC Treatment on Locomotion and Depressive-Like Behavior in the Diabetic Rats

The locomotor activity and depressive behavior were examined using the OFT and the TST, respectively. In the OFT, the total distance moved, movement time, and velocity were compared among the groups. As shown in Fig. 6a–c, there was a significant reduction in all parameters in the STZ + TAC group compared with those of the other groups. In addition, the immobile time was significantly increased in the TAC group compared with that in the VH group, and was further increased in the STZ + TAC group compared with that in the STZ group (Fig. 6d). This finding suggested a significant influence of TAC treatment in diabetic rats, resulting in lower locomotor activity and evident depressive–like behavior (Fig. 7).

Effects of TAC Treatment on the mRNA Expression of GABA and 5-HT Receptor Subunits in the Hippocampus of Diabetic Rats

RT-qPCR analysis revealed that the mRNA level of the $GABA_{A1}$ receptor was significantly deceased in the STZ and STZ + TAC groups compared with that in the VH and TAC groups; the STZ + TAC group showed the lowest level of $GABA_{A2}$ receptor mRNA expression (Fig. 7 a–b). In addition, the mRNA levels of $GABA_{B1}$ and $GABA_{B2}$ receptors were substantially decreased in the TAC group compared with those in the VH group, and were further decreased in the STZ and STZ + TAC groups compared with those in the TAC group (Fig. 7 c–d). Moreover, the level of $5\text{-}HT_{1A}$ receptor was the lowest in the STZ + TAC group among all groups (Fig. 7 e).

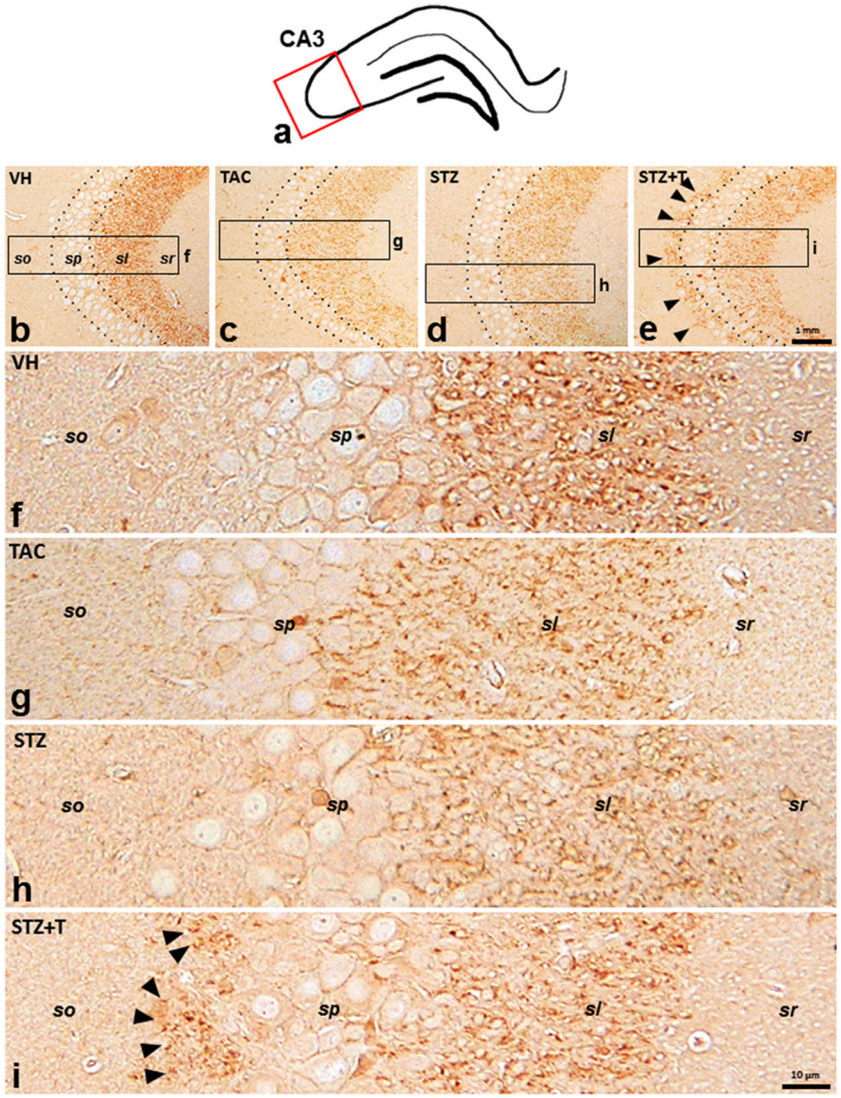

Fig. 3 Spatial difference of BDNF expression in the CA3 region of the diabetic hippocampus with TAC treatment. **a** Schematic diagram indicating the areas of observation for BDNF immunolabeling. Boxed areas denote the CA3 region in the hippocampus. **b**–**e** BDNF expression exhibited a spatial difference in the CA3 between the groups. In the stratum lucidum of the CA3 region, intense BDNF immunoreactivity was observed in the VH group (**b**) compared with the other groups (**c**–**e**). **f**–**i** Higher-magnification images of the boxed areas in **b**–**e**, respectively. Note that the punctate BDNF–labeled profiles were distributed in the stratum lucidum, which are associated with the mossy fiber tract. In the stratum oriens of the CA3 region, prominent BDNF immunoreactivity was exclusively detected in the STZ + TAC group (arrowheads in **e**, **i**). In the stratum pyramidale, BDNF expression was not detectable in any of the groups. *so*, stratum oriens; *sp*, stratum pyramidale; *sl*, stratum lucidum; *sr*, stratum radiatum. Scale bars = 1 mm for **b**–**e**; 10 μm for **f**–**i**

Discussion

Several studies have shown that TAC treatment or a diabetic condition could stimulate reactive oxygen species production. Therefore, it is well known that oxidative stress is a common pathway in TAC-induced organ injury (Damiano et al. 2015; Yoon and Yang 2009), and also plays a major role in the pathogenesis of DM (Baynes 1991; Baynes and Thorpe 1999; Ceriello 2000). The brain is particularly vulnerable to oxidative processes, and accumulation of oxidative damage to biomolecules in the brain causes many neurodegenerative diseases such as schizophrenia and bipolar disorder (Hong et al. 2014; Kapczinski et al. 2008; Kelly 1999; Liu and Mori 1999; Zhang et al. 2015). Moreover, clinical studies have shown that increased oxidative stress is associated with lower BDNF levels under pathological conditions such as in patients with acute mania (Kapczinski et al. 2008; Zhang et al. 2015). However, the present study is the first to demonstrate that TAC treatment could induce a change of BDNF regulation in the hippocampus in a diabetic condition. Either the TAC treatment or the diabetic condition decreased BDNF expression in the hippocampus, but TAC treatment in the diabetic condition caused further injury. This finding suggests that TAC treatment may cause further neurological complications in patients with diabetes.

In TAC-treated or diabetic rats, there was a significant decrease of BDNF expression both in the DG and CA3 region compared with that of the VH group. Specifically, differential expression of BDNF occurred in two distinct regions of the diabetic hippocampus compared with the control hippocampus: in the GCL of the DG region, and in the *sl* and *so* of the CA3 region. Together with previous studies, our data raise the intriguing possibility that the increased oxidative stress in TAC treatment or diabetes could mechanistically contribute to a decrease of BDNF expression in the DG and CA3 regions.

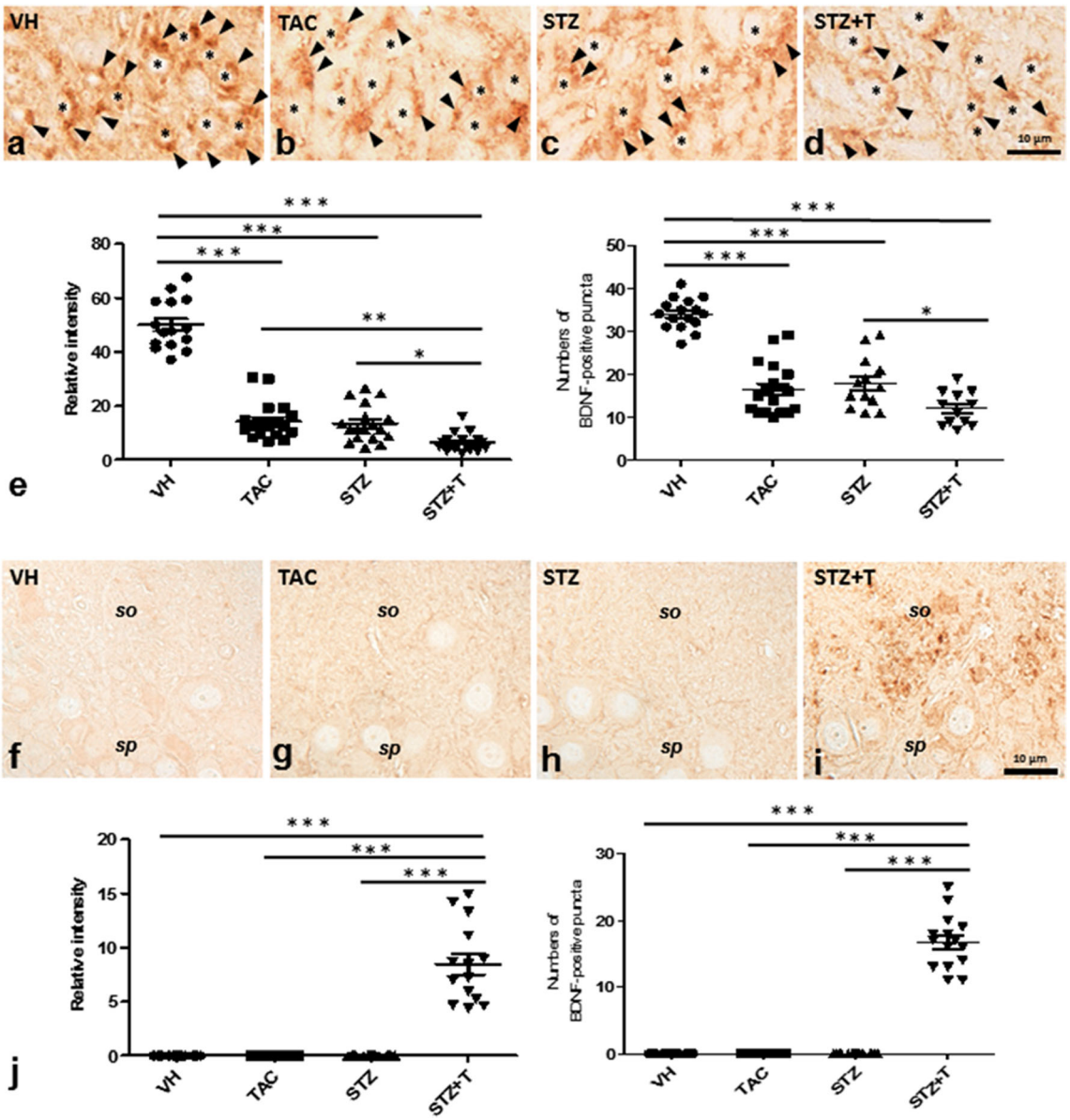

Fig. 4 Quantitative analysis of BDNF expression in the CA3 region. **a**–**c** In the stratum lucidum of the CA3 region, punctate BDNF immunoreactivity surrounded the dendrite-like profiles (asterisks in **a**–**d**). **e** Scatterplot of BDNF staining in the stratum lucidum. The relative intensity and numbers of the BDNF-positive profiles were significantly decreased in the TAC or STZ group compared with those of the VH group, and a further decrease of BDNF expression in the STZ + TAC group was observed. **f**–**i** In the stratum oriens of the CA3 region, BDNF immunoreactivity was exclusively detected in the STZ + TAC group. **j** Quantification of the relative intensity and numbers for BDNF staining in the stratum oriens. Data are presented as means ± standard errors of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *so*, stratum oriens; *sp*, stratum pyramidale. Scale bars = 10 μm for **a**–**d**, **f**–**i**

TAC treatment in diabetic rats further decreased the BDNF expression in the DG and CA3 region. TAC is an important risk factor of NODAT, a serious metabolic complication after kidney transplantation (Cosio et al. 2002; Hjelmesaeth et al. 2006; Kasiske et al. 2003; Weir and Fink 1999). TAC-induced NODAT is related to its direct toxic effect on pancreatic β cell dysfunction and injury. Previous studies indicated that a high level of TAC-induced oxidative cellular damage was closely associated with hyperglycemia (Kaneto et al. 1996; Kim et al. 2005; Robertson et al. 2003; Tanaka et al. 1999). Furthermore, pre-clinical studies have shown that transplantation of islets in diabetic animals could reverse the diabetic state when combined with immunosuppression or immune tolerance induction to prevent rejection (Contreras et al. 2003; Fu et al. 2002; Kenyon et al. 1999). Interference of a calcineurin inhibitor such as TAC in graft function has been observed, which was related to the diabetogenic side effects of the compound (Sai et al. 1988; Stegall et al. 1989). These findings, together with our observations, led us to speculate that TAC treatment may induce a further decrease in the BDNF level in diabetic rats

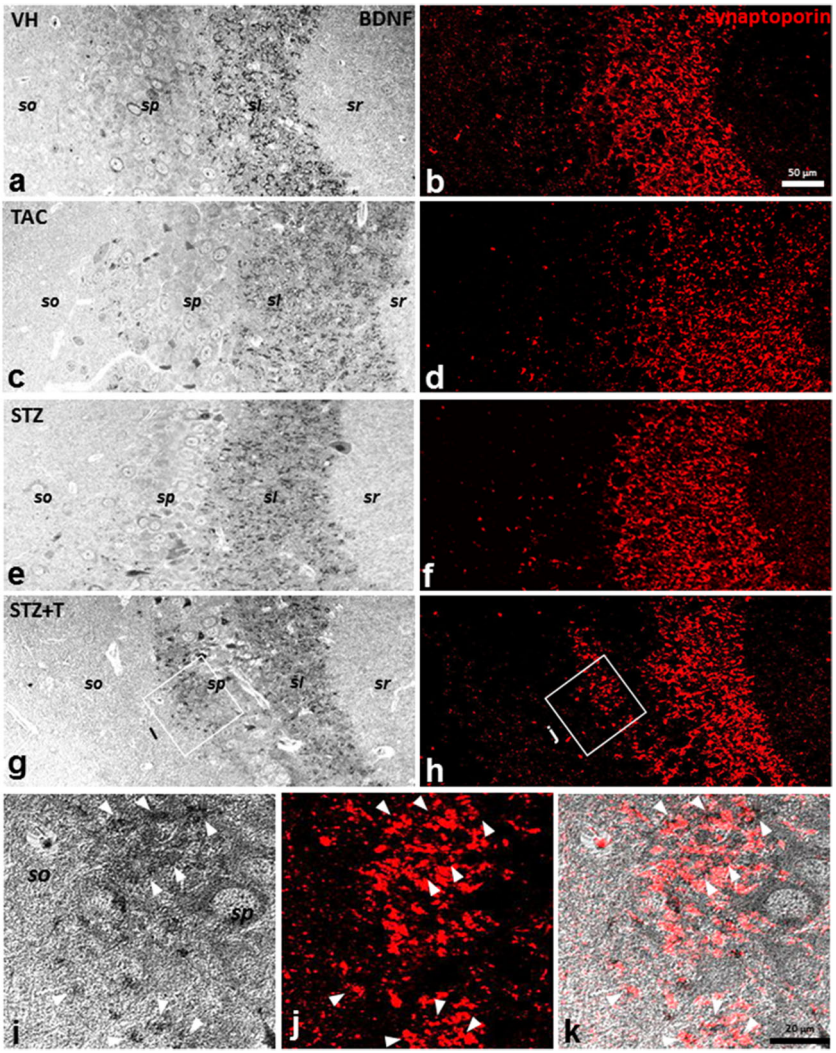

Fig. 5 BDNF immunoreactivity was observed in the mossy fiber sproutings in the CA3 region. **a**–**f** Double labeling for BDNF and synaptoporin, a specific marker for mossy fibers, showing that the majority of BDNF-labeled profiles were along the synaptoporin-positive mossy fiber tract of the CA3. **g**–**i** High-magnification images of the boxed areas in **e**–**f**, respectively. Arrowheads denote the BDNF-labeled mossy fiber sprouting in the stratum oriens of the CA3 region in the STZ + TAC group. *so*, stratum oriens; *sp*, stratum pyramidale; *sl*, stratum lucidum; *sr*, stratum radiatum. Scale bars = 50 μm for **a**–**f**; 20 μm for **g**–**i**

due to its diabetogenic potential. In addition, these processes may be affected by oxidative stress in TAC-induced pancreas dysfunction.

In the trisynaptic circuit, information from the DG is directed to the pyramidal cells of the CA3 through mossy fibers. The hippocampal mossy fiber pathway contains the highest levels of BDNF protein in the central nervous system (Conner et al. 1997; Yan et al. 1997), and BDNF has been shown to regulate synaptic efficacy (Korte et al. 1995, 1998; Patterson et al. 1996). Furthermore, BDNF immunoreactivity was found to be localized in mossy fiber giant boutons, and neuronal activity increased the pool of BDNF-expressing GC presynaptic terminals contacting CA3 pyramidal cells (Danzer and McNamara 2004). These findings, together with our observations, led us to speculate that BDNF plays a role in regulation of synaptic control via the perforate pathway and mossy fiber projections from the DG to the CA3 region for hippocampal circuits. Thus, this hippocampal synaptic regulation may be disturbed by BDNF reduction due to TAC treatment or a diabetic condition.

Interestingly, mossy fiber sprouting, demonstrated by prominent punctate immunolabeling of BDNF with synaptoporin, was observed in the STZ + TAC group and was localized at the *so* of the CA3 region. Mossy fiber sprouting is a common pathological hallmark in individuals with temporal lobe epilepsy (Mathern et al. 1995; Proper et al. 2000). Several experimental studies that mimic epilepsy in vivo and epileptiform seizures in vitro have replicated this mossy fiber sprouting (Buckmaster 2010). In addition, BDNF has been shown to be highly upregulated in some hyperactivity conditions such as temporal lobe epilepsy (Koyama et al. 2004; Scharfman et al. 2002), which may induce abnormalities in mossy fiber sprouting (Koyama and Ikegaya 2005). Considered together, our results suggest that TAC treatment may lead to irregular mossy fiber sprouting in the diabetic hippocampus via BDNF modulation.

In addition, TAC treatment resulted in the lowest locomotor activity, demonstrated by total distance moved, movement time, and velocity, in the diabetic rats compared with those of the other groups. This finding suggests that the highest values

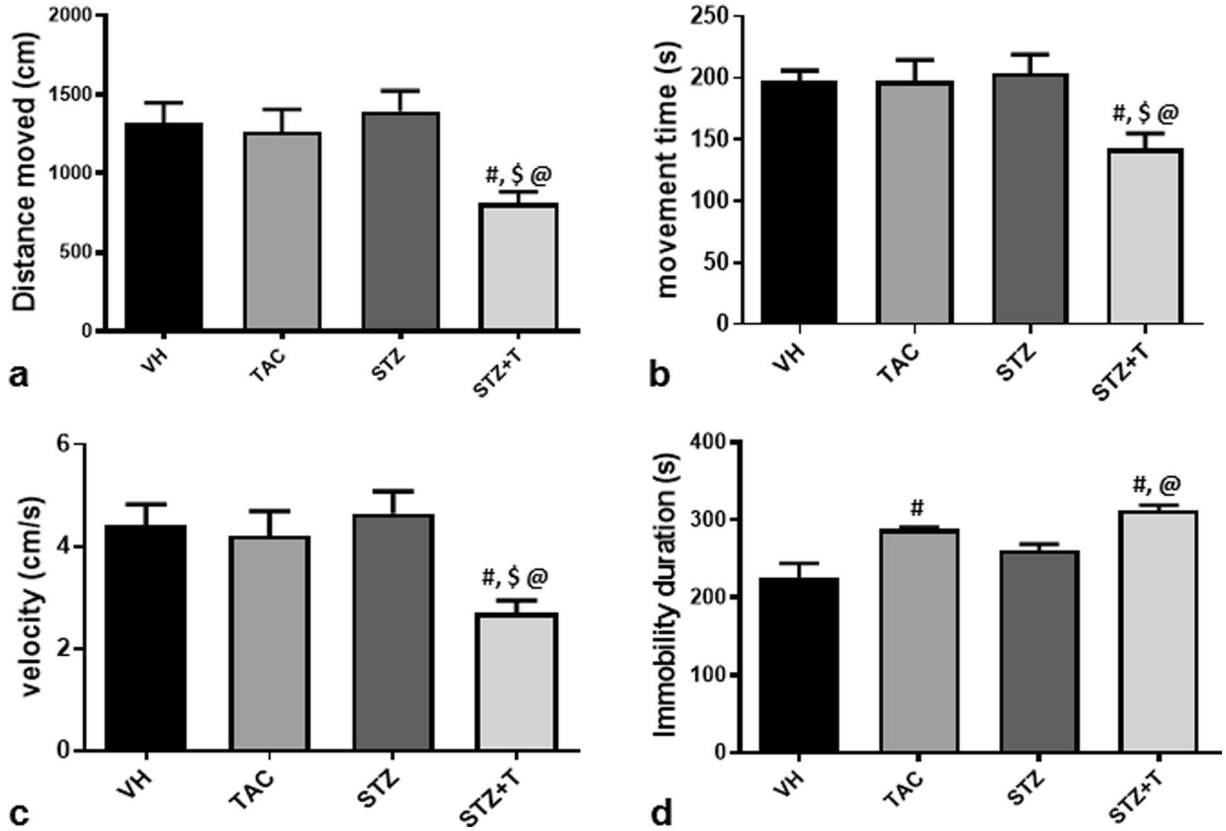

Fig. 6 Effects of TAC treatment on locomotor activity and depression level in diabetic rats. **a**–**c** Distance moved (cm), movement time (s), and velocity (cm/s) were examined for assessing locomotor activity using an open-field test. All parameters were significantly decreased in the STZ + TAC group compared with those of the other groups. **d** Immobility duration(s) was examined with respect to the depression level using a tail suspension test. The values shown are the mean ± SE ($n = 7$). $^{\#}P < 0.05$ vs. VH; $^{\$}P < 0.05$ vs. TAC; $^{@}P < 0.05$ vs. STZ

of all functional basic parameters in TAC-treated diabetic rats (Table 1) were associated with the negative effect of TAC on locomotion. Furthermore, the depressive-like behavior, demonstrated by increased immobility duration, was observed in the TAC and STZ + TAC groups. Several clinical studies demonstrated adverse neurologic effects of TAC, including depression after renal transplantation (Bechstein 2000; Cadavid-Aljure et al. 2012; Chegounchi et al. 2006; Kastrup et al. 2012, 2015; Mayer et al. 1997; Neylan et al. 1998; Veroux et al. 2002; Wu et al. 2013). Interestingly, the diabetic rats treated with TAC showed the lowest mRNA expression levels of GABA and serotonin (5-HT) receptor subunits. The level of the GABA neurotransmitter is decreased in the synaptic cleft in diabetic rats (Gomez et al. 2003) and in depressed patients (Sanacora et al. 1999), suggesting that a GABAergic imbalance is crucial to the etiology of depression (Brambilla et al. 2003; Petty 1995; Sanacora et al. 1999, 2002). In addition, the mRNA levels of 5-HT_{1A} receptors were reported to be decreased in the hippocampus of depression further suggesting an essential role in the regulation of depressive-like behaviors (Lopez et al. 1998).

There are several reports showing that BDNF is directly involved in the pathophysiology of depression, and that its restoration may underlie the therapeutic efficacy of antidepressant treatment. Reduced BDNF levels in the prefrontal cortex and hippocampus were found in post-mortem tissues of patients with major depressive disorder (MDD) (Dwivedi et al. 2003), whereas MDD patients medicated with antidepressants showed elevated BDNF levels in the hippocampus post-mortem. Similarly, the serum BDNF was found to be decreased in depressed patients and elevated following medication (Karege et al. 2005; Sen et al. 2008; Shimizu et al. 2003). In experimental studies, the BDNF level decreased in the rodent hippocampus following exposure to various stressors (Altar et al. 2003; Barrientos et al. 2003; Gervasoni et al. 2005; Nibuya et al. 1995; Pizarro et al. 2004; Roceri et al. 2004; Smith et al. 1995; Tsankova et al. 2006; Ueyama et al. 1997; Vaidya et al. 1997) and increased following antidepressant treatment (Altar et al. 2004; Coppell et al. 2003; Dias et al. 2003; Hoshaw et al. 2005; Muller et al. 2000; Neeper et al. 1996; Russo-Neustadt et al. 1999; Van Hoomissen et al. 2003; Xu et al. 2003). These findings, together with our observations, led us to speculate that TAC treatment may induce a depressive-like behavior in diabetic rats, and that these processes may be affected by BDNF reduction in the TAC-treated diabetic hippocampus.

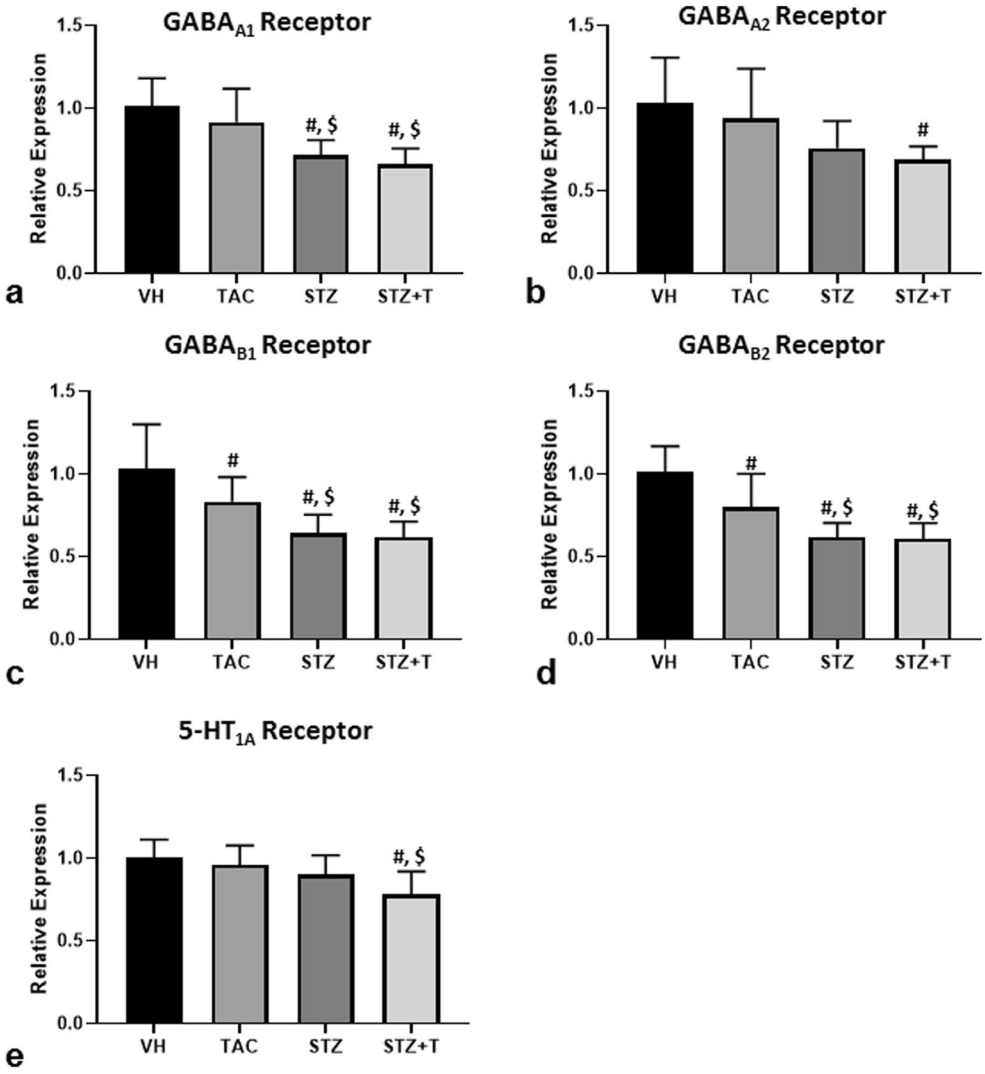

Fig. 7 Quantitative analysis of the mRNA expression of GABA receptor and 5-HT receptor subunits in the TAC-treated diabetic hippocampus. GABA receptors (**a**–**d**) and 5-HT receptor (**e**) subunits were detected by qPCR in the hippocampus. Relative mRNA expression levels were calculated after normalization to *Gapdh* expression. The data are presented as the mean ± SE ($n = 4$). $^{\#}P < 0.05$ vs. VH; $^{\$}P < 0.05$ vs. TAC

Several studies have reported that TAC showed a narrow therapeutic index: underdosing is associated with an increased risk of rejection episodes, while overdosing may exacerbate drug-related toxicity, leading to side effects, including neurotoxicity (Dhar and Human 2011). Because calcineurin is abundant in the brain, its inhibition could form the basis for the pathophysiology of the neurotoxicity observed in patients on calcineurin inhibitor therapy. In addition, neurotrophin-dependent elongation of neuronal processes requires an intact calcineurin/nuclear factor of activated T-cells (NFAT) pathway (Graef et al. 2003), and the neuronal growth effects of BDNF are regulated via a calcineurin-dependent pathway (Rauskolb et al. 2010). It has also been reported that TAC-induced encephalopathy is related to its direct cytotoxic effect on brain capillary endothelial cells and an enhancement of permeability across the blood–brain barrier (Kochi et al. 1999).

The main limitation of our study is that we did not evaluate the therapeutic effects of BDNF on the hippocampal function in TAC-treated diabetic rats. Therefore, further evaluation is required to determine the therapeutic efficacy of BDNF in the TAC-induced pathophysiological effects on emotional behaviors.

In conclusion, our data demonstrate that BDNF may contribute to neuronal circuitry in the hippocampus via its regulation of pathophysiological status; i.e., TAC treatment in the diabetic rat brain. These findings suggest that TAC treatment may cause further psychiatric and neurological complications in patients with diabetes.

Authors' Contributions Y-J Shin, Y-T Chun, S-W Lim, and C-W Yang: designed the research and wrote the report; K Luo, Y Quan, and C Sheng: conducted the animal experiments; E-J Ko, J Lee, and S Hong: performed the histological experiments; Y-J Shin, Y-T Chun, B-H Chung, M-Y Lee, H-G Kang, and C-W Yang.: analyzed the data and edited the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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