RESEARCH ARTICLE

Structural and functional damage to the hippocampal neurovascular unit in diabetes-related depression

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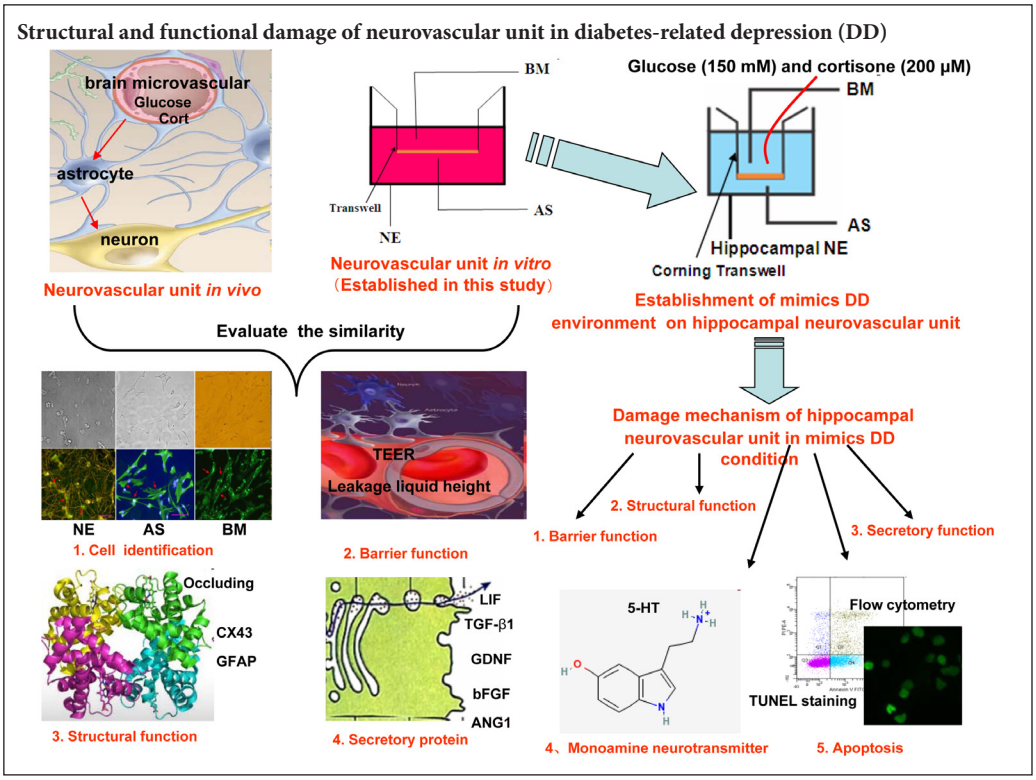
Graphical Abstract



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Abstract

Previous studies have shown that models of depression exhibit structural and functional changes to the neurovascular unit. Thus, we hypothesized that diabetes-related depression might be associated with damage to the hippocampal neurovascular unit. To test this hypothesis, neurons, astrocytes and endothelial cells were isolated from the brain tissues of rat embryos and newborn rats. Hippocampal neurovascular unit co-cultures were produced using the Transwell chamber co-culture system. A model of diabetes-related depression was generated by adding 150 mM glucose and 200 μM corticosterone to the culture system and compared with the neuron + astrocyte and astrocyte + endothelial cell co-culture systems. Western blot assay was used to measure levels of structural proteins in the hippocampal neurovascular unit co-culture system. Levels of basic fibroblast growth factor, angiogenic factor 1, glial cell line–derived neurotrophic factor, transforming growth factor β1, leukemia inhibitory factor and 5-hydroxytryptamine in the hippocampal neurovascular unit co-culture system were measured by enzyme-linked immunosorbent assay. Flow cytometry and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling staining was used to assess neuronal apoptosis in the hippocampal neurovascular unit. The neurovascular unit triple cell co-culture system had better barrier function and higher levels of structural and secretory proteins than the double cell co-culture systems. In comparison, in the model of diabetes-related depression, the neurovascular unit was damaged with decreased barrier function, poor structural integrity and impaired secretory function. Moreover, neuronal apoptosis was markedly increased, and 5-hydroxytryptamine levels were reduced. These results suggest that diabetes-related depression is associated with structural and functional damage to the neurovascular unit. Our findings provide a foundation for further studies on the pathogenesis of diabetes-related depression.

Key Words: *nerve regeneration; hippocampus; neurovascular unit; neurons; astrocytes; brain microvascular cells; cell culture; co-culture; diabetes-related depression; hyperglycemia; corticosterone; neural regeneration*

Chinese Library Classification No. *R459.9; R364; Q2*

Introduction

According to the world health organization, approximately 422 million people worldwide suffer from diabetes mellitus. Diabetes mellitus is a chronic metabolic disease with numerous complications. People with diabetes mellitus have a high risk of mental disorder and a 15.3–36% risk of depressive symptoms (Islam et al., 2015), which is higher than in non-diabetics. Moreover, diabetics have 31% of the symptoms associated with clinical depression (Roy and Lloyd, 2012) and are two to three times more likely to have depression. The morbidity was higher in females, with 11% of patients showing severe depressive symptoms. Its recurrence rate is eight times that of non-diabetic patients (Pouwer, 2009). Furthermore, the quality of life of people with diabetes-related depression (DD) is seriously affected, and it also imparts a great economic and social burden.

The pathogenesis of DD is complex, but most studies have focused on the hypothalamic-pituitary-adrenal axis (Zahn et al., 2015). Furthermore, research on DD has generally been limited to clinical studies. In our previous studies, the expression of pro-apoptotic proteins, including Bax, Caspase-8 and c-Fos, in the hippocampus of rats with DD were dramatically increased, while levels of the anti-apoptotic protein Bcl-2 were decreased (Wang et al., 2015a; Du et al., 2016). These findings indicate that apoptosis of hippocampal neurons may be an important pathogenetic event in DD. Moreover, our previous study showed that functional proteins in the blood-brain barrier, AQP4 and CX43, were decreased in the hippocampus of rats with DD and that the expression levels of brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) were decreased as well. Furthermore, we observed dysregulated expression of excitatory amino acid transporter 3 and vesicular glutamate transporter 2, as well as immune system hyperactivation (Wang et al., 2015b). These observations suggest that structural and functional damage to microvascular endothelial cells and astrocytes causes neuronal apoptosis, which may be an important pathogenetic event in DD.

Iadecola (2004) proposed the concept of the neurovascular unit (NVU), which refers to a unit consisting of neurons, neurogliocytes and brain microvascular endothelial cells with extracellular matrix. The NVU plays an important role in maintaining the brain microenvironment and in signal transduction, ion transport and metabolic regulation (Han and Suk, 2005; Shi et al., 2017). The majority of studies on the NVU have used *in vivo* models (Friedrichs et al., 2017; Hosoo et al., 2017), and have focused on neurogenesis and the blood-brain barrier (Pan et al., 2015). There are very few *in vitro* studies (Xue et al., 2013; Khilazheva et al., 2015). Furthermore, most studies on the NVU have focused on cerebral cortical neurons rather than hippocampal neuron, although the latter play a critical role in emotional regulation, and learning and memory.

We hypothesized that structural and functional damage to the NVU may be involved in the pathogenesis of DD. We used an *in vitro* Transwell chamber co-culture system to examine the effect of simulated DD conditions on the structure and function of the hippocampal NVU.

Materials and Methods

Animals

Specific-pathogen-free Sprague-Dawley rats (E16–18 embryos from five pregnant females, six 2–3-day-old male rats and five 10-day-old male rats) were provided by Hunan Slac Jingda Laboratory Animal Co., Ltd., China (license No. SCXK 2016 0002). All rats were housed at 26 ± 3°C under a 12-hour light/dark cycle, with lights on at 6:00 a.m., and allowed free access to food and water. All experiments were approved by the Laboratory Animal Ethics Committee at the Hunan University of Chinese Medicine (approval number: HN-LL-KY-2016-004-01).

Establishment of the hippocampal NVU triple cell co-culture system

Separation and cultivation of neurons

E18 pregnant rats were anesthetized with 4 mL/kg 10% chloral hydrate before removal of the embryos. The hippocampus was carefully excised from the brain and cut into pieces. The tissue was then digested with 0.25% trypsin and 0.2% collagenase at 37°C for 15 minutes. The digestion was terminated by adding Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin. After centrifugation at 250 × *g* for 5 minutes, cells were collected, resuspended and filtered through a 200-mesh sieve. The cells were then resuspended in DMEM/F12 containing 10% fetal bovine serum, 1% L-glutamine, 1% B27 (Gibco) and 1% penicillin/streptomycin. The cells density was adjusted to 3.0×10^5/mL and seeded on cell culture plates pre-coated with poly-L-lysine. After 4 hours of incubation at 37°C, 5% CO_2, the medium was replaced by maintenance medium, containing 96% Neurobasal medium (Gibco), 2% B27, 1% glutamine and 1% penicillin/streptomycin. Half of the culture medium was replaced with fresh medium every 3 days.

Separation and cultivation of astrocytes

Astrocytes were obtained from the brains of newborn Sprague-Dawley rats aged 2–3 days. After the pups were sterilized with 75% alcohol, the meninges and blood vessels were carefully removed. The cerebral cortices were cut into pieces and digested in 0.25% trypsin and 0.2% collagenase at 37°C for 20 minutes. The digestion was terminated by adding DMEM/F12 medium containing 15% fetal bovine serum. The cell suspension was then centrifuged at 250 × *g* for 5 minutes, and the supernatant was discarded. The cells were then resuspended in DMEM/F12 containing 20% FBS, and filtered through the 200-mesh sieve. Cell density was adjusted to 3×10^6/mL. Afterwards, cells were seeded on a 25 cm^2 cell culture flask pre-coated with poly-L-lysine and cultured at 37°C, 5% CO_2, for 1 hour for differential adhesion. The cells were then transferred to another flask. Half of the culture medium was replaced every 3 days with fresh medium until confluent. Differential adhesion/agitation was used to purify the cells.

Separation and cultivation of brain microvascular endothelial cells (BMECs)

Brain endothelial cells were obtained from the brains of newborn Sprague-Dawley rats aged 10 days. After the rats were sterilized with 75% alcohol, the brains were carefully removed and placed in a petri dish with D-Hank's medium. White matter, residual vessels and pia matter were removed under a stereomicroscope (Leica, Wetzlar, Germany). Subsequently, the cerebral cortex was cut into pieces and rinsed in D-Hank's medium. The cell suspension was then centrifuged at 250 × *g* for 3 minutes, the supernatant was discarded, and a 1:1 ratio of 25% FBS was added. A 1:2 volume of 0.1% collagenase II was added and incubated for 40 minutes. The cell density was adjusted to 1×10^4 cells/mL and cultured at 37°C, 5% CO_2. The medium was changed after 24 hours. Afterwards, half of the culture medium was replaced every 3 days until confluence.

Generation of the NVU co-culture system and cell identification

Briefly, as shown in **Figure 1**, neurons were seeded into a 24-well culture plate and cultured for 5–7 days. According to a previous study (Xue et al., 2013), the density of purified astrocytes was adjusted to 2.5×10^5/mL, and 100 μL of the cell suspension was seeded in the outer side of the matching well inserts. After 45 minutes, the unattached astrocytes were washed away with phosphate buffered saline (PBS), and the well inserts were placed into the wells of a 24-well dish, where neurons were cultured for 5–7 days with 1 mL astrocyte culture medium. After 2 days of co-culture, the density of brain microvascular endothelial cells was adjusted to 1.5×10^5/mL, and 150 μL of the cell suspension was seeded in the inner side of the well inserts with brain microvascular endothelial cell culture medium. The medium was changed every 3 days until confluence. The NVU, primarily consisting of neurons, astrocytes and brain microvascular endothelial cells, was successfully established after 3 days of co-culture.

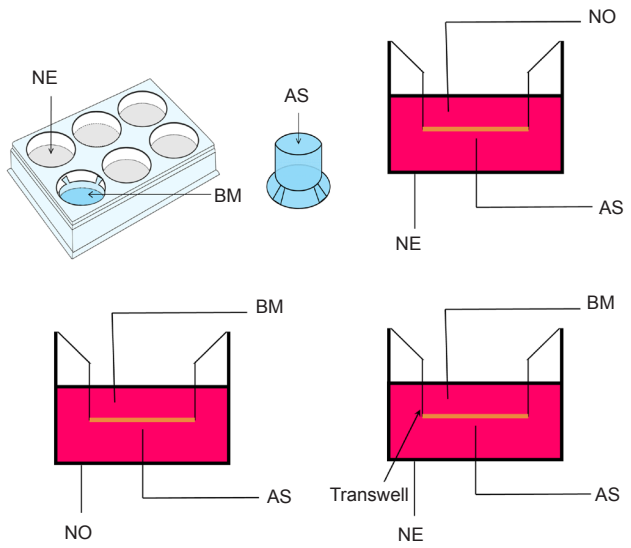

Figure 1 Diagrams of the *in vitro* NVU system and the double cell co-culture systems.

Astrocytes were seeded in the outer side of the matching Transwell inserts after neurons were seeded into a 24-well culture plate and cultured for 5–7 days. After 2 days of co-culture, endothelial cells were seeded in the inner side of the matching well inserts. The NVU triple cell system and the double cell co-culture systems were successfully established after 3 days of culture. NE: Neuron; AS: astrocyte; BM: brain microvascular endothelial cell; NO: no cell; NVU: neurovascular unit.

The medium inside the transwell was removed before the cells were washed with 0.01 M PBS, three times, and fixed with 4% paraformaldehyde for 30 minutes. Neurons were treated with 0.25% Triton X-100 for 15 minutes, blocked in 5% bovine serum albumin for 30 minutes, and incubated with rabbit anti-neuron specific enolase (NSE) (1:100; Boster, Wuhan, China) and mouse anti-β-tubulin (1:100; Proteintech, Chicago, IL, USA) antibodies at 4°C overnight. The cells were then incubated with anti-rabbit FITC (1:200; Proteintech) and anti-mouse RP-E (1:200, Proteintech) secondary antibodies at 37°C for 30 minutes. The cells were then stained with DAPI (Abcam, Cambridge, UK) and analyzed with a high content analysis system (PerkinElmer, Boston, MA, USA). Astrocytes and brain microvascular endothelial cells were labeled with rabbit anti-glial fibrillary acidic protein (GFAP) (1:100; Boster) and rabbit anti-PECAM-1/CD31 (1:100; Boster) antibodies.

Neurons and astrocytes were separately seeded in a 96-well culture plate (PerkinElmer) until 60–80% confluency, and then washed with 0.01 M PBS and fixed with 4% paraformaldehyde for 30 minutes. Afterwards, the cells were incubated in 0.25% Triton X-100 for 15 minutes and blocked in 5% bovine serum albumin for 30 minutes. The neurons were incubated with rabbit anti-NSE (Boster) and mouse anti-β-tubulin (Proteintech) antibodies, while the astrocytes were incubated with rabbit anti-GFAP (Boster) antibody at 4°C overnight. After washing with 0.01 M PBS, the cells were incubated with anti-rabbit FITC-conjugated secondary antibodies (Proteintech) at 37°C for 30 minutes. Nuclei were stained with DAPI for 20 minutes. Images were captured with a high content analysis system and an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Modeling of hippocampal NVU under DD conditions and pharmacological intervention in vitro

Double cell co-culture systems, including neurons + astrocytes and astrocytes + brain microvascular endothelial cells, were set up and used as controls (**Figure 1**). DD conditions were simulated according to a previous study (Liu et al., 2016). Briefly, 150 mM glucose and 200 μM corticosterone were added to the insert wells for 18 hours. Metformin, 20 mM, and 10 μM fluoxetine were added to the transwell to assess apoptosis and 5-hydroxytryptamine (5-HT) levels in neurons in the NVU triple cell co-culture system.

Simulation of hippocampal DD-induced NVU damage *in vitro*

Determination of hippocampal NVU barrier function

Transendothelial electrical resistance (TEER) was measured to determine barrier function in the hippocampus using a Millipore resistor (Millipore, Billerica, MA, USA). TEER

in insert wells was measured three times, and the average was calculated as follows: TEER = (TEER sample − TEER blank)Ω × effective area of the membrane. Culture medium was added inside the transwell, and the liquid level outside the transwell was adjusted lower than the inside by 0.5 cm. After 4 hours of incubation at 37°C, 5% CO_2, the difference between the inner and outer levels was measured using a vernier caliper (CHIIB, Shanghai, China).

Detection of hippocampal NVU structural proteins

Astrocytes and brain microvascular endothelial cells were collected after mimicking DD conditions, and radioimmunoprecipitation assay buffer containing phenylmethyl sulfonylfluoride was added to extract total protein. Proteins were then electrophoretically resolved on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The blots were blocked with skimmed milk and incubated with rabbit anti-occludin (1:1000; Cell Signaling Technology, Boston, MA, USA), rabbit anti-GFAP (1:1000; Cell Signaling Technology), rabbit anti-CX43 (1:1000; Cell Signaling Technology) or rabbit anti-GAPDH (1:1000; Cell Signaling Technology) antibody overnight at 4°C. The blots were washed twice with Tris-buffered saline containing Tween 20, and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Boster). Finally, the signals were visualized with an enhanced chemiluminescence kit (Amersham, Shanghai, China).

Detection of NVU secreted proteins

The cell culture supernatant from the triple cell co-culture system was collected after simulated DD. The levels of leukemia inhibitory factor (LIF) in brain microvascular endothelial cells, basic fibroblast growth factor (bFGF), angiogenesis factor-1 (ANG-1), GDNF and transforming growth factor-β1 (TGF-β1) in astrocytes were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN, USA).

Detection of 5-HT content in the hippocampal NVU

Neurons and the culture medium were collected separately. Cells were resuspended in PBS and centrifuged at 3000 r/min for 15 minutes. The supernatant was collected, and 5-HT levels in the supernatant and culture medium were measured by ELISA (R&D).

Detection of apoptosis in the hippocampal NVU

After simulated DD, cells were digested in 0.25% trypsin for 2 minutes, followed by centrifugation at 250 × *g* for 5 minutes. The cells were resuspended in 100 μL 1× Annexin binding buffer and incubated with 5 μL propidium iodide and 5 μL FITC-Annexin V (BD, Franklin, NJ, USA). After incubation at room temperature in the dark for 20 minutes, 400 μL Annexin binding buffer was added, and fluorescence intensities of propidium iodide and Annexin V were detected by flow cytometry (BD).

After simulated DD, the transwell was removed, and the medium was discarded. Cells were fixed with 4% paraformaldehyde and treated with 0.2% Triton X-100 for 5 minutes and 0.3% H_2O_2 for 2 minutes. Afterwards, cells were incubated in 50 μL terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction mix (Roche, Basel, Switzerland) for 60 minutes at 37°C in the dark. Fluorescence microscopy (Olympus) was used to observe apoptosis.

Statistical analysis

The experimental data, expressed as the mean ± SD, were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and analyzed by one-way analysis of variance, followed by Dunnett's *post hoc* test. Values of $P < 0.05$ and $P < 0.01$ were considered statistically significant.

Results

Cell morphological characteristics and immunofluorescence of the hippocampal NVU

The morphological characteristics and immunofluorescence identification of neurons, astrocytes and brain microvascular endothelial cells in the hippocampal NVU co-culture system were assessed by optical microscopy and high content analysis/microscopy, respectively. As shown in **Figure 2A**, cell somas of neurons were clear with crisscrossing dendrites and a rich neural network. The refractivity of astrocytes was low, with slender processes. Brain microvascular endothelial cells exhibited a typical cobblestone organization. Further-

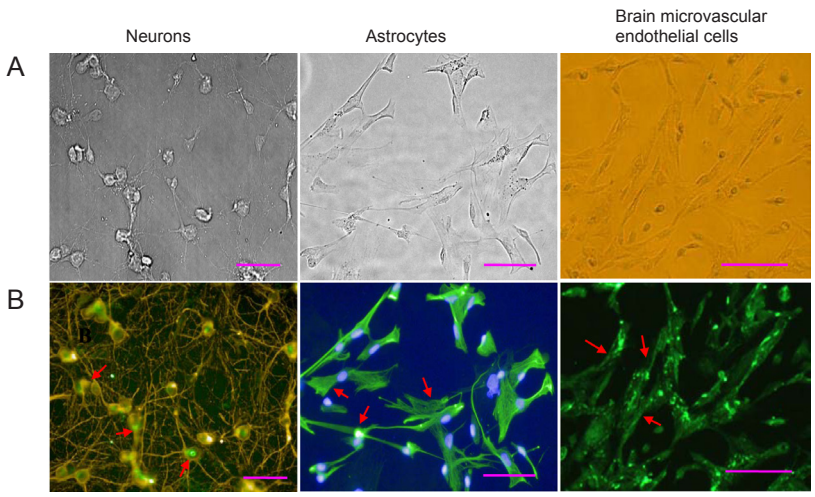

Figure 2 Morphological and immunocytochemical characteristics of the hippocampal NVU.
(A) Hippocampal neurons, astrocytes and microvascular endothelial cells under an inverted light microscope. (B) Neurons are positive for NSE (arrows), astrocytes express GFAP (arrows), and brain microvascular endothelial cells are positive for CD31 (arrows). Scale bars: 100 μm. NVU: Neurovascular unit; NSE: neuron-specific enolase; GFAP: glial fibrillary acidic protein.

more, the cytoplasms of neurons, astrocytes and brain microvascular endothelial cells were positive for NSE, GFAP and PECAM-1/CD31, respectively, as shown in **Figure 2B**.

Effect of DD on cerebral vascular endothelial cells in the hippocampal NVU co-culture system

Structural protein expression in brain microvascular endothelial cells decreases after DD

As shown in **Figure 3**, occludin protein expression in the NVU was decreased after DD compared with the control ($P < 0.01$). However, expression was higher in the NVU group compared with the astrocytes + brain microvascular endothelial cells group after exposure to hyperglycemia and corticosterone. Thus, occludin, the structural protein of brain microvascular endothelial cells was reduced in the co-culture systems after simulated DD. The expression of the structural protein was greater in the NVU triple cell co-culture system than in the astrocytes + brain microvascular endothelial cells group ($P < 0.05$).

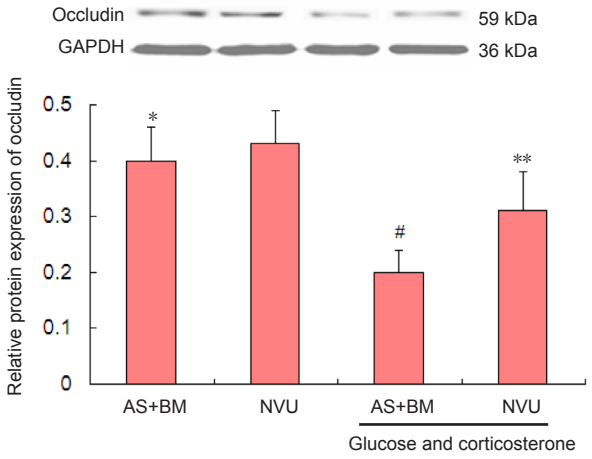

Figure 3 Expression of occludin protein in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), assessed by western blot assay.

Results are shown as the mean ± SD ($n = 3$; one-way analysis of variance followed by Dunnett's *post hoc* test). *$P < 0.05$, **$P < 0.01$, *vs.* NVU group; #$P < 0.05$, *vs.* NVU model group (NVU + glucose + corticosterone). Western blot assay was performed in triplicate. GAPDH was used as loading control. The numbers on the right are the molecular weights of the proteins (kDa). AS: Astrocyte; BM: brain microvascular endothelial cell; NVU: neurovascular unit; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Barrier function in the NVU is impaired by DD conditions and strongly associated with damage to brain microvascular endothelial cells

The barrier function of the NVU was assessed by TEER and permeability assays. As shown in **Figure 4A**, the TEER was reduced in the astrocytes + brain microvascular endothelial cells group, as well as in the NVU triple cell co-culture group after DD. However, the difference between neurons + astrocytes and neurons + astrocytes exposed to hyperglycemia and corticosterone was not clear. Nonetheless, we can conclude that simulated DD conditions negatively impact barrier function of the NVU and that this effect is strongly associated with damage to brain microvascular endothelial cells. Furthermore, the permeability within 4 hours was increased in the groups exposed to DD conditions compared with the corresponding control groups (**Figure 4B**). These findings suggest that hyperglycemia and corticosterone significantly impair NVU barrier function.

Secretory protein expression in brain microvascular endothelial cells declines after DD

As shown in **Table 1**, expression of the secretory protein LIF in brain microvascular endothelial cells was decreased in the astrocytes + brain microvascular endothelial cells group and NVU group after DD, compared with the corresponding controls ($P < 0.01$). Moreover, LIF expression was lowest in the astrocytes + brain microvascular endothelial cells group exposed to hyperglycemia and corticosterone ($P < 0.01$). Therefore, the secretory function of brain microvascular endothelial cells is reduced by simulated DD. Secretory function was better in the NVU co-culture system compared with the astrocytes + brain microvascular endothelial cells system.

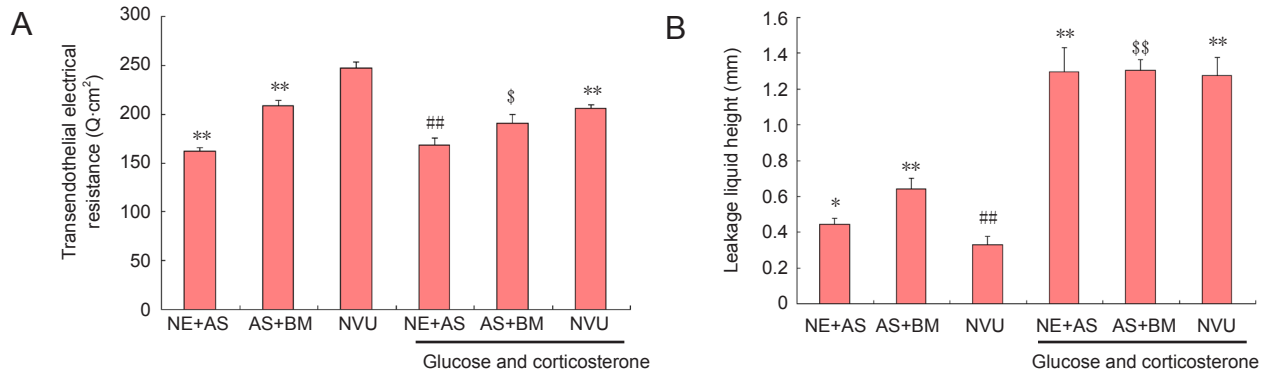

Figure 4 Transendothelial electrical resistance (A) and leakage liquid height (B) in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), as detected by Millipore resistor and vernier caliper.

Quantitative results are shown as the mean ± SD ($n = 3$; one-way analysis of variance followed by Dunnett's *post hoc* test). *$P < 0.05$, **$P < 0.01$, *vs.* NVU group; ##$P < 0.01$, *vs.* NVU model group (NVU + glucose + corticosterone); $$P < 0.05$, $$$P < 0.01$, *vs.* AS + BM group. Experiments were performed in triplicate. NE: Neuron; AS: astrocyte; BM: brain microvascular endothelial cell; NVU: neurovascular unit; TEER: transendothelial electrical resistance.

Effect of DD on astrocytes in the NVU co-culture system

Astrocyte structural proteins CX43 and GFAP are downregulated by DD

As shown in **Figure 5**, the expression of the structural proteins CX43 and GFAP were reduced after exposure to DD conditions compared with the corresponding control group ($P < 0.01$ or $P < 0.05$). CX43 and GFAP expression levels were markedly decreased in the neurons + astrocytes and astrocytes + brain microvascular endothelial cells groups after exposure to the DD conditions. The resistance to damage was greater in the NVU triple cell co-culture system than in the other double cell co-culture systems ($P < 0.05$).

Secretory protein expression in astrocytes decreases after DD

Table 2 shows levels of various secretory proteins, including TGF-β1, GDNF, bFGF and ANG1 in astrocytes. Secretory protein levels in astrocytes were downregulated by DD compared with the corresponding controls. The expression levels of these proteins were highest in the NVU group compared with the other double co-culture systems after DD. These findings suggest that the secretory function of astrocytes is impaired by DD. Secretory function in the NVU system was better than in the other co-culture systems (neurons + astrocytes, astrocytes + brain microvascular endothelial cells).

Table 1 Levels of LIF, a BM secretory protein, in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM)

Group	LIF (pg/mL)
AS+BM	86.96±4.34**
NVU	97.99±10.44
AS+BM+G&P	63.76±7.48##$$
NVU+G&P	85.24±4.68**

Data are expressed as the mean ± SD (one-way analysis of variance followed by Dunnett's *post hoc* test). **$P < 0.01$, *vs.* NVU group; ##$P < 0.01$, *vs.* NVU model group (NVU + G & P, NVU + glucose + corticosterone, AS + BM + G & P); $$$P < 0.01$, *vs.* AS + BM group. AS: Astrocyte; BM: brain microvascular endothelial cell; NVU: neurovascular unit; G & P: glucose (150 mM) and corticosterone (200 μM); LIF: leukemia inhibitory factor.

Impact of DD on hippocampal neurons in the NVU co-culture system

Levels of 5-HT in the hippocampus are reduced after DD

Figure 6 shows the levels of 5-HT in the supernatant of the NVU co-culture system and in neurons after DD. The levels of 5-HT in the neurons + astrocytes co-culture system was decreased after DD exposure compared with the corresponding control. Compared with the simulated DD conditions group, the levels of 5-HT in both neurons and in the supernatant of the triple cell co-culture system were higher in the non-DD groups. The levels of 5-HT in the supernatant and cells were increased in the NVU group treated with metformin and fluoxetine. This suggests that metformin and fluoxetine enhance 5-HT expression. Our findings are in agreement with previous studies showing that monoamine neurotransmitter levels are diminished in depression.

DD strongly promotes neuronal apoptosis in the hippocampal NVU

Apoptosis was detected by flow cytometry and TUNEL assay after DD and treatment with fluoxetine and metformin. As shown in **Figure 7**, apoptosis in neurons co-cultured with

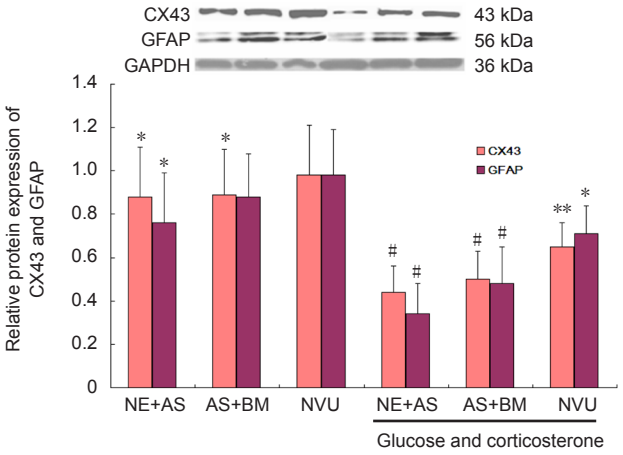

Figure 5 Expression of CX43 and GFAP proteins in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), as detected by western blot assay.

Results are shown as the mean ± SD ($n = 3$; one-way analysis of variance followed by Dunnett's *post hoc* test). *$P < 0.05$, **$P < 0.01$, *vs.* NVU group; #$P < 0.05$, *vs.* NVU model group (NVU + glucose + corticosterone). Western blot assay was performed in triplicate. GAPDH was used as loading control. Numbers on the right indicate the molecular weights of the proteins (kDa). NE: Neuron; AS: astrocyte; BM: brain microvascular endothelial cell; NVU: neurovascular unit; CX43: connexin 43; GFAP: glial fibrillary acidic protein.

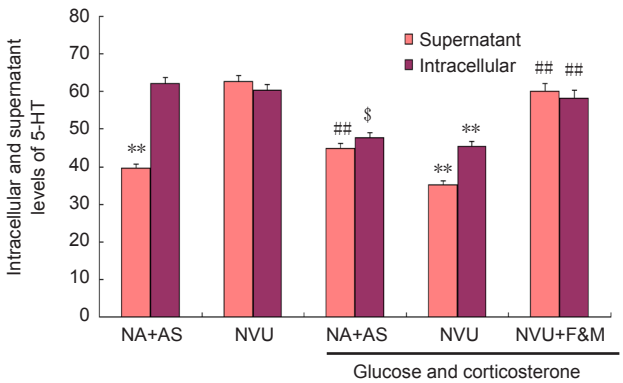

Figure 6 Intracellular and supernatant levels of 5-HT in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), as measured by enzyme-linked immunosorbent assay.

Results are shown as the mean ± SD ($n = 3$; one-way analysis of variance followed by Dunnett's *post hoc* test). **$P < 0.01$, *vs.* NVU group; ##$P < 0.01$, *vs.* NVU model group (NVU + glucose + corticosterone); $$P < 0.05$, *vs.* AS + BM group. Experiments were performed in triplicate. NE: Neuron; AS: astrocyte; BM: brain microvascular endothelial cell; NVU: neurovascular unit; F&M: fluoxetine and metformin; 5-HT: 5-hydroxytryptamine.

Table 2 Protein levels (pg/mL) of TGF-β1, GDNF, bFGF and ANG1 (astrocyte secretory proteins) in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), as detected by enzyme-linked immunosorbent assay

Group	TGF-β1	bFGF	GDNF	ANG1
NE+AS	5.24±2.25*	9.25±0.44*	13.74±0.68*	19.50±2.57*
AS+BM	7.18±2.69*	11.14±2.97*	13.68±1.01*	10.97±4.13*
NVU	13.07±2.10	16.72±2.59	25.74±1.50	27.00±2.13
NE+AS+G&P	4.58±1.43#	6.43±1.32	12.50±2.04	11.32±2.13
AS+BM+G&P	5.19±3.20#	9.53±0.75	12.84±0.42	14.38±4.69#
NVU+G&P	7.62±1.71*	9.31±2.88**	13.57±4.86*	7.57±2.67**

*$P < 0.05$, **$P < 0.01$, *vs.* NVU group; #$P < 0.05$, *vs.* NVU model group (NVU + G & P, NVU + glucose + corticosterone). NE: Neuron; AS: astrocyte; BM: brain microvascular endothelial cell; NVU: neurovascular unit; G & P: glucose and corticosterone; TGF-β1: tumor growth factor beta 1; GDNF: glial cell line-derived neurotrophic factor; bFGF: basic fibroblast growth factor; ANG1: angiogenesis factor-1.

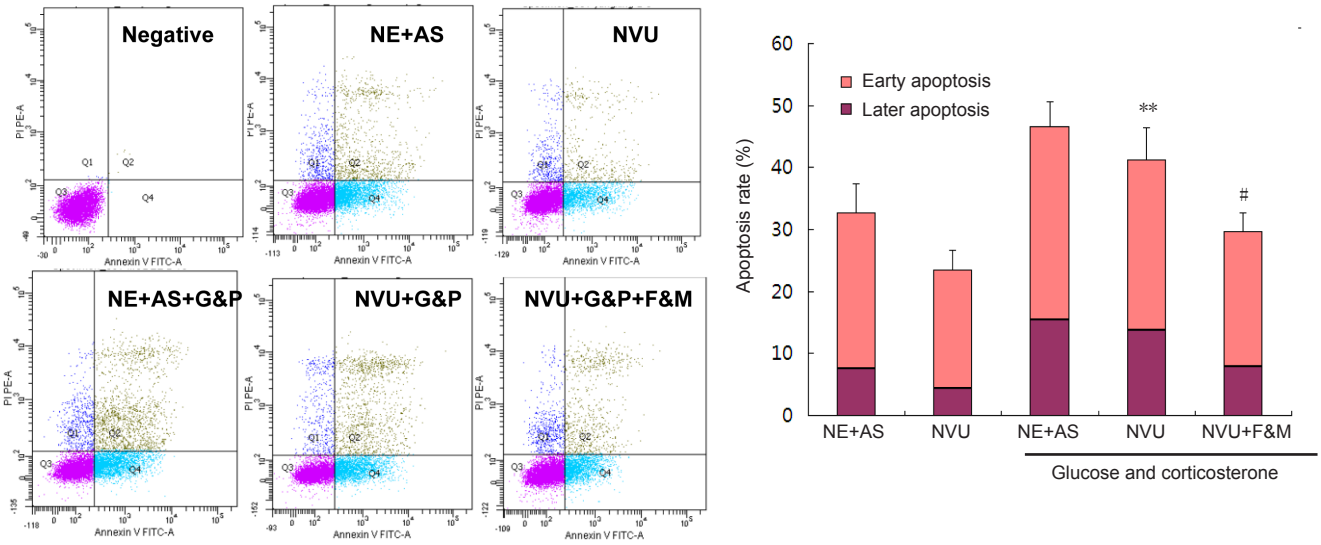

Figure 7 Apoptosis in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), as detected by flow cytometry.
Results are shown as the mean ± SD ($n = 3$; one-way analysis of variance followed by Dunnett's *post hoc* test). **$P < 0.01$, *vs.* NVU group; #$P < 0.05$, *vs.* NVU model group (NVU + G & P, NVU + glucose + corticosterone). All apoptotic cells were in the Q2 and Q4 quadrants. Early apoptotic cells were in Q4, while late apoptotic cells were in Q2. Experiments were performed in triplicate. NE: Neuron; AS: astrocyte; NVU: neurovascular unit; G&P: glucose and corticosterone; F&M: fluoxetine and metformin.

astrocytes in the triple cell co-culture system was increased after simulated DD compared with the corresponding controls. The apoptosis rate was decreased after treatment with fluoxetine and metformin. Furthermore, the apoptosis rate in neurons co-cultured with astrocytes was higher than in the triple cell co-culture system after simulated DD. Apoptosis was substantially reduced after treatment with fluoxetine and metformin (**Figure 8**). These findings suggest that DD aggravates apoptosis ($P < 0.01$) and that fluoxetine and metformin are anti-apoptotic in our system. Moreover, the apoptosis rate in the triple cell co-culture system was lower than in neurons co-cultured with astrocytes.

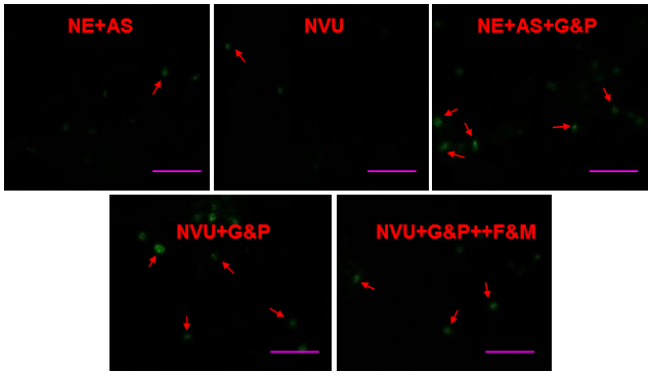

Figure 8 Apoptosis in the hippocampal NVU triple cell co-culture system and in the AS + BM co-culture system, with or without glucose (150 mM) and corticosterone (200 μM), as detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling staining.
Arrows point to apoptotic cells. NE: Neuron; AS: astrocyte; NVU: neurovascular unit; G & P: glucose and corticosterone; F & M: fluoxetine and metformin. Scale bars: 100 μm.

Discussion

In a seminal paper, Dalkara and Moskowilz emphasized that neurons, neurogliocytes and brain microvascular endothelial cells in the NVU were connected and influenced by each other, and play a critical role in cranial nerve vascular

system-related disease (Lo et al., 2003). However, previous studies were hampered by difficulties in establishing and evaluating the NVU system *in vitro*. Furthermore, there are only a few studies on the NVU in which hippocampal neurons were used. Thus, establishing an *in vitro* system that effectively mimics the *in vivo* hippocampal NVU is key to studying its role in diseases such as depression.

In this study, we successfully established a hippocampal *in vitro* NVU system consisting of a triple cell co-culture system (brain microvascular endothelial cells, astrocytes and neurons). The structure, function and cellular viability of this hippocampal NVU were assessed and compared with the two different double cell co-culture system (neurons + astrocytes, astrocytes + brain microvascular endothelial cells). Our major findings are as follows: (a) A relatively higher TEER and lower leakage liquid height indicate better barrier function in the hippocampal NVU system compared with the double cell co-culture system. Several studies have shown that TEER and leakage liquid height are important indicators of the integrity and permeability of the hippocampal NVU (Nakagawa et al., 2009; Xue et al., 2016). A higher TEER with low permeability usually occurs when cells form strong contacts with each other. (b) The expression of GFAP, CX43 and occludin were higher in the hippocampal NVU system compared with the double cell co-culture system. Occludin is a tight junction-associated protein in the blood-brain barrier, GFAP is a cytoskeletal protein in astrocytes, and CX43 is a component of the gap junctions between brain microvascular endothelial cells and astrocytes. Strong structural stability is usually accompanied with increased expression of occludin, CX43 and GFAP (Kurata et al., 2012; Salmina et al., 2014). (c) Expression of secretory proteins by astrocytes, including TGF-β1, GDNF, bFGF and ANG1, and expression of LIF by brain microvascular endothelial cells were increased in the hippocampal NVU system compared with the double cell co-culture system. Several studies have shown that the growth of cells is promoted by these secretory proteins (Levy et al., 2014; Seo et al., 2014) and that reduced levels of these secretory proteins impairs the normal functions of the NVU. (d) The proportion of apoptotic neurons in the hippocampal NVU system was lower than that in the double cell co-culture system. Neurons, astrocytes and brain microvascular endothelial cells in the hippocampal NVU depend on these factors for viability and to interact with each other. Our observations suggest that we successfully established the *in vitro* hippocampal NVU system and that it is similar to the *in vivo* NVU in terms of structure, function and viability.

DD is a major complication of diabetes mellitus and is associated with a high risk of suicide and relapse (Gemeay et al., 2015). However, the pathogenesis is not yet clear. Previous studies on DD have mainly focused on the hypothalamic-pituitary-adrenal axis (Zahn et al., 2015), especially the hippocampus. DD is a disease with a complex pathogenesis, involving perturbations in endocrine and neuronal function. A previous study showed that the permeability of the hippocampal blood-brain barrier was increased and that the structure and function of microvascular endothelial cells were impaired by exposure to high glucose levels (Aggarwal et al., 2015). Moreover, corticosterone in the plasma enters astrocytes after crossing the microvascular endothelium, and high levels affect the structure and function of astrocytes, as well as the function and viability of neurons after blood-brain barrier disruption. Additionally, the expression levels of excitatory amino acid transporter-1 and vesicular glutamate transporter in astrocytes are influenced by corticosterone binding to the glucocorticoid receptor on astrocytes (Cisneros and Ghorpade, 2012), while the uptake and concentration of glutamate in astrocytes are affected by perturbed expression of excitatory amino acid transporter-1 and vesicular glutamate transporter. Glutamate is upregulated in the intracellular space and causes abnormal activation of mGluR2/3 (Battaglia et al., 2009), which then leads to hippocampal neuronal damage and apoptosis through excitotoxicity. Based on these observations from previous studies, we hypothesized that the mechanisms underlying DD may be associated with damage to the NVU, involving morphological and functional damage to brain microvascular endothelial cells, astrocytes and neurons.

In the present study, compared with the normal NVU group, TEER in brain microvascular endothelial cells in the NVU groups was decreased, indicating increased permeability after DD, consistent with a previous study (Xue et al., 2013; Liu et al., 2016). Furthermore, expression levels of structural proteins in astrocytes, including GFAP and CX43 and of the brain microvascular endothelial cell protein occludin were downregulated after exposure to simulated DD conditions. Simultaneously, levels of the astrocyte secretory proteins TGF-β1, GDNF, bFGF and ANG1 and of the brain microvascular endothelial cell protein LIF were decreased. Moreover, the levels of secreted and intracellular 5-HT in neurons was reduced, and the number of apoptotic neurons was markedly increased. Previous studies have shown that depressive symptoms are worsened by low 5-HT levels. Interestingly, fluoxetine and metformin in the present study increased 5-HT levels and reduced apoptosis. Thus, NVU barrier function, as well as the structural and functional integrity of cells were perturbed by exposure to conditions associated with depression. These findings provide further support for the concept that damage to the hippocampal NVU may be an important pathogenetic event in DD.

Abnormal expression of barrier-related proteins, including ZO-1, GFAP, AQP4 and Cx43, was found in rats with DD in our previous studies (Yang et al., 2012; Wang et al., 2015b). These and our current findings suggest that the structural and functional characteristics of the *in vitro* hippocampal NVU model are consistent with the characteristics of the hippocampal NVU *in vivo*.

In summary, a stable and reliable *in vitro* hippocampal NVU system was established using neurons, astrocytes and brain microvascular endothelial cells and was similar to the *in vivo* hippocampal NVU in structure and function. Furthermore, conditions associated with DD caused structural and functional damage to the NVU. Our findings provide

insight into the pathogenesis of DD and lay the foundation for further *in vitro* studies on the mechanisms of DD and other neurological disorders. Additional studies are needed to further clarify the role of NVU damage in the pathogenesis of DD.

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