

# Endothelin-1 Inhibition Improves the mBDNF/proBDNF Ratio in Endothelial Cells and HT22 Neurons Under High Glucose/Palmitate Growth Conditions

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## Summary

Diabetes increases the risk and worsens the progression of cognitive impairment. The hippocampus is an important domain for learning and memory. We previously showed that endothelin-1 (ET-1) reduced diabetes-induced inflammation in hippocampal neurons, suggesting a neuroprotective effect. Given that neurons and endothelial cells within the neurovascular unit depend on each other for proper function, we investigated the effect of ET-1 on brain-derived neurotrophic factor (BDNF) synthesis, a key neurotrophin and prosurvival factor, in neuronal (HT22 hippocampal neurons) and brain microvascular endothelial (BMEC-5i) cells under normal and diabetes-mimicking (high glucose plus palmitate) conditions. Cells were treated with exogenous ET-1 or ET receptor antagonists including ET<sub>B</sub> receptor selective antagonist BQ788 (1 μM) or dual-receptor antagonist bosentan (10 μM). Mature (m)BDNF, proBDNF and caspase-3 levels were measured by Western blotting. Diabetic conditions reduced the prosurvival mBDNF/proBDNF ratio in both HT22 and BMEC-5i cells. Addition of exogenous ET-1 had no effect on the BDNF system in HT22 cells in diabetic conditions. Both HT22 and BMEC-5i cells had an increase in the mBDNF/proBDNF ratio when grown in diabetes-simulating conditions in the presence of endothelin receptor inhibition. These data suggest that blockade of ET-1 may provide neuroprotection to hippocampal cells through the modulation of the BDNF system.

## Key words

Diabetes • Mature BDNF • proBDNF • Endothelin

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## Introduction

Diabetes is becoming a rapidly increasing problem, affecting 422 million people worldwide (WHO 2016). In addition to accepted complications associated with the disease, such as nephropathy and retinopathy, it is now known that diabetes elevates the risk and worsens progression of cognitive impairment (CDC 2017, Hardigan *et al.* 2016, McCrimmon *et al.* 2012). Learning and memory deficits as well as executive functions such as decision making are the most prominently observed form of cognitive impairment in diabetes (Kodl and Seaquist 2008, McCrimmon *et al.* 2012). Currently, therapeutic options are limited and as recently reviewed, one potential reason of slow progress towards finding more effective treatments due to focus on neuronal protection while neglecting neighboring cells within the brain (Iadecola 2017). Over time that led to the development of neurovascular unit concept, which includes microvascular endothelial cells, neurons, and glial cells as one unit. The effect of diabetes on close communication within this unit at the cellular level has not been well studied.

The hippocampus is a major contributor to learning and memory, contributing to critical functions such as spontaneous exploration of novel environment, formation of episodic memories and spatial memory. It is

comprised of the hippocampus proper (CA1-4), subiculum and dentate gyrus, and it is highly sensitive to injury (Knierim 2015). The hippocampus has been shown to be a target for neurovascular changes in diabetes. Reductions in microvasculature have been reported in GK rats as well as mice fed a high fat diet (Beauquis *et al.* 2010, Tucsek *et al.* 2014). Furthermore, numerous studies in type 1 and type 2 diabetes have shown loss of dendritic spines, impaired synaptic plasticity and reduced neurogenesis in the hippocampus that are associated with cognitive impairment (Han *et al.* 2016, Stranahan *et al.* 2008, Wongchitrat *et al.* 2016, Zhang *et al.* 2016). Reductions in cerebral blood flow (CBF) as a result of increased vascular tone has been noted in diabetic models. This decrease in CBF can negatively affect hippocampal neurons and therefore contribute to cognitive impairment. Patients and experimental models have shown endothelin-1 (ET-1), the most potent vasoconstrictor, to be elevated in diabetes (Matsumoto *et al.* 2004, Takahashi *et al.* 1990). Increased ET-1 can affect CBF and impact neuronal survival and cognition. Previously, we reported that ET-1 reduces diabetes-induced inflammation in hippocampal neurons, suggesting a neuroprotective effect (Ward and Ergul 2016). However, direct effects of ET-1 on neuroprotective pathways in hippocampal neurons in control and diabetic conditions are unknown.

Brain-derived neurotrophic factor (BDNF) has been implicated in learning and memory (Bekinschtein *et al.* 2014, Tyler *et al.* 2002). High expression of BDNF has been observed in the limbic system, which includes the hippocampus (Leibrock *et al.* 1989). BDNF can exert neurotrophic and angiogenic effects (Guan *et al.* 2012, Hempstead 2015, Liu *et al.* 2006). The neurotrophic roles of BDNF include development, brain plasticity and neuronal maturation. Administration of collagen-binding BDNF showed increased angiogenesis after middle cerebral artery occlusion in rats (Guan *et al.* 2012). Within the neurovascular unit, BDNF secreted from endothelial cells has been shown to reduce hypoxia-induced cell death in the neighboring neurons (Guo *et al.* 2008). Low BDNF levels in diabetic patients has been associated with cognitive impairment (Zhen *et al.* 2013). In experimental models, reduced BDNF contributes to cognitive dysfunction and synaptic plasticity in both obese (Wosiski-Kuhn *et al.* 2014) and diabetic models (Kariharan *et al.* 2015). Synthesis of BDNF occurs in the endoplasmic reticulum as the precursor form pre-proBDNF (Hashimoto 2016). The signal peptide is cleaved to become proBDNF. This protein can be further cleaved by proteases to become mature BDNF (mBDNF).

While mBDNF exerts neuroprotective effects through tropomyosin receptor kinase B (TrkB) receptor signaling, proBDNF has been suggested to induce neuronal apoptosis *via* p75<sup>NTR</sup> receptor (Benarroch 2015, Hempstead 2015). Intracerebroventricular administration of an endothelin ET<sub>B</sub> agonist elevated BDNF mRNA levels as well as expression of the mature protein in the caudate putamen (Koyama *et al.* 2003). Furthermore, ET-1 increases BDNF mRNA levels and release of BDNF in astrocyte culture (Koyama *et al.* 2005). While studies have shown positive effects in astrocytes, little is known about the effect of ET-1 on mBDNF and proBDNF synthesis in endothelial cells and neurons. Therefore, the goal of the current study was to determine the effect of ET-1 on this major neurotrophic factor with angiogenic properties within the neurovascular unit cells under normal and disease conditions.

## Methods

### Cell culture

Immortalized mouse HT22 hippocampal neurons were cultured as described previously (Ward and Ergul 2016). Briefly, HT22 hippocampal cells were grown in DMEM media, supplemented with 10 % FBS and 1 % penicillin streptomycin cocktail in a humidified incubator at 37 °C with 5 % CO<sub>2</sub>. Due to their higher metabolic demands, HT22 cells require higher levels of glucose in normal growth media (25 mM glucose) (Ward and Ergul 2016). HBEC-5i cells (ATCC), which originate from human cerebral microvascular endothelial cells, were cultured in DMEM: F-12 medium, supplemented with 10 % FBS and 1 % penicillin streptomycin cocktail in a humidified incubator at 37 °C with 5 % CO<sub>2</sub>. To mimic a high fat diet induced *in vivo* model of diabetes, we used high glucose plus palmitate combination in *in vitro* experiments. Control media included 25 mM and 5 mM glucose for HT22 cells and HBEC-5i cells, respectively. High glucose/palmitate media included palmitate (200 μM in 50 % ethanol) plus 50 mM and 12.5 mM glucose for HT22 cells and HBEC-5i cells, respectively. 12.5 mM glucose corresponds to ~225 mg/dl blood glucose levels in diet-induced model of diabetes (data not shown). As discussed above normal growth medium for HT22 cells requires 25 mM glucose. This concentration was doubled to mimic diabetes conditions. Before all experiments, cells were serum starved for 6 h. HT22 cells were treated with ET-1 (10, 50, 100 nM) for 24 h. In a second set of experiments, activation of ET receptors by endogenous ET-1 was blocked by either the dual-receptor antagonist

bosentan (10  $\mu$ M) or the ET<sub>B</sub> selective antagonist BQ788 (1  $\mu$ M) in both HT22 and HBEC-5i cells.

#### Western blot analysis

Cells were harvested after experiments and lysed using a modified RIPA buffer (Millipore Billerica, MA, USA). Equal protein loads were boiled in sample buffer and separated on a 4-15 % Mini-PROTEAN TGX gel by electrophoresis, then transferred by Trans-Blot Turbo system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) onto a nitrocellulose membrane. Membranes were probed for caspase-3 (Cell Signaling, Danvers, MA, USA), mBDNF and proBDNF (ThermoFisher Scientific, Waltham, MA, USA) at a concentration of 1:500. Expression was standardized using  $\beta$ -actin. Band intensity was analyzed using ImageJ software.

#### Statistical analysis

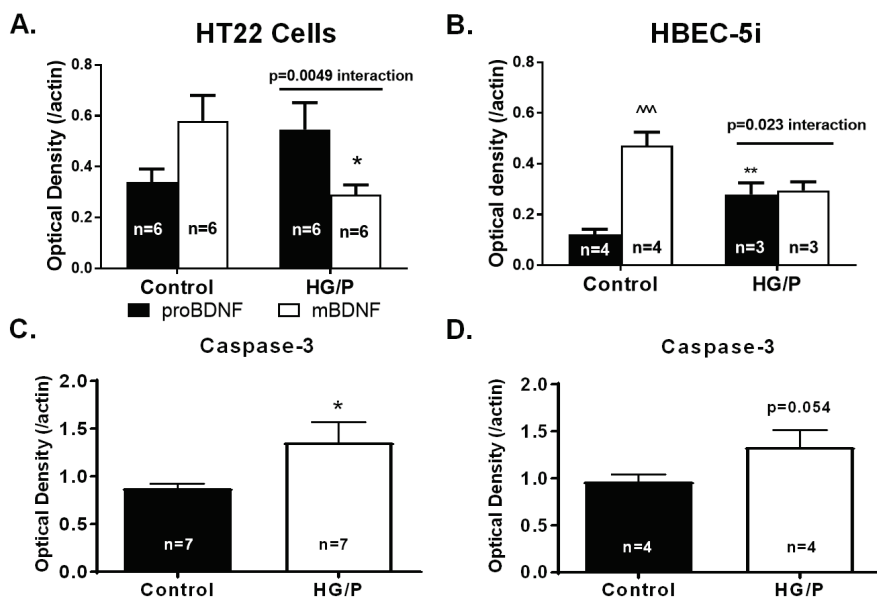
All data points were expressed as mean  $\pm$  SEM. Prism7 was used for all analyses. Interaction of condition

(control, diabetes) and either exogenous ET-1 (0, 10, 50, 100  $\mu$ M) or inhibition (no treatment, bosentan, BQ788) on BDNF or caspase-3 expression was analyzed by two-way ANOVA. Bonferroni's *post hoc* test was used to compare means from significant ANOVAs. Statistical significance was determined at  $\alpha < 0.05$ .

## Results

### High glucose/palmitate growth conditions alter the BDNF system and increase apoptosis in neuronal and endothelial cell lines

Diabetes-mimicking conditions increased proBDNF with a concomitant decrease in mBDNF as determined by immunoblotting in both HT22 (Fig. 1A) and endothelial cells (Fig. 1B). Caspase-3 was increased in HT22 cells grown in diabetic conditions (Fig. 1C). While no significance was observed in HBEC-5i cells, there was a trend towards elevated caspase-3 in diabetic conditions ( $p = 0.054$ ; Fig. 1D).



**Fig. 1.** Diabetes-mimicking growth conditions (HG/P) increase proBDNF and decrease mBDNF protein levels in both HT22 (**A**:  $P_{\text{interaction}} = 0.0049$ ,  $F(1,22) = 9.796$ ;  $P_{\text{diabetes}} = 0.6054$ ,  $F(1,22) = 0.2747$ ;  $P_{\text{BDNF}} = 0.9301$ ,  $F(1,22) = 0.0079$ ) and HBEC-5i (**B**:  $P_{\text{interaction}} = 0.0023$ ,  $F(1,10) = 16.42$ ;  $P_{\text{diabetes}} = 0.8208$ ,  $F(1,10) = 0.0541$ ;  $P_{\text{BDNF}} = 0.0012$ ,  $F(1,10) = 19.77$ ) cells as indicated by the quantification of corresponding bands on Western blots. Furthermore, caspase-3 was increased in high glucose/palmitate conditions (**C-D**). Control media included 25 mM and 5 mM glucose for HT22 cells and HBEC-5i cells, respectively. High glucose/palmitate media included palmitate (200  $\mu$ M in 50 % ethanol) plus 50 mM and 12.5 mM glucose for HT22 cells and HBEC-5i cells, respectively. \* $p < 0.05$  vs. control; <sup>^^</sup> $p < 0.001$  vs. proBDNF.

### Effects of exogenous endothelin on HT22 hippocampal cells

Since we previously showed reduced inflammation after treatment with exogenous ET-1 in HT22 cells (Ward and Ergul 2016), we next wanted to investigate the effect of ET-1 on BDNF synthesis and cell death. ET-1 had no effect on proBDNF levels in either control or diabetes-mimicking conditions (Fig. 2A). Under control conditions, there was a trend for decreased mBDNF (Fig. 2B) along with increased apoptotic marker caspase-3 (Fig. 2C). While exogenous ET-1 did not

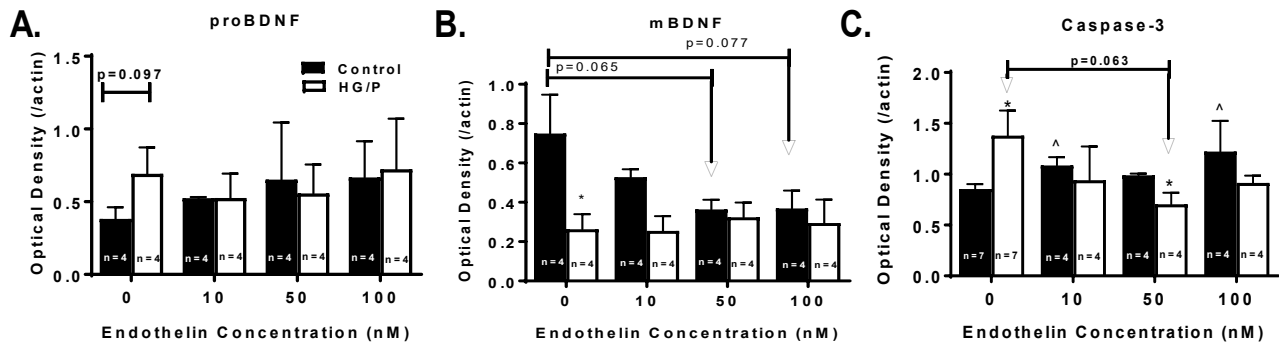
change mBDNF levels in high glucose/palmitate growth conditions, there was a trend for reduced apoptosis with ET-1 (Fig. 2C).

### Effects of endothelin receptor antagonism in HT22 and HBEC-5i cells

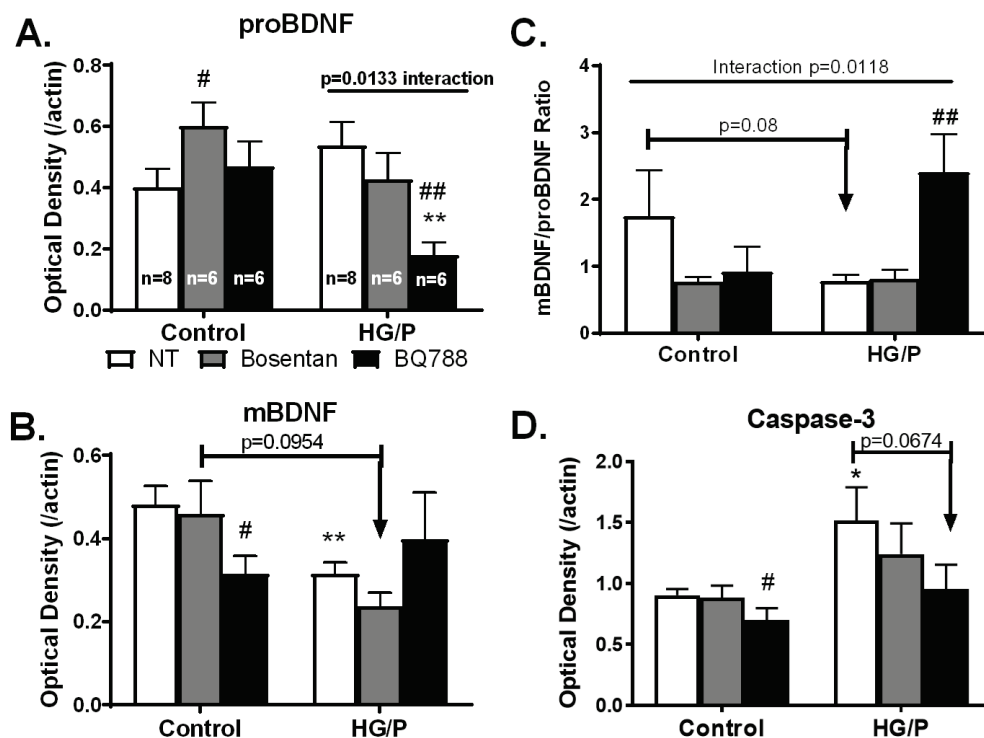
The next experiments aimed to understand the role of endogenous ET-1 on the endothelial and neuronal BDNF synthesis. HT22 cells treated with the dual-receptor antagonist bosentan showed no significant differences in the BDNF system expression grown under

diabetes-mimicking conditions. Cells grown under control plus bosentan conditions had increased proBDNF expression without subsequent changes to mBDNF (Figs 3A and 3B). Unlike the dual receptor blockade, inhibition of the ET<sub>B</sub> receptor alone with BQ788

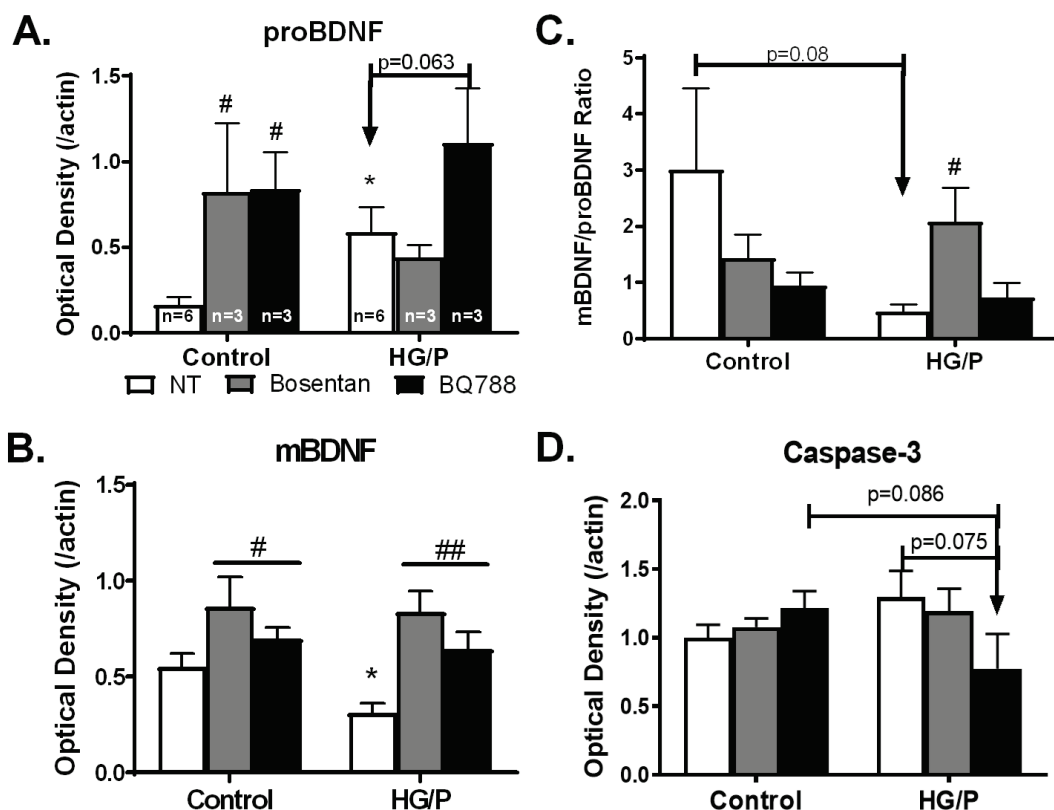
improved the mBDNF/proBDNF ratio in HT22 neurons (Fig. 3C). Furthermore, treatment with BQ788 showed a trend towards decreased caspase-3 expression ( $p=0.0674$ ; Fig. 3D).



**Fig. 2.** Exogenous ET-1 has no effect on the BDNF system in HT22 cells. While diabetes-mimicking conditions (HG/P) alone reduced mBDNF, addition of exogenous ET-1 in increasing concentrations did not change protein expression of proBDNF (A) or mBDNF as determined by immunoblotting (B). Band intensity corresponding to caspase-3 expression was increased in high glucose/palmitate conditions. Control media included 25 mM and 5 mM glucose for HT22 cells and HBEC-5i cells, respectively. High glucose/palmitate media included palmitate (200  $\mu$ M in 50 % ethanol) plus 50 mM and 12.5 mM glucose for HT22 cells and HBEC-5i cells, respectively. \* $p<0.05$  vs. control; ^ $p<0.05$  vs. 0  $\mu$ M ET-1.



**Fig. 3.** Protein expression of proBDNF (A:  $P_{\text{Interaction}}=0.0133$ ,  $F(2,34)=4.917$ ;  $P_{\text{diabetes}}=0.0781$ ,  $F(1,34)=3.301$ ;  $P_{\text{treatment}}=0.0463$ ,  $F(2,34)=3.369$ ) and mBDNF (B:  $P_{\text{Interaction}}=0.0579$ ,  $F(2,29)=3.148$ ;  $P_{\text{diabetes}}=0.0666$ ,  $F(1,29)=3.632$ ;  $P_{\text{treatment}}=0.7113$ ,  $F(2,29)=0.3447$ ) was measured after 24 h of ET receptor inhibition in HT22 cells by quantification of immunoblots. Dual-receptor antagonism with bosentan increased expression of proBDNF in control conditions. Inhibition of ET<sub>B</sub> with BQ788 (1  $\mu$ M), but not dual-receptor antagonism with bosentan (10  $\mu$ M), increased the mBDNF/proBDNF ratio in diabetes-mimicking conditions (C:  $P_{\text{Interaction}}=0.0118$ ,  $F(2,29)=5.194$ ;  $P_{\text{diabetes}}=0.5492$ ,  $F(1,29)=0.3673$ ;  $P_{\text{treatment}}=0.0806$ ,  $F(2,29)=2.749$ ). Cell death marker caspase-3 was elevated in high glucose/palmitate growth conditions and had a decreased trend after ET receptor inhibition (D:  $P_{\text{Interaction}}=0.5722$ ,  $F(2,26)=0.5722$ ;  $P_{\text{diabetes}}=0.0079$ ,  $F(1,26)=8.276$ ;  $P_{\text{treatment}}=0.1071$ ,  $F(2,26)=2.438$ ). Control media included 25 mM and 5 mM glucose for HT22 cells and HBEC-5i cells, respectively. High glucose/palmitate media included palmitate (200  $\mu$ M in 50 % ethanol) plus 50 mM and 12.5 mM glucose for HT22 cells and HBEC-5i cells, respectively. \* $p<0.05$ , \*\* $p<0.01$  vs. control; # $p<0.05$ , ## $p<0.01$  vs. NT.



**Fig. 4.** Protein expression of proBDNF (**A**:  $P_{\text{Interaction}}=0.1294$ ,  $F(2,18)=2.296$ ;  $P_{\text{diabetes}}=0.5384$ ,  $F(1,18)=0.3934$ ;  $P_{\text{treatment}}=0.0186$ ,  $F(2,18)=5.016$ ) and mBDNF (**B**:  $P_{\text{Interaction}}=0.3893$ ,  $F(2,17)=0.9978$ ;  $P_{\text{diabetes}}=0.1691$ ,  $F(1,17)=2.062$ ;  $P_{\text{treatment}}=0.0004$ ,  $F(2,17)=12.65$ ) was measured after 24 h of ET receptor inhibition in HBEC-5i cells by quantification of immunoblots. In diabetes-mimicking conditions, dual-receptor blockade (10  $\mu\text{M}$ ) improved mBDNF to proBDNF ratio (**C**:  $P_{\text{Interaction}}=0.0947$ ,  $F(2,12)=2.888$ ;  $P_{\text{diabetes}}=0.2391$ ,  $F(1,12)=1.535$ ;  $P_{\text{treatment}}=0.3350$ ,  $F(2,12)=1.2$ ). Caspase-3 expression had no significant changes in diabetic or ET receptor inhibition in HBEC-5i cells (**D**:  $P_{\text{Interaction}}=0.0855$ ,  $F(2,18)=2.828$ ;  $P_{\text{diabetes}}=0.9445$ ,  $F(1,18)=0.00498$ ;  $P_{\text{treatment}}=0.5776$ ,  $F(2,18)=0.566$ ). Control media included 25 mM and 5 mM glucose for HT22 cells and HBEC-5i cells, respectively. High glucose/palmitate media included palmitate (200  $\mu\text{M}$  in 50 % ethanol) plus 50 mM and 12.5 mM glucose for HT22 cells and HBEC-5i cells, respectively. \* $p<0.05$  vs. control; # $p<0.05$ , ## $p<0.01$  vs. NT.

While proBDNF expression was increased after treatment with bosentan or BQ788 in HBEC-5i cells under control conditions, in diabetes-mimicking *in vitro* conditions proBDNF was increased even in untreated cells. Bosentan had no effect but BQ788 further increased proBDNF levels. High glucose/palmitate growth conditions reduced mBDNF and endothelin inhibition increased mBDNF (Figs 4A and 4B). In BMEC-5i cells, bosentan improved the mBDNF/proBDNF ratio in cells grown under diabetes-mimicking conditions (Fig. 4C). While no significant difference was found in caspase-3 expression in these cells, endothelin inhibition trended towards reduced expression ( $p=0.075$ ; Fig. 4D).

## Discussion

The current study provides novel insights into the regulation of BDNF synthesis by ET-1 within cells of the neurovascular unit. Our results showed that in diabetes-mimicking conditions 1) mBDNF/proBDNF

ratio was reduced in both hippocampal neurons and brain endothelial cells; 2) exogenous ET-1 had opposite effects on neuronal apoptosis compared to control, and 3) inhibition of endogenous ET-1 signaling improved the mBDNF/proBDNF ratio.

Cognitive impairment is increasingly considered as a diabetic complication, yet remains one of the less understood complications associated with the disease. With a greater number of younger patients being diagnosed with diabetes each year (Benjamin *et al.* 2017, D'Adamo and Caprio 2011, Eehalt *et al.* 2008, Kumar *et al.* 2008), this problem needs to be a target of study. In type 2 diabetic patients, the most common deficits have been observed in the areas of attention, memory, executive function and information processing (Palta *et al.* 2014, van den Berg *et al.* 2009). These deficits have been noted in early diabetes and even in pre-diabetic patients (Crichton *et al.* 2012, Ruis *et al.* 2009). As a critical domain of learning and memory, the hippocampus has been the focus of numerous studies

relating to diabetes and cognition. Experimental diabetic models such as Goto-Kakizaki (GK) rats, high fat diet plus low dose streptozotocin, Zucker rats and db/db mice have all been shown to develop hippocampal-dependent cognitive deficits (Li *et al.* 2002, Ramos-Rodriguez *et al.* 2013, Stranahan *et al.* 2008). Loss of dendritic spine, impaired synaptic plasticity and low neurogenesis rates in the hippocampus have been reported to contribute to the cognitive impairment seen in diabetes (Li *et al.* 2013, Stranahan *et al.* 2008, Wongchitrat *et al.* 2016, Zhang *et al.* 2016). ET-1 can contribute to these changes *via* regulation of blood flow in hippocampus as well as direct effects on neuronal survival and function. While some studies suggested that stimulation of the endothelin B receptor by IRL-1620 reduces neuronal damage after ischemic injury as well as improving cognitive deficits in type 1 diabetic rats and in a rat model of Alzheimer's disease (AD) in which disease is induced by injection of amyloid B (A $\beta$ 1-40) in the lateral cerebral ventricles (Briyal *et al.* 2015, Briyal *et al.* 2014, Leonard *et al.* 2011, Leonard *et al.* 2012), other studies suggested that inhibition of the ET system was beneficial in improving outcomes. For example, ET<sub>A</sub> receptor antagonists prevented cognitive dysfunction in AD while the dual ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist TAK-044 had no effect in this model (Briyal *et al.* 2011). Treatment with another dual receptor blocker bosentan has been reported to improve learning and memory in a similar yet slightly different model of AD (Singh and Prakash 2017). Hung *et al.* (2015) observed severe spatial learning and memory deficits after stroke in transgenic mice overexpressing astrocytic ET-1 as well as cognitive dysfunction in animals in which ET-1 is overexpressed in endothelial cell (Zhang *et al.* 2013). In support of these studies, lower levels of plasma ET-1 was associated with better cognition in children (Calderon-Garciduenas *et al.* 2013). Understanding how ET-1 modulates growth factors that are critical for the survival of different cell types within the hippocampus in health and disease conditions may provide critical insights to novel treatments for this complication. In the current study, we show that ET inhibition with bosentan modulates BDNF synthesis while decreasing caspase-3 protein expression in HT22 and BMEC-5i cells grown in high glucose/palmitate containing growth conditions to simulate diabetic conditions. This suggests that blocking endothelin signaling may improve cell survival.

Neurotrophins, such as BDNF, are important to the central nervous system and play a role in neuronal

survival. BDNF signaling is critical in learning and memory through neuronal maturation and plasticity (Iadecola 2017, Wosiski-Kuhn *et al.* 2014). The hippocampus in particular has robust expression of BDNF and its main receptor TrkB. Mature BDNF binds to TrkB, which leads to autophosphorylation of the receptor and downstream signaling cascades such as the MAPK pathway, phosphatidylinositol 3-kinase-Akt pathway, phospholipase 3 pathway and CaMK pathway. The proBDNF form can activate p75<sup>NTR</sup> and promote apoptosis (Harte-Hargrove *et al.* 2013, Hempstead 2015). BDNF and its receptors are synthesized not only in neurons, but also in endothelial cells, smooth muscle cells and glial cells (Donovan *et al.* 2000, Ejiri *et al.* 2005, Tasci *et al.* 2012). Interestingly, within the neurovascular unit, BDNF secreted by endothelial cells has been shown to protect neurons from hypoxia or amyloid-induced cell death (Guo *et al.* 2008). Reductions in BDNF have been noted in diabetic patients and experimental models that also present with cognitive dysfunction but the relative ratio of mBDNF/proBDNF has not been well studied (Boyuk *et al.* 2014, Kariharan *et al.* 2015, Reus *et al.* 2016, Utkan *et al.* 2015, Zhen *et al.* 2013). In the present study, we confirm reductions of mBDNF in neuronal and endothelial cell lines grown in diabetes-simulating growth conditions. Furthermore, we show a subsequent increase in protein expression of proBDNF, which may play a role in increased cell death in the diabetic brain. Here, we provide additional evidence that inhibition of the endothelin receptors improves the mBDNF/proBDNF ratio. This suggests that perhaps blocking ET-1 action in the diabetic brain may improve neuronal function and decrease cell death within the neurovascular unit by improving vasotrophic coupling. Further *in vivo* studies need to be conducted to determine if endothelin blockade would prevent diabetes-induced pathological neurovascular remodeling of the hippocampus and improve cognitive function.

The current study has several limitations that should be identified. First, in order to study the regulation of mBDNF/proBDNF in different cells of the neurovascular unit, we only used an *in vitro* approach in cell lines rather than *in vivo* studies. Secondly, we measured solely caspase-3 as a marker of cell death and did not perform additional cell viability experiments. Third, we did not investigate endothelin receptor subtype expression under control and diabetes-simulating conditions. However, in a related study, we observed that endothelial cells used in this current study (HBEC-5i)

possess both ET<sub>A</sub> and ET<sub>B</sub> receptors and inhibition by dual blockade reduces regulated cell death (Abdul *et al.* 2018). Nevertheless, our data showing that inhibition of endothelin receptors improves the mBDNF/proBDNF ratio in both neurons and endothelial cells grown in diabetic conditions and supports the notion that ET-1 may contribute to the development and progression of cognitive dysfunction in diabetes by modulating the BDNF synthesis to promote cell death rather than survival.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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